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Genetic relationships between obesity and osteoporosis in LGXSM recombinant inbred mice

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Summary

Obesity and osteoporosis affect millions of Americans. While phenotypically, obesity is negatively correlated with fracture risk, research on a genetic basis for this relationship is lacking. We used males and females from 16 LGXSM recombinant inbred (RI) mouse strains to investigate the genetically mediated relationship between obesity and osteoporosis-related traits. First, heritabilities were estimated for (1) bone morphology properties determined by microCT (femoral and radial diaphyseal bone cross-sectional area and moments of inertia, as well as proximal tibial trabecular bone volume, connectivity density, structure model index, trabecular number, trabecular thickness and trabecular separation), (2) mechanical properties determined by bending tests (femoral and radial rigidity, yield moment, ultimate moment, fracture displacement and post-yield displacement), and (3) effective material properties (femoral and radial modulus of elasticity and ultimate tensile strength). All femoral ($H^2 = 43$–$74\,\%$) and tibial traits ($H^2 = 31$–$56\,\%$) were heritable; as were 8 of 10 radial traits ($H^2 = 21$–$33\,\%$). Eighteen significant genetic correlations were discovered between obesity- and osteoporosis-related phenotypes. Genetic correlations indicate that gene effects associated with increased fat mass and leptin levels are also associated with larger, stronger femora. Gene effects associated with larger, stronger radii and with denser tibiae were also associated with increased fat mass but not with leptin levels. Furthermore, quantitative trait loci (QTLs) previously reported for obesity and leptin levels also had effects on bone morphology, mechanical and material properties. Our results support the use of the LG/J-by-SM/J mouse intercross populations as models for normal, complex genetic variation in obesity, bone properties and their interrelationship.

1. Introduction

Both osteoporosis and obesity are serious health concerns that have been increasing in prevalence over the past two decades. Fifty-five percent of individuals of at least 50 years of age have either osteoporosis or osteopenia (National Osteoporosis Foundation, 2002). Obesity is also a common condition in the US population; nearly 66 % of US adults aged 20 or older are either overweight or obese (Hedley *et al.*, 2004) based on their body mass index (BMI). Secondary health implications of obesity include an increased incidence of high cholesterol levels, coronary artery disease, gallbladder disease, hypertension, Type II diabetes mellitus and osteoarthritis (Must *et al.*, 1999; Mokdad *et al.*, 2003).

These two diseases have usually been considered separately, but more recently both clinical and molecular linkages between them have become evident (Rosen & Bouxsein, 2006). Epidemiological studies show a positive correlation between BMI and bone mineral density (BMD) in humans (Castro *et al.*, 2005). In addition, positive correlations have been reported between an increase in fat mass and BMD at different skeletal locations in both females and males (Reid *et al.*, 1992; Stewart *et al.*, 2002). However, a twin study suggested that the correlation between BMD and BMI may be primarily due to environmental ($r_E \sim 0{\cdot}60$) rather than genetic factors ($r_G \sim 0{\cdot}20$) (Nguyen *et al.*, 1998).

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One recently delineated physiological link between osteoporosis and obesity occurs via leptin, a hormone produced by adipocytes that serves as a satiety signal, triggering the brain to stop food acquisition and increase metabolism (Halaas *et al*., 1995; Considine *et al*., 1996). Interestingly, obese individuals may suffer from hyperleptinaemia and leptin resistance (Frederich *et al*., 1995; Hamrick *et al*., 2004), resulting in satiety signals not being properly interpreted by the brain and allowing obese individuals to consume more calories than necessary (Frederich *et al*., 1995). Leptin also affects BMD, having different effects on different skeletal locations. Leptin deficiency in mice leads to shorter femora and decreased femoral BMD, cortical area and amount of trabecular bone as compared with wild-type controls. In contrast, in vertebrae, leptin-deficient mice have increased vertebral length and increased BMD and trabecular bone area (Hamrick *et al*., 2004). Research also shows increased osteoclast activity in the femora of leptin-deficient mice and increased osteoclast activity in the spine of leptin-treated mice (Hamrick *et al*., 2004, 2005). In addition to the role of leptin, there is strong evidence that other adipokines, including adiponectin, also affect bone turnover (Lenchik *et al*., 2003; Shinoda *et al*., 2006; Jürimäe & Jürimäe, 2007; Richards *et al*., 2007) along with other factors, such as neuropeptide Y (NPY) and neuromedin U, affecting energy metabolism and bone through hypothalamic processing (Allison & Herzog, 2006; Panuccio *et al*., 2007; Sato *et al*., 2007).

Several additional factors may affect the relationship between bone and obesity. First, there is the relationship between obesity, mechanical loading and bone morphology that predicts stronger bones as a result of higher mechanical loading in heavier individuals. Furthermore, bone and fat cells originate from the same progenitor cell populations but develop through alternative, mutually exclusive pathways (Wan *et al*., 2006). This is indicated in young adult humans where there is an inverse relationship between bone marrow adiposity and the amount of bone in the axial and appendicular skeleton (Iorgi *et al*., 2008).

Lee *et al*. (2007) show that osteoblasts also produce factors with substantial effects on metabolism and obesity, through the effects of osteocalcin on the pancreas and other tissues. Bone helps regulate metabolism, and therefore has an effect on obesity. Beta cells and adipocytes were known to regulate bone but it seems that bone is also talking back (Lee *et al*., 2007; Semenkovich & Teitelbaum, 2007). Hence, there are a variety of physiological mechanisms connecting bone and obesity with feedback between adipose and osseous tissues.

More general genetic analyses can contribute to our understanding of the relationship between obesity and osteoporosis outside of the single gene leptin deficiency models commonly used in this research. Many quantitative trait loci (QTLs) have been reported separately for obesity- and osteoporosis-related traits. The 2004 Obesity Gene Map Update by Perusse *et al*. (2005) describes 221 obesity-related QTLs found in mouse models and 204 QTLs found in human studies. Studies have found loci for obesity-related traits on all human and mouse autosomes and on the X chromosome (Perusse *et al*., 2005). These genes affect fatness, BMI, body weight (Dionne *et al*., 2002), insulin levels, and leptin levels (Arya *et al*., 2002). Studies of mouse models also indicate that bone properties can be affected by many loci. Additionally, QTLs for femoral and vertebral BMD as well as femoral bone area, moment of inertia, rigidity, ultimate moment, and energy to fracture have been found on all mouse chromosomes except chromosomes 3 and 17 (Beamer *et al*., 1999, 2001; Klein *et al*., 2002; Koller *et al*., 2003). We are unaware of genetic studies that have examined the relationships between obesity and osteoporosis in a variable mouse population.

In this paper, we describe the relative contribution of genetic effects on bone morphology, mechanical and material properties in relation to obesity-related traits by estimating genetic correlations between obesity- and osteoporosis-related traits in LGXSM recombinant inbred (RI) lines of mice. In addition, Cheverud *et al*. (2004*a*) found six fat mass QTLs, four of which also affected leptin levels, in a genome scan of the LGXSM RI lines. Therefore, we will test whether these previously identified obesity and leptin QTLs also affect bone morphology, mechanical and material properties.

2. Materials and methods

(i) *Specimens*

The 16 LGXSM RI strains of mice used in the present study were derived from the intercross of LG/J females and SM/J males, obtained from the Jackson Laboratories (Bar Harbor, ME, USA). LG/J and SM/J were developed in the 1940s and have since been maintained by brother–sister mating. Both strains resulted from artificial selection experiments in which large and small body weight phenotypes were selected (Goodale, 1938, 1941; MacArthur, 1944). Genetic studies on these mice have revealed that the extreme phenotypes result from genotypic differences at many loci of small effect and their interactions (Chai, 1956).

Three males and three females from each RI strain were used in the present study (four mice were included for LGXSM 38 females), totalling 97 mice. All mice were between 20·3 and 31·6 weeks old at the time of necropsy (average: 25 weeks; SD = 2·43), at which point they had already reached skeletal maturity

(Ehrich *et al*., 2003). We detected no effects of age at necropsy on the osseous phenotypes and ages were randomly distributed relative to strain membership. All animals were fed high-fat diets *ad libitum* as part of a dietary obesity experiment (Cheverud *et al*., 2004*a*) (catalogue no. TD88137; Harlan Teklad, Madison, WI, USA). We first examined animals on a high-fat diet because they have more extreme obesity-related trait values and higher levels of interstrain variation than low-fat-fed animals. Later studies will focus on the effects of dietary fat on bone properties and QTL effects. We used fat mass (a combined measure of reproductive, renal, mesenteric and inguinal fat pad weights at the time of necropsy) and serum leptin levels obtained from the plasma of blood samples acquired from mice that had been fasted for 4 h as obesity-related traits. Fat mass data for one mouse and leptin levels for four mice were not available for this analysis. The average obesity phenotypes for all mice of each sex-strain cohort are provided in Cheverud *et al*. (2004*b*) and were used to represent mice of the same sex and strain that lacked fat mass and leptin data. All mice were stored in freezers maintained at −20 °C until used in the present study.

A single femur, radius and tibia were removed from each mouse. Most bones were from the right anatomical side, although some were taken from the left when the right side was damaged or unavailable. Bilateral bones are morphologically and mechanically similar to one another and assumed to be interchangeable (Margolis *et al*., 2004). Due to dissection errors there was no available femur for one mouse, no radius for ten mice, and no tibia for four mice. Immediately after removal, bones were wrapped in tissue soaked in 0·9 % saline solution. Each wet bone was subsequently wrapped in plastic wrap and then placed in a plastic tube and stored in a freezer at −20 °C. Bones remained in the freezer until they were required for scanning or mechanical testing.

(ii) *Phenotypic data collection*

Bone morphology was assessed using micro-computed tomography. In preparation for scanning, bones were thawed at room temperature and embedded in 1·5 % agarose gel to ensure moistness and prevent motion. Bones were scanned using a commercial system (μCT 40, SCANCO Medical AG, Bassersdorf, Switzerland) at 16 μm isotropic resolution. Femora and radii were scanned at their mid-diaphysis. Four sets of three slices were taken for each bone; the sets were located ±1·5 and ±0·5 mm from the midpoint. These scans were then exported and converted to binary images using a simple threshold midway between the values of bone and background (ImageJ; NIH, Bethesda, MD, USA). Femoral images were rotated such that the medial–lateral bone axis was horizontal (*x*-axis); this was the bending axis for subsequent mechanical testing. Images of the radii were likewise rotated so that the axis of bending was horizontal. Using a custom macro (Excel; Microsoft Corporation, Redmond, WA, USA), the coordinates of the bone voxels were used to determine bone area (Area) and moments of inertia with respect to the *x*-axis and *y*-axis (I_{xx} and I_{yy}, respectively). For each morphological property, the average value calculated from the 12 slices of each bone was used for data analysis. Following scanning, femora and radii were placed back into storage at −20 °C until mechanical testing.

The morphological properties of tibial trabecular bone were obtained by scanning the proximal region of each tibia. Thirty slices of the metaphysis, spanning 0·48 mm and starting just distal to the growth plate, were used in analysis. Trabecular bone regions were segmented using the Scanco analysis software's contouring function and manually corrected to ensure that the appropriate regions were enveloped. The regions were analysed by the software to measure trabecular bone volume per total volume (BV/TV), connectivity density (ConnD), structure model index (SMI), trabecular number (TbN), trabecular thickness (TbTh) and trabecular separation (TbSp).

Mechanical testing on femora and radii was done using a three-point bending test. Prior to mechanical testing, bones were thawed in 0·9 % phosphate-buffered saline solution. The mid-diaphysis of each bone was then measured and marked. Tests were conducted using an Instron 1331/8500R and 8841 for femora and radii, respectively (Instron Corporation, Canton, MA, USA). The support span (*L*) for the femora was 7 mm; the span for radii was 8 mm. For both bones, the rate of vertical displacement of the load point was 0·03 mm/s. The applied force and bone displacement were recorded at 60 Hz using Labview7.0 (National Instruments, Austin, TX, USA). The output from the three-point bending tests was a force (*F*) versus displacement (*d*) graph, which, by taking into account the span, was converted to a moment (*M*) versus normalized displacement (*d*′) graph by the equations (Brodt *et al*., 1999):

$$M = (F \times L)/4,$$

$$d' = d \times 12/L^2.$$

Rigidity (Rig), yield moment (M_y), ultimate moment (M_u), fracture displacement (d'_{fx}), and post-yield displacement (d'_{py}) were calculated from these graphs.

Two material properties were derived from the morphological and mechanical properties of the femur and radius: (i) Young's modulus, also called the modulus of elasticity (*E*), and (ii) the ultimate tensile strength (σ_{ult}). These properties were calculated using simple beam theory equations and thus represent

estimates rather than direct measures (Turner & Burr, 1993; Brodt *et al.*, 1999):

$$E = \mathrm{Rig}/I_{xx},$$

$$\sigma_{\mathrm{ult}} = (M_{\mathrm{u}} \times Y_{\mathrm{max}})/I_{xx}.$$

(iii) *Statistical analysis*

Heritabilities were calculated using the general linear model (GLM) of the statistical program Systat11 (Systat Software, Point Richmond, CA, USA). The GLM function performs analysis of variance (ANOVA) tests. A two-way ANOVA was performed using the model:

$$Y_{ijk} = \mu + \mathrm{sex}_i + \mathrm{strain}_j + (\mathrm{sex}_i \times \mathrm{strain}_j) + e_{ijk},$$

where Y_{ijk} is the phenotype of the kth animal modelled by the overall phenotypic mean (μ), the deviation from the mean due to the ith sex and the jth strain, the interaction between the ith sex and jth strain, and the residual or environmental effect (e_{ijk}). Sex was treated as a fixed factor, while strain was considered a random factor (Sokal & Rohlf, 1995). When a group of traits were considered jointly, we used multivariate ANOVA (MANOVA).

Using the ANOVA output for strain differences, the phenotypic variation attributable to the different strains (σ^2_{str}) was calculated based on the following equations, where MS is the mean square value, the subscript r denotes the residual value and n is the number of specimens per strain:

$$\sigma^2_{\mathrm{str}} = (\mathrm{MS}_{\mathrm{str}} - \mathrm{MS}_{\mathrm{r}})/n,$$

$$\sigma^2_{\mathrm{r}} = \mathrm{MS}_{\mathrm{r}},$$

$$H^2 = \sigma^2_{\mathrm{str}}/(\sigma^2_{\mathrm{str}} + \sigma^2_{\mathrm{r}}).$$

Based on the understanding that all individuals of a given strain are genetically identical, strain variances are genetic variances. All genetic factors contributing to genetic variance are passed from generation to generation, including the dominance and epistatic relationships in inbred strains. The heritability calculated is thus the broad-sense heritability (Falconer & Mackay, 1997).

Genetic correlations were calculated separately for all phenotypes of the femur, radius and tibia; between the same phenotypes measured on each bone; and between the obesity-related phenotypes and the bone phenotypes. The data used for fat mass and leptin levels were obtained from Cheverud *et al.* (2004*b*); only the data from the specimens included in bone analysis were used in determining the correlations.

All correlations were directly computed by the GLM as matrices of residual correlations. The genetic correlations were calculated from the strain mean phenotypic values using the model (Sokal & Rohlf, 1995):

$$Y_{ij} = \mu + \mathrm{sex}_i + e_{ij}.$$

This takes into account the mean phenotype of the jth strain and the variance due to the ith sex and the residual (e_{ij}). Environmental correlations were calculated from the individuals' phenotypes based on the residuals from the ANOVA model described above with sex, strain and their interaction as factors (Sokal & Rohlf, 1995). The phenotypic correlations were based on individual data using the model:

$$Y_{ij} = \mu + \mathrm{sex}_i + e_{ij}$$

such that the residual correlations represent the relationship between phenotypes after removing differences due to sex (Sokal & Rohlf, 1995). The significance of the correlations was computed using a t-distribution, and correlations whose P-values were 0·05 or less were considered significant.

Table 1. *Markers associated with effects on fat mass (F) and leptin (L) levels as well as their confidence intervals, physical positions and LOD scores (Cheverud et al., 2004a)*

Obesity QTL	Traits affected	Begin (Mb)	End (Mb)	Begin (cM)	End (cM)	LOD
D1Mit421	F, L	91·11	127·30	56·6	65·0	3·68
D8Mit89	F, L	63·82	116·32	32·0	59·0	3·21
D10Mit47N	F	100·41	126·04	59·0	70·0	3·58
D11Mit349	F	34·00	80·56	17·0	46·2	2·34
D18Mit80	F, L	76·19	84·97	47·0	55·0	2·91
DXMit121	F, L	142·68	142·68	69·0	69·0	3·50

Genetic markers have been scored for 506 microsatellite loci in the LGXSM RI strains (Hrbek *et al.*, 2006). Markers found to be associated with fat mass and leptin levels (Table 1) (Cheverud *et al.*, 2004*a*) were tested for their potential association with femoral, radial and tibial morphological properties and femoral and radial mechanical and material properties. In order to test these hypothesized associations more efficiently, we first performed a data reduction analysis by separately obtaining the principal components of the femoral, radial and tibial traits. Principal component scores were obtained for each strain for each principal component accounting for more than 10% of the total variance in femoral (three components), radial (two components) and tibial (two components) traits. Thus, these principal components are based on the genetic correlations between traits. A two-way mixed model ANOVA was run using the model:

$$y_{ijkl} = \mu + \mathrm{sex}_i + \mathrm{genotype}_j + (\mathrm{sex}_i \times \mathrm{genotype}_j) + \mathrm{strain}_k \times (\mathrm{sex}_i \times \mathrm{genotype}_j) + e_{ijkl},$$

where Y_{ijkl} is the principal component score of the lth animal described by the overall phenotypic mean (μ), the deviation from the mean due to the ith sex, the jth genotype and the kth strain, the interaction between sex and genotype, the nested effect of strain within the sex-by-genotype interaction, and the residual (e) (Sokal & Rohlf, 1995). Multivariate tests were performed using a comparable MANOVA model.

Statistical significance was determined using F-ratios, the ratio of $MS_{genotype}$ to $MS_{strain\,(sex\times genotype)}$; a significant effect suggests that a QTL affecting bone biomechanical properties is located near the marker. An F-ratio of the $MS_{sex\times genotype}$ over the $MS_{strain\,(sex\times genotype)}$ term was used to determine if the sex-by-genotype interaction is significant at a marker. Significant interactions represent a QTL with different effects on males and females (Sokal & Rohlf, 1995). Multivariate tests of QTL effects on bone phenotypes were considered significant at the nominal 5% level. They were not corrected for multiple comparisons because the locations examined were chosen *a priori* due to an earlier finding of an effect on obesity and/or serum leptin level. Since these locations were chosen based on earlier analyses, they are considered protected from multiple comparisons problems when applied to bone traits. However, QTL effects on fat mass and serum leptin level were, themselves, adjusted for multiple comparisons (Cheverud *et al*., 2004*a*).

Table 2. *Femoral and radial P-values for the effects of sex and sex-by-strain interaction*

	Sex		Sex-by-strain *interaction*	
Trait[a]	Femoral	Radial	Femoral	Radial
Area	<0·001	0·033	0·061	0·779
I_{xx}	<0·001	0·046	0·104	0·963
I_{yy}	<0·001	<0·001	0·088	0·718
Rig	0·168	0·149	0·610	0·558
M_y	0·002	0·125	0·277	0·976
M_u	0·002	0·126	0·532	0·922
d'_{fx}	0·400	0·278	0·017	0·513
d'_{py}	0·039	0·261	0·048	0·520
E	<0·001	0·820	0·015	0·853
σ_{ult}	0·440	0·585	0·384	0·744

[a] Area: cortical bone area; I_{xx}: horizontal moment of inertia; I_{yy}: vertical moment of inertia; Rig: rigidity.

Table 3. *Tibial P-values for the effects of sex and sex-by-strain interaction*

Trait	Sex	Sex-by-strain
BV/TV	0·002	0·002
ConnD	0·011	0·048
SMI	<0·001	0·052
TbN	0·728	0·150
TbTh	0·525	0·103
TbSp	0·817	0·013

3. Results

(i) *Effects of sex*

ANOVA results for phenotypes pooled across the strains show sexual dimorphism at $P<0{\cdot}05$ (Tables 2 and 3); phenotypic means, standard deviations and sample sizes for all femoral, radial and tibial phenotypes are presented in Online Appendix 1. Overall, for the femur, males showed greater phenotypic values for bone area ($P=6{\cdot}9\times10^{-4}$), inertia (I_{xx}) ($P=2{\cdot}5\times10^{-6}$), inertia (I_{yy}) ($P=2{\cdot}0\times10^{-9}$), M_y ($P=2{\cdot}5\times10^{-3}$) and M_u ($P=2{\cdot}0\times10^{-3}$), whereas females were greater for d'_{py} ($P=3{\cdot}9\times10^{-2}$) and E ($P=9{\cdot}8\times10^{-6}$). Regarding the radius, bone area ($P=3{\cdot}3\times10^{-2}$), inertia (I_{xx}) ($P=4{\cdot}6\times10^{-2}$) and inertia (I_{yy}) ($P=8{\cdot}2\times10^{-4}$) were greater in males than in females. For the tibia, bone volume ($P=2{\cdot}2\times10^{-3}$) and ConnD ($P=1{\cdot}1\times10^{-2}$) were larger in females, whereas male trabeculae had a higher SMI (i.e. more rod-like and less plate-like) than that of females ($P=6{\cdot}1\times10^{-4}$).

The sex-by-strain interaction term measures genetic variation in sexual dimorphism between strains and was significant for properties of the femur and tibia (Tables 2 and 3), including femoral d'_{fx} ($P=1{\cdot}7\times10^{-2}$), post-yield displacement ($P=4{\cdot}8\times10^{-2}$) and E ($P=1{\cdot}5\times10^{-2}$). Significant genetic variation in sexual dimorphism for tibial trabecular bone volume ($P=2{\cdot}0\times10^{-3}$), ConnD ($P=4{\cdot}8\times10^{-2}$) and TbSp ($P=1{\cdot}3\times10^{-2}$) was also discovered. For these traits, the level of sexual dimorphism was different in different strains, indicating that some genes are likely to have sex-specific effects on these traits.

(ii) *Heritabilities*

The effects of strain (Table 4), when pooled across the sexes, were significant for all femoral, radial and tibial traits except radial d'_{fx} and post-yield displacement, which were not different between strains. There were significant strain effects for femoral bone area ($P=1{\cdot}8\times10^{-12}$), inertia ($I_{xx}$) ($P<1{\cdot}0\times10^{-17}$), inertia ($I_{yy}$) ($P=8{\cdot}3\times10^{-13}$), rigidity ($P=5{\cdot}0\times10^{-8}$), M_y ($P=6{\cdot}8\times10^{-7}$), M_u ($P=3{\cdot}1\times10^{-8}$), d'_{fx} ($P=2{\cdot}0\times10^{-8}$), post-yield displacement ($P=2{\cdot}2\times10^{-8}$), E ($P=4{\cdot}4\times10^{-10}$) and tensile strength ($P=1{\cdot}7\times10^{-8}$). Strain effects were noted for radial bone area ($P=1{\cdot}9\times10^{-3}$), inertia (I_{xx}) ($P=4{\cdot}8\times10^{-3}$), inertia (I_{yy}) ($P=5{\cdot}7\times10^{-4}$), rigidity ($P=1{\cdot}4\times10^{-4}$), M_y ($P=1{\cdot}0\times10^{-4}$), M_u ($P=1{\cdot}6\times10^{-4}$), E ($P=2{\cdot}6\times10^{-4}$) and tensile strength ($P=8{\cdot}6\times10^{-4}$). Tibial bone volume ($P=3{\cdot}7\times10^{-10}$), ConnD

Table 4. *Femoral, radial and tibial P-values for the effects of strain, as well as strain variances* (σ^2_{str}), *residual variances* (σ^2_r) *and heritabilities* (H^2).

Trait	P	σ^2_{str}	σ^2_r	H^2
Femoral				
Area	<0·001	0·009	0·006	62·2%
I_{xx}	<0·001	<0·001	<0·001	73·5%
I_{yy}	<0·001	0·003	0·002	63·0%
Rig	<0·001	33499·967	37201·200	47·4%
M_y	<0·001	31·578	42·652	42·5%
M_u	<0·001	36·235	38·944	48·2%
d'_{fx}	<0·001	<0·001	<0·001	49·0%
d'_{py}	<0·001	<0·001	<0·001	48·8%
E	<0·001	1836941·667	1501650·000	55·0%
σ_{ult}	<0·001	362·512	373·129	49·3%
Radial				
Area	0·002	<0·001	<0·001	24·2%
I_{xx}	0·005	<0·001	<0·001	21·3%
I_{yy}	<0·001	<0·001	<0·001	27·9%
Rig	<0·001	755·678	1609·270	32·0%
M_y	<0·001	0·841	1·728	32·7%
M_u	<0·001	0·903	1·959	31·5%
d'_{fx}	0·054	<0·001	0·007	12·2%
d'_{py}	0·062	<0·001	0·006	11·5%
E	<0·001	7410183·333	17053200·000	30·3%
σ_{ult}	<0·001	653·237	1781·990	26·8%
Tibial				
BV/TV	<0·001	0·003	0·002	56·0%
ConnD	<0·001	404·518	406·081	49·9%
SMI	<0·001	0·106	0·166	39·0%
TbN	<0·001	0·095	0·098	49·2%
TbTh	<0·001	<0·001	<0·001	30·8%
TbSp	<0·001	<0·001	<0·001	43·9%

Table 5. *Bone–obesity genetic correlations, with significant correlations in boldface*

Trait	Total fat	Leptin
Femoral		
Area	**0·45**	0·16
I_{xx}	0·19	−0·13
I_{yy}	0·30	−0·01
Rig	**0·64**	**0·42**
M_y	**0·58**	**0·35**
M_u	**0·59**	0·31
d'_{fx}	−0·19	−0·12
d'_{py}	−0·16	−0·11
E	0·33	**0·45**
σ_{ult}	**0·37**	**0·48**
Radial		
Area	**0·44**	0·07
I_{xx}	**0·56**	0·33
I_{yy}	**0·45**	0·11
Rig	**0·46**	0·25
M_y	**0·47**	0·28
M_u	**0·45**	0·23
d'_{fx}	0·20	0·26
d'_{py}	0·20	0·26
E	0·06	0·12
σ_{ult}	0·05	0·11
Tibial		
BV/TV	**0·35**	0·20
ConnD	0·13	0·06
SMI	**−0·55**	−0·29
TbN	0·03	0·01
TbTh	**0·40**	0·19
TbSp	−0·12	−0·08

($P = 1{\cdot}7 \times 10^{-8}$), SMI ($P = 4{\cdot}7 \times 10^{-6}$), TbN ($P = 2{\cdot}5 \times 10^{-8}$), TbTh ($P = 1{\cdot}5 \times 10^{-4}$), and TbSp ($P = 4{\cdot}4 \times 10^{-7}$) were also affected by strain.

Heritabilities varied widely (Table 4). For the femur, inertia (I_{xx}) showed the greatest heritability at 73·5% and M_y had the lowest heritability at 42·5%; all other femoral heritabilities were between 47·0 and 63·0%. The greatest radial heritability was only 32·7% for M_y, the lowest significant heritability was 21·3% for inertia (I_{xx}), and other significant radial heritabilities lay within this range. Bone volume showed the greatest heritability in the tibia at 56·0%, TbTh was the least heritable at 30·8%, and all other tibial phenotypes had heritabilities between 39·0 and 50·0%.

(iii) *Genetic correlations*

Genetic correlations are provided between femoral phenotypes, between radial phenotypes and between tibial phenotypes (Online Appendix 2). They are also provided between these bone phenotypes and obesity-related traits (Table 5) and across the femoral, radial and tibial phenotypes (Online Appendix 2). All correlations stronger than $r_G = \pm 0{\cdot}35$ ($r^2_G = 0{\cdot}12$) were significant at the $P = 0{\cdot}05$ level.

There were many significant intra-bone and inter-bone genetic correlations. Within a bone, no bone property varied independently of all others, although femoral d'_{fx}, E and tensile strength; radial d'_{fx} and post-yield displacement; and tibial TbTh were not correlated with most other traits measured on the same bone. Across the bones, femoral tensile strength varied independently of all radial and tibial phenotypes; femoral d'_{fx}, post-yield displacement and E varied independently of all tibial traits; radial tensile strength varied independently of all femoral phenotypes; radial d'_{fx} and post-yield displacement varied independently of all tibial traits; and tibial TbN varied independently of all radial traits. There were also many phenotypes that, when compared across bones, showed lack of dependence on most phenotypes of other bones.

Bone phenotypes were also correlated with fat mass and leptin levels. Fat mass was positively correlated with femoral bone area, rigidity, M_y, M_u and tensile strength ($r^2_G = 0{\cdot}14$–$0{\cdot}41$); positively correlated with radial bone area, inertia (I_{xx} and I_{yy}), rigidity, M_y and

Table 6. *Femoral, radial and tibial principal components*

Trait	Femur 1	Femur 2	Femur 3	Radius 1	Radius 2	Trait	Tibia 1	Tibia 2
Area	0·932	0·056	0·283	0·735	0·576	BV/TV	0·965	0·203
I_{xx}	0·863	−0·418	0·147	0·621	0·689	ConnD	0·929	−0·239
I_{yy}	0·903	−0·326	0·175	0·756	0·418	SMI	−0·841	−0·403
Rig	0·498	0·701	0·287	0·957	−0·01	TbN	0·849	−0·432
M_y	0·904	0·281	0·037	0·922	0·182	TbTh	0·491	0·819
M_u	0·892	0·322	0·258	0·967	0·102	TbSp	−0·884	0·394
d'_{fx}	−0·602	−0·212	0·75	−0·437	0·742			
d'_{py}	−0·644	−0·085	0·758	−0·434	0·735			
E	−0·391	0·873	0·043	0·588	−0·711			
σ_{ult}	−0·143	0·902	−0·007	0·538	−0·675			
% Total variance	52	26	14	52	31		71	21

Table 7. *Probabilities of obesity QTLs having no effects on bone property principal components. The 'Overall' column is a multivariate test for each locus separately, whereas the 'Overall' row is the probability for each bone property principal component. The intersection of the 'Overall' row and the 'Overall' column provides the overall probability of obesity QTLs having no effect on bone property principal components. 'ns' indicates not significant at the 0·10 level*

		Principal components						
Obesity QTL	Overall	Femur 1	Femur 2	Femur 3	Radius 1	Radius 2	Tibia 1	Tibia 2
D1Mit421	0·078	ns	ns	ns	ns	0·035	ns	ns
D8Mit89	0·020	0·012	ns	0·082	0·049	0·049	ns	ns
D10Mit47N	0·247	0·027	ns	ns	ns	ns	ns	ns
D11Mit349	0·005	0·018	ns	0·056	0·018	0·039	ns	ns
D18Mit80	0·002	ns	ns	ns	0·104	0·003	ns	ns
DXMit121	0·041	0·004	ns	ns	0·005	ns	ns	0·105
Overall	0·008	0·027	0·405	0·488	0·037	0·016	0·157	0·293

M_u ($r^2_G = 0{\cdot}19$–$0{\cdot}31$); and positively correlated with tibial bone volume and TbTh and negatively correlated with SMI ($r^2_G = 0{\cdot}12$–$0{\cdot}31$). Leptin levels were positively correlated with femoral rigidity, M_y, E and tensile strength ($r^2_G = 0{\cdot}12$–$0{\cdot}23$). Leptin did not correlate with any radial or tibial traits.

(iv) *Principal components*

Three femoral, two radial and two tibial principal components were obtained, jointly accounting for 92, 83 and 91% of the total variance between strains in these trait sets, respectively (Table 6). The first femoral principal component (52% of variance) primarily contrasts size (area, I_{xx} and I_{yy}) and structural stiffness and strength (M_y and M_u) with measures of ductility (d'_{fx} and d'_{fy}). E and σ_{ult} have relatively small, negative loadings. The second femoral component (26%) is positively related to rigidity, E and σ_{ult}, in contrast with negative coefficients for the moments of inertia (I_{xx} and I_{yy}), while the third component (14%) is essentially a measure of ductility (d'_{fx} and d'_{fy}).

The first radial principal component (52%) is similar to the first femoral component (vector correlation between Femur 1 and Radius 1 is 0·82), contrasting size (area, I_{xx} and I_{yy}), structural stiffness and strength (rigidity, M_y and M_u) with ductility (d'_{fx} and d'_{fy}), but with E and σ_{ult} having positive coefficients instead of negative ones. The second radial component (31%) contrasts size (area, I_{xx} and I_{yy}) and ductility (d'_{fx} and d'_{fy}) with E and σ_{ult}.

The first tibial principal component (71%) contrasts the measures related to bone volume and trabecular number, thickness and connectivity (BV/TV, TbN, TbTh and ConnD) with TbSp and SMI. The second component (21%) contrasts TbTh and TbSp with TbN and SMI.

(v) *QTLs*

Fat mass and leptin QTLs have significant effects on bone morphology and material properties (see Table 7). A MANOVA using all seven principal components as dependent variables and all six QTL

genotypes as independent variables indicates only an overall 0·008 probability of obtaining the observed effects under the null hypothesis of no influence of fat mass/leptin loci on bone properties. The genotypes jointly have significant effects on Femur 1 ($P=0·027$), Radius 1 ($P=0·037$) and Radius 2 ($P=0·016$). Four of the six obesity/leptin QTLs had multivariate significant effects on overall bone properties and all six had significant effects on at least one bone property principal component (see Table 7).

4. Discussion

(i) *Heritabilities and genetic correlations*

The data obtained from the LGXSM RI mouse strains for osteoporosis-related traits in the present study showed that a substantial proportion of the phenotypic variance in these traits is due to heritable genetic causes. The greatest heritabilities were found for femoral and tibial morphological properties and, with the exception of three radial phenotypes, all bone phenotypes investigated showed a significant proportion of heritable genetic variation. Clearly, this strain set displays substantial genetic variation for bone morphological, mechanical and material characteristics.

The correlations with serum leptin levels present further insights into the relationship between leptin and various osseous characteristics. Femoral results were in agreement with the conclusions of Hamrick *et al.* (2004, 2005), supporting the link between the increased levels of this hormone and stronger femora. In contrast, no radial or tibial phenotypes were significantly correlated with leptin. The absence of genetic correlations with leptin indicates a lack of genetic relationship between serum leptin levels and radial or tibial trabecular properties, at least in our mouse population. In contrast with our lack of association between leptin levels and tibial trabecular mass, other murine studies have discovered such relationships involving vertebral bodies and the femur. Even so, the effects of leptin may differ for different osseous elements and for different bone compartments (cortical and trabecular) (Hamrick *et al.*, 2004), and may indeed be specific to the leptin-deficient mutant mice utilized in this earlier research. Li *et al.* (2008) find no relationship between leptin levels and bone mass across mouse strains and that the fat–bone mass relationship may differ depending on diet and genotype.

The adiposity data show that there are strong genetic relationships between fat mass and bone density, ductility, size and strength. Fat mass was correlated with more bone phenotypes in all three bones than was leptin. Some human quantitative genetic studies have found that the bone–obesity relationship is not genetic but largely environmental (Nguyen *et al.*, 1998) and that genetic correlations and pleiotropic QTLs between bone and obesity (Tang *et al.*, 2007) are mediated through general body weight factors or muscle mass, which, when controlled for, eliminate the positive association between bone mass and obesity (Zhao *et al.*, 2007). This is not the case in our set of LGXSM RI lines. The genetic correlation between fat mass and body weight at necropsy is 0·91, while their environmental correlation is 0·85 (Cheverud *et al.*, 2004*b*). These high correlations may be due, in part, to the fact that all animals were reared on a high-fat diet. Fat mass, as measured here, on average makes up about 20% of the total body weight. Given the part–whole relationship of body weight and fat mass and the very high genetic and environmental correlations between these two traits in our population, statistical separation of the effects of lean and fat mass on bone properties is not informative. We have no independent data on muscle mass in these mice. Given the observed correlations between fat mass and bone properties, and assuming our findings extend to other mammals, it is evident that genes that contribute to more physically obese individuals are inherited with those that help reduce the likelihood of fracture in the femur, radius and tibia.

(ii) *Principal components*

Principal components were calculated to describe the overall pattern of variations in bone morphology, mechanical and material properties in the LG/J-by-SM/J intercross. Measures of cross-sectional morphology and bone biomechanical properties were taken on the femur and radius. The first component for both femoral and radial traits was quite similar ($r=0·82$) and contrasted the measures of size and structural stiffness and strength with the measures of ductility. This contrast can be interpreted as representing the differences between large, stiff, strong, more brittle bones (femora or radii) versus smaller, less stiff, weaker, more ductile bones. This grouping of traits is generally consistent with the paradigm described by Jepsen *et al.* (2003), whereby bone size was positively associated with stiffness and failure load across eight inbred mouse strains.

The second femoral and radial principal components were similar in that they both contrasted estimated material properties (E and σ_{ult}) with moments of inertia. This grouping is consistent with the beam theory equations used to compute the material properties (see section 2), as moment of inertia (I_{xx}) is the denominator in the E and σ_{ult} equations. Recalling that principal components are statistically independent of each other, the contrast of moments of inertia with material properties should be considered

among femora (or radii) with equal scores on the first principal component. In other words, this contrast is between femora of the same stiffness, strength and ductility that have relatively high material properties and small moments of inertia versus those that have relatively low material properties and large moments of inertia.

The third femoral principal component is a scale that runs from brittle to ductile. The fact that measures of ductility (d'_{fx}, d'_{fy}) weigh most strongly in this component without strong weightings from other traits suggests that ductility is largely independent of morphology and whole-bone stiffness and strength. This is consistent with the findings of Jepsen *et al.* (2003). Also related to ductility, the first two femoral and radial principal components each contrast measures of ductility against measures of material properties. It is possible that bone composition, specifically ash content (which we did not measure), would explain this contrast. Bone tissue that is more highly mineralized will have greater material stiffness and strength, but less ductility (Currey, 1984; Jepsen *et al.*, 2003).

Measurements were also taken on the trabecular bone in the proximal tibia. The first tibial principal component, which captures 71% of the total variance, is a contrast between measures that increase with trabecular bone density (BV/TV, ConnD, TbTh and TbN) and measures that decrease with increased trabecular bone density (TbSp and SMI). Tibia that have high scores on this first component have many, closely spaced, thick, interconnected, plate-like trabeculae leading to high trabecular bone volume, while those with low scores have relatively few, sparsely distributed, thin, unconnected, rod-like trabeculae leading to low trabecular bone volume.

(iii) *QTLs*

Analysis of the effects of obesity and leptin QTLs on bone properties indicates that obesity loci also have effects on bone morphology, biomechanical and material properties and are consistent with the observed genetic correlations between obesity and bone traits. While for any one specific obesity locus we cannot eliminate the possibility that the osseous effects are due to a separate, closely linked locus, it seems highly unlikely that this would be true for all six obesity/leptin loci examined. Four of the six loci affected the first femoral and radial principal components and the second radial principal component. Overall, the most prominent effects of obesity QTLs were on these three components. Thus, obesity QTLs at *D8Mit89*, *D10Mit47N*, *D11Mit349* and *DXMit121* affect the difference between large, stiff, strong, brittle bones and smaller, less stiff, weaker, more ductile ones. Obesity QTLs (*D8Mit89*, *D11Mit349*, *D18Mit18* and *DXMit121*) also affect the second radial principal component. Two of the obesity QTLs that affected the second radial principal component (*D8Mit89* and *D11Mit349*) also had marginally significant affects on the related third femoral component. No obesity QTLs affected the second femoral or first tibial principal components and only a marginally significant effect was detected at a single obesity QTL (*DXMit121*) for the second tibial component.

These results confirm the known human epidemiological relationships between obesity and osteoporosis in our mouse model population. Genetic correlations demonstrate that genes influencing phenotypes related to obesity and osteoporosis are inherited together, and QTL analysis reveals potential genomic loci associated with these phenotypic relationships. However, it must be noted that we can only crudely map bone QTLs in this population of mice. It is possible that separate but linked genetic factors may be responsible for the apparent bone – obesity pleiotropic effects reported here. Further studies in populations with enhanced levels of recombination will be necessary to separate the possibilities of genetic correlation due to close linkage from those due to pleiotropy.

Furthermore, obesity QTLs that affect bone properties allow for identification of candidate genes at each locus responsible for the relationships observed; candidate genes can be found at http://www.ensemble.org (Hubbard *et al.*, 2005). Examples include *Npy1r* and *Npy5r*, genes that encode NPY receptors Y1 (NPY1-R) and Y5 (NPY5-R) on chromosome 8 (Hubbard *et al.*, 2005). These two positional candidates are found in the genomic region associated with obesity, femoral principal components 1 and 3 and radial components 1 and 2. NPY affects hypothalamic control of food and energy consumption. Interestingly, NPY and leptin may regulate energy intake and expenditure in a homeostatic loop in which NPY production is reduced by leptin and NPY stimulates leptin production (Wang *et al.*, 1997). In mice, NPY leads to increased body weight and body fat when it binds to either NPY1-R or NPY5-R (Henry *et al.*, 2005); these effects could also influence bone morphology and mechanics (Reid *et al.*, 1992; Stewart *et al.*, 2002; Castro *et al.*, 2005; Hassa *et al.*, 2005). Moreover, mice lacking the NPY1-R receptor have elevated bone and adipose tissue mass (Baldock *et al.*, 2007).

Another interesting example of genes that may influence the obesity–osteoporosis relationship is *Igfbp2* and *Igfbp5* on chromosome 1. These tightly linked genes are approx. 20 Mb from an obesity QTL that also affects the second radial principal component. *Igfbp2* and *Igfbp5* encode insulin-like growth factor-binding proteins (IGFB2 and IGFB5) that affect bone formation. Whereas IGFBP2 inhibits bone formation

(Fisher *et al*., 2005), IGFBP5 stimulates it (Richman *et al*., 1999). When compared with control subjects, a study showed osteoporotic individuals to have increased levels of IGFBP2 and decreased levels of IGFBP5 (Jehle *et al*., 2003); another study found both IGFBP2 and IGFBP5 in decreased levels in Type II diabetes mellitus patients, but only IGFBP2 in decreased levels in obese diabetic patients (Mohan *et al*., 1995). Further investigation of this locus may be important in elucidating the genetic relationship between obesity and osteoporosis.

(iv) *Conclusions*

We have shown that there is substantial genetic variation in a number of osteoporosis-related phenotypes in LGXSM RI strain panel. More importantly, the present study also indicates that genetic effects on obesity and osteoporosis are related to one another and likely map to many of the same QTLs. Thus, the LG/J-by-SM/J intercross mice provide a new model for studying normal genetic variation in the complex traits of obesity, osteoporosis and their interrelationships. Further study of populations derived from this intercross will allow us to more precisely define these relationships and identify the genetic variations responsible for them.

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