Growth of C57Bl/6 Mice and the Material and Mechanical Properties of Cortical Bone from the Tibia

J. M. Somerville,[1,2] R. M. Aspden,[1] K. E. Armour,[2] K. J. Armour,[2] D. M. Reid[2]

[1]Department of Orthopaedic Surgery, University of Aberdeen, IMS Building, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK
[2]Department of Medicine & Therapeutics, University of Aberdeen, IMS Building, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK

Abstract. Murine models are becoming increasingly important for studying skeletal growth and regulation because of the relative ease with which their genomes can be manipulated. This study measured the changes in cortical bone of tibiae from one of the more common models, the C57Bl/6, as a function of aging. A total of 97 mice, male and female, were studied at the ages of 1, 2, 3, 6, 9, and 12 months. The body weight of the animals, the length of the tibiae, the composition (in terms of mineral and organic mass fractions), and the density and modulus of the bone were measured. Peripheral quantitative computed tomography was also used to measure bone mineral density (BMD), total and cortical areas, and the cross-sectional moment of inertia. Most parameters measured followed a growth-like curve, which leveled off some time before 6 months of age. Bone composition and modulus were the same at maturity in both sexes, but there were sex-related differences in the modulus with aging. Dimensional measurements and the density of the bone showed significant differences between male and female animals at all ages, with the male mice having larger values. Skeletal maturity for most factors in C57Bl/6 mice has been reached before the age of 6 months.

Key words: Mouse — Bone — Growth — Material properties — Mechanical properties

Murine models are becoming increasingly important for studying growth, aging, and disease, where these can be difficult to investigate in human subjects. Targeted modification of gene expression in these murine models then enables the resulting effects on the skeleton to be investigated. For proper comparisons to be made, it is important to first know the normal pattern of development, and there are few studies of this in mice. Most lacking are studies of mechanical properties. The most

detailed study appears to be that by Brodt et al. [1] who measured the mechanical properties of femora from C57Bl/6 mice from the ages of 4 to 24 weeks (6 months). They showed that peak bone strength was not achieved before 20 weeks, although the ash fraction and dry density of the bone matrix from the corresponding tibiae did not change significantly throughout the study period. The increase in strength was attributed to changes in geometry, which were measured, and alterations in material properties, which were calculated from the mechanical test data and remain somewhat in conflict with the apparent lack of change in density and mineral content.

Other studies have compared geometric or histomorphometric properties at various time-points by using microcomputed tomography. In C57Bl/6 J mice, it was found that cancellous bone volume decreased continuously from the age of 6 weeks: a combination of decreasing trabecular number and increased trabecular spacing. However, trabecular thickness and cortical bone area both increased up to 6 months of age but then changed little [2]. Beamer et al. measured the bone density of several strains of mouse at 2, 4, 8, and 12 months, and they suggested that in all strains, peak bone density was achieved by 4 months, although the magnitude of that peak was different in each strain [3]. Some strain comparisons used only microcomputed tomographic (pQCT) measurements and did not extend beyond 6 months [4, 5], whereas others used only a single (16-week) time-point by using histomorphometric, biomechanical, and serum measurements [6].

This study investigates, in more detail, the growth of male and female C57Bl/6 mice by using laboratory measurements of density, modulus, and composition of cortical bone from the tibiae, as well as geometrical properties by using pQCT scanning. The study was designed to form the baseline for future studies comparing wild-type animals with those in which genetic alterations have been introduced.

Table 1. Number of mice used at each time-point

	Age (months)						
	1	2	3	6	9	12	Total
Male	11	8	13	7	6	3	48
Female	14	8	6	6	6	9	49

Materials and Methods

Animals

A colony of C57Bl/6 mice were kept in standard conditions in accordance with UK Home Office guidelines. Animals were euthanized at the ages of 1, 2, 3, 6, 9, and 12 months. The numbers of animals studied at each age and sex are shown in Table 1. The mice were weighed, and both tibiae were dissected and cleaned of soft tissue. The right tibiae were wrapped in tissue soaked in phosphate-buffered saline (PBS) and stored frozen at −20°C until measurements were made. Because this study concentrates on the biomechanical properties, only data from cortical bone of the diaphysis are reported. The left tibiae were fixed in 4% formaldehyde and subjected to pQCT scanning.

Material Properties

Before further preparation, the right tibiae were thawed overnight at room temperature. After the total length was measured by using a micrometer screw gauge, each tibia was divided with use of a mineralogical saw (Struers Accutom-2, Struers Ltd, Glasgow) fitted with an aluminium oxide cut-off wheel cooled with distilled water. First the proximal metaphysis was removed, as judged visually, cutting where the shaft became more uniform. An adjacent slice, approximately 2-mm thick, was then cut from the proximal diaphysis for ultrasonic measurement. The distal metaphysis was then removed, leaving the main part of the diaphysis for density and composition measurements. A syringe filled with PBS was used to wash the marrow from within the diaphysis.

The density of the diaphyseal cortical bone was measured by using Archimedes' principle [7, 8]. A small hole was drilled near one end of the bone with a 0.25-mm drill so that the bone could be suspended from a thin wire. A syringe was used to ensure that the marrow cavity of the bone was filled with distilled water, and then the bone was weighed while suspended in distilled water. This process was repeated several times to ensure consistency. If the marrow cavity was not filled with water, then the mass was considerably lighter, a result of buoyancy of the air within the medulla, and these readings were discounted. The immersed mass, m_b, was taken as the mean of the valid readings. The bone was then lightly dried using a dampened tissue to remove excess water from the surface, and a syringe was used to ensure that the marrow cavity was empty. Because the bone dehydrated very rapidly in air, the mass of each bone was determined by weighing the bone in a preweighed beaker of water. Again this process was repeated several times and the mean value of the bone mass calculated, m_a. The density was then found from $\rho = m_a\rho_b/(m_a − m_b)$, where ρ_b is the density of the distilled water.

To determine the composition of the bone, the specimens were dehydrated in predried crucibles at 105°C for 24 hours and then weighed again. The water content was calculated as the difference between the wet weight and the dry weight. The bone specimens were then ashed at 600°C for 24 hours and reweighed to determine the ash weight, which was taken to be the mineral content. The organic content was calculated by subtracting the ash weight from the dry weight. The mass of each component was then expressed as a fraction of the wet

mass of the sample. Mineral mass fractions, for comparison with pQCT data, were calculated from the mass of mineral and the bone volume, as determined by using Archimedes' principle as mentioned previously.

An ultrasonic method was used to determine the elastic modulus of the bone in the 2-mm slice that was cut from the end of the diaphysis [9–11]. Ultrasound, of 10 MHz frequency, was obtained using a pulsar/receiver (Model 5052 PR, Panametrics Inc) and a straight-beam contact transducer (Panametrics Inc.). Each specimen was pressed firmly onto the wear plate of the transducer with a drop of distilled water to ensure good acoustic coupling. In this method, pulses of ultrasound are transmitted through the sample and the transit time, T, of the pulses measured by a "pitch-and-catch" method using a separate transmitter and receiver and a dual-beam oscilloscope (Hitachi V-665A, Japan). The thickness, d, of the sample was measured by using an electronic micrometer screw-gauge (Mitutoya, RS Ltd, Corby, Northamptonshire). The longitudinal sonic plesio-velocity was calculated using $v = d/T$. The effective path length for sound in an anisotropic, inhomogeneous medium, such as bone, is not known, and will generally be longer than the specimen. The estimation of longitudinal velocity will, therefore, probably be an underestimate and has been termed the plesiovelocity [12]. The longitudinal elastic modulus, E, was calculated from the formula $E = \rho v^2$, where ρ is the density of the specimen [12].

Computed Tomography

Left tibial bones were scanned in a Stratec XCT Research M pQCT densitometer (Stratec Medizintechnik, Pforzheim, Germany) using a pixel size of 100×100 μm^2, a slice thickness of 0.5 mm, and software version 5.14. Care was taken to insert all of the bones into the scanner in the same orientation. A scout view of the length of each bone was obtained so that the exact position of the transverse diaphyseal scan could be located, which was obtained from halfway along the length of the whole bone. From this image, the total cross-sectional area, the cross-sectional area of the cortical bone, and the polar cross-sectional moment of inertia (CSMI) were calculated. The polar moment was used in this study, as it is a function of the moments of inertia about the perpendicular axes and with its use one can avoid defining a bending axis in the bone. The volumetric bone mineral density ("cortical density") was determined by using a bone threshold of 570 mg/cm^3 to identity the cortex. The same threshold was used for all samples to ensure that the same density of bone would be detected in all samples.

Repeatability

Repeatability of the density and speed-of-sound measurements was assessed by making duplicate measurements on 21 samples. The standard deviation of the differences between the pairs of measurements was determined, and the coefficient of repeatability taken to be twice this figure [13]. Repeatability of the pQCT measurement was assessed by measuring the bones from the 1 month and 12 month groups twice without repositioning. Repeatability was determined in each case using analysis of variance (ANOVA) of the differences between repeated measurements and expressed as 95% confidence limits [13].

Statistical Analyses

Comparisons between the sexes and ages of mice were performed using ANOVA. This was performed on ranked data if the raw data were not normally distributed. Normality of the distributions was assessed using a Kolmogorov-Smirnov test with the significance level set at 0.05. Post hoc pair-wise comparisons were made using Tukey's test. Growth curves were fitted using a three-parameter exponential "growth to a maximum" equation of the form $y = y_0 + a(1 − \exp(−bx))$, where
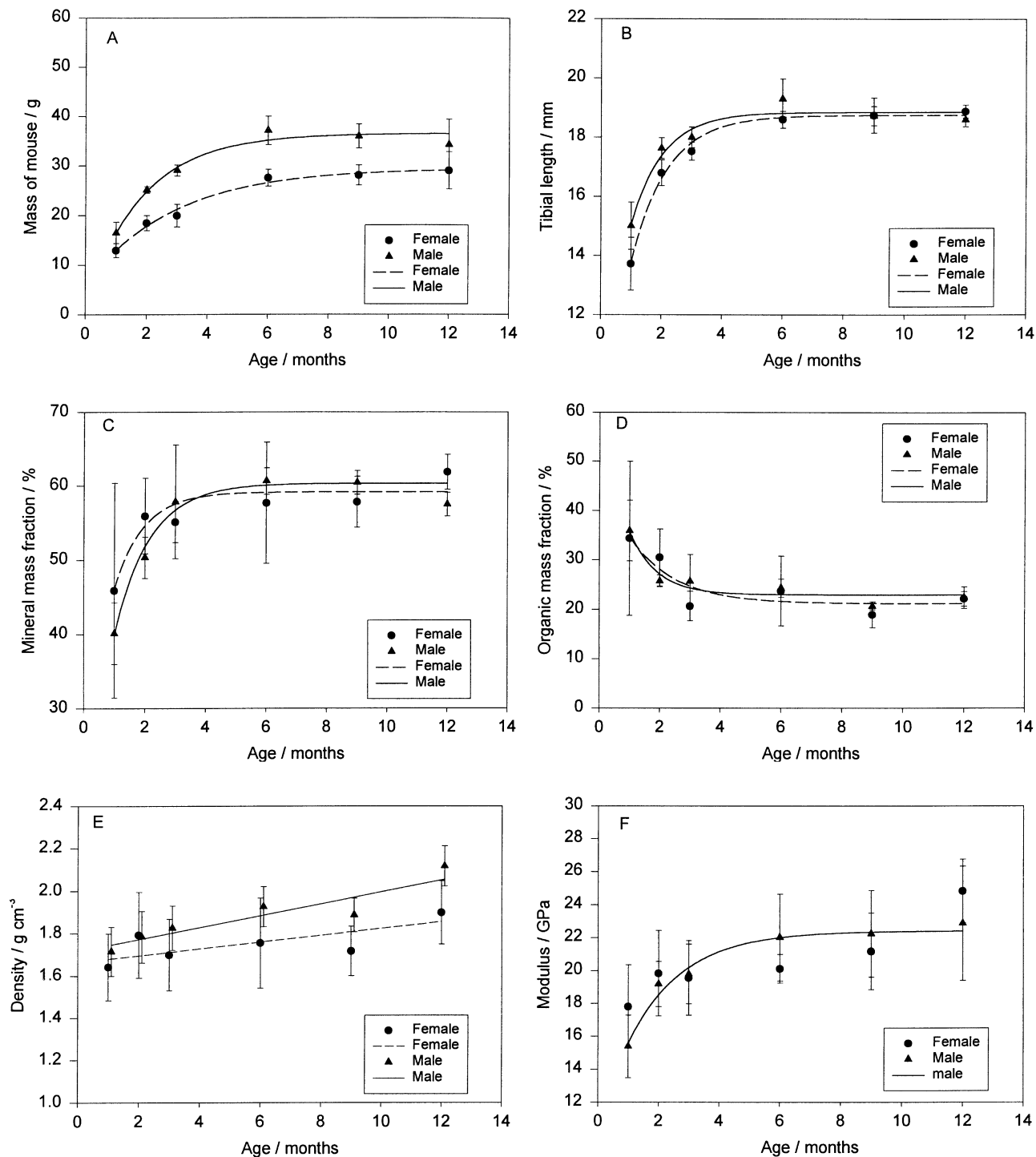
Fig. 1. Growth (mean and standard deviation) of male and female C57Bl/6 mice results in an increase in (**A**) the mass of the animal and (**B**) the length of the tibia. Within the tibia there were changes in (**C**) the mass fraction of mineral, (**D**) the mass fraction of organic material (both expressed as fractions of the wet mass), (**E**) the density, and (**F**) the modulus of the bone matrix which were different in male and female animals.

y_0, a, and b were determined by the fitting procedure. Although this is a plausible growth curve, six time-points are not adequate to properly describe such a model with three coefficients, and standard errors on the exponent were very large. Hence the growth curves were used more to indicate the growth pattern than for quantitative purposes. R^2 values are shown for males R^2_m and females R^2_f to indicate the goodness of fit. Either no curve or linear regression was used where appropriate.

Results

Growth of C57Bl/6

Male C57Bl/6 mice were heavier than the corresponding female mice, (two-way ANOVA, $P < 0.001$) (Fig. 1A, $R^2_m = 0.91$, $R^2_f = 0.89$). Similarly, tibial lengths were

Table 2. Values of parameters (mean ± standard deviation) for mature C57Bl/6 mice derived from the mean value over the 6, 9, and 12-month time-points. Significance values are those obtained by ANOVA for all time-points after allowing for the effects of age

Parameter	Male	Female	P
Mass (g)	36.2 ± 3.2	28.4 ± 2.8	< 0.001
Tibial length (mm)	18.93 ± 0.56	18.75 ± 0.38	< 0.001
Density (g/cm^3)	1.95 ± 0.12	1.80 ± 0.17	< 0.001
Modulus/GPa	22.3 ± 2.6	23.2 ± 2.8	0.55
Mineral%/wet mass	60.0 ± 2.0	59.4 ± 5.2	0.099
Organic%/wet mass	22.6 ± 2.3	21.7 ± 4.4	0.11

Fig. 2. The relationship between modulus and density was not strong and was similar in male and female animals. The regression lines show that, on average, for a given density, the female mice have a higher modulus.

greater in the male than the female mice ($P < 0.001$) (Fig. 1B, $R^2_m = 0.87$, $R^2_f = 0.93$). There was a significant interaction between age and sex, with the female mice growing relatively more slowly than the male mice in the early months, but both mass and tibial length appeared to reach their maximum by about 6 months of age for both sexes. Similar trends were seen in the mineral and organic mass fractions (Fig. 1C, $R^2_m = 0.74$, $R^2_f = 0.31$ and D, $R^2_m = 0.58$, $R^2_f = 0.29$); mineral content increased and organic content decreased, and both appeared to have reached a steady value by about 3 to 4 months of age. There was no significant difference in the organic content of the male and female tibiae after controlling for age, although an interaction between age and sex showed that the mineral mass fraction in the male mice was significantly ($P < 0.05$) lower than in the female mice at 1, 2, and 12 months of age.

This pattern was not fully reflected in the density or modulus (Fig. 1E and F). The density of the bone showed a significant difference with age and sex (both $P < 0.001$, two-way ANOVA). However, pair-wise comparisons showed that only the 12-month-old animals had a density that was significantly greater than the densities at 1, 2, 3, and 9 months. Linear regression was deemed to fit better to the data in Fig. 1E but the correlations were low, $R^2_m = 0.41$ and $R^2_f = 0.13$. Despite the increase in mineral content—expressed as a fraction of the dry mass—density and mineral content were poorly correlated. The modulus of the bone was found to vary significantly with age ($P < 0.001$), but there was no significant difference between the sexes ($P = 0.55$). The bone modulus in the male mice appeared to follow a growth-like increase with age in a similar way to, the properties described previously ($R^2_m = 0.59$). However, inspection of Fig. 1F does not show such a clear pattern for the female mice, and no attempt was made to fit a curve to the data. The mature values of all the parameters are shown in Table 2.

Linear correlations with similar gradients were found between modulus and density for both male and female mice, but with the female mice having a greater modulus than the males for a given density (Fig. 2). The gradients
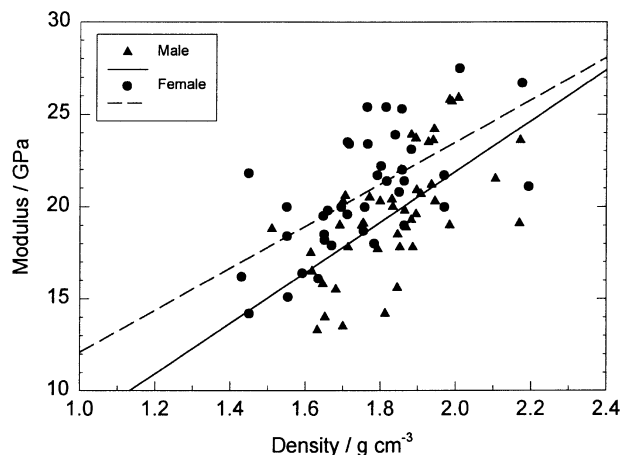
and correlation coefficients were 13.8 GPa cm^3 g^{-1} and $R^2_{adj} = 0.37$ for the male mice, and 14.1 GPa cm^3 g^{-1} and $R^2_{adj} = 0.44$ for the female mice, and the residuals were normally distributed in both cases, showing that a linear fit is adequate.

pQCT Measurements

Measurements were made from a single slice at the center of the diaphysis. There was an increase in volumetric bone mineral density (BMD) that was similar to the growth pattern described previously, the peak being reached before the age of 6 months (Fig. 3A, $R^2_m = 0.94$, $R^2_f = 0.90$), and there was no difference between male and female mice. Sex differences were found, though, for total and cortical cross-sectional areas (Fig. 3B, $R^2_m = 0.89$, $R^2_f = 0.89$ and C, $R^2_m = 0.92$, $R^2_f = 0.92$) and for the cross-sectional moment of inertia (Fig. 3D, $R^2_m = 0.85$, $R^2_f = 0.88$) (all $P < 0.001$) with male mice always having greater values than females. For both sexes, peak values were achieved by 3 months of age or soon after, and the stable, mature values are given in Table 3.

Repeatability

The repeatability of the measurements was determined as described. That for the density measurements was 0.28 g cm^3 and for the modulus was 3.36 GPa. The repeatability for total mineral density, total area, and CSMI as measured by pQCT are 22.6 mg/cm^3, 0.10 mm^2, and 0.02 mm^4, respectively.

Discussion

The objective of this study was to describe in more detail the age-related maturation of the mechanical and ma-
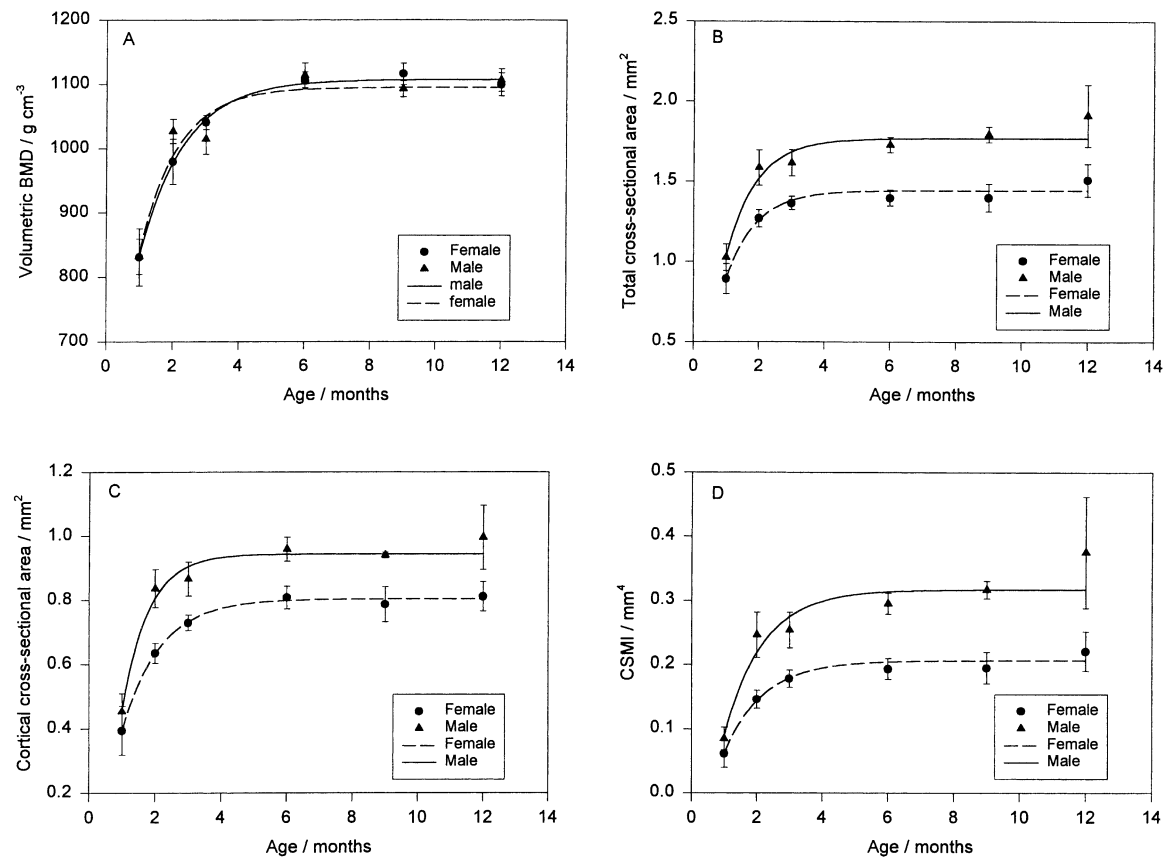
Fig. 3. Peripheral QCT measurements (mean and standard deviation) from the midshaft of the tibia. (**A**) Volumetric BMD, (**B**) total cross-sectional area, (**C**) cortical cross-sectional area, and (**D**) the cross-sectional moment of inertia.

Table 3. Values of parameters (mean $\pm$ standard deviation) for mature C57Bl6 mice derived from the values over the 6, 9, and 12 month time-points

	Male	Female	P
vBMD (g/cm³)	1103 $\pm$ 18	1105 $\pm$ 17	0.69
Total cross-sectional area (mm²)	1.79 $\pm$ 0.11	1.45 $\pm$ 0.10	< 0.001
Cortical cross-sectional area (mm²)	0.958 $\pm$ 0.049	0.804 $\pm$ 0.045	< 0.001
CSMI (mm⁴)	0.320 $\pm$ 0.046	0.205 $\pm$ 0.028	< 0.001

CSMI, cross-sectional moment of inertia; vBMD, volumetric bone mineral density

terial properties of cortical bone in C57Bl/6 mice, which have become one of the standard laboratory models for studies of bone. Nearly all the parameters measured from the bones reached their maximum value at between 3 and 6 months of age, probably earlier in this time span rather than later, but it is not possible to be more precise given the study design. The body weight of the mice followed the same pattern. These data would support those of Beamer et al. [3] which indicated skeletal maturity being reached at about 4 months.

The exceptions to this growth pattern were the density of the bone, in animals of both sexes, and the modulus in the female animals. The density of the bone in both male and female mice increased slowly over the whole study period, though slightly more rapidly in the

male animals. This was not reflected in the modulus of the bone which, in the male mice, showed a similar increase to that of the other parameters and, in the females, seemed still to be increasing even at 12 months. Mineral content might be expected to be one of the major factors determining the density of the tissue, given that its density is approximately three times greater than the other components, but the correlations between density and mineral mass fraction, either of the hydrated material or as a fraction of the dried matrix, were low. This suggests that the packing of the mineral within the matrix plays an important part and not simply the amount of mineral present. Similarly, the correlations between modulus and density were low, indicating that the stiffness of the material is dependent on the nature of
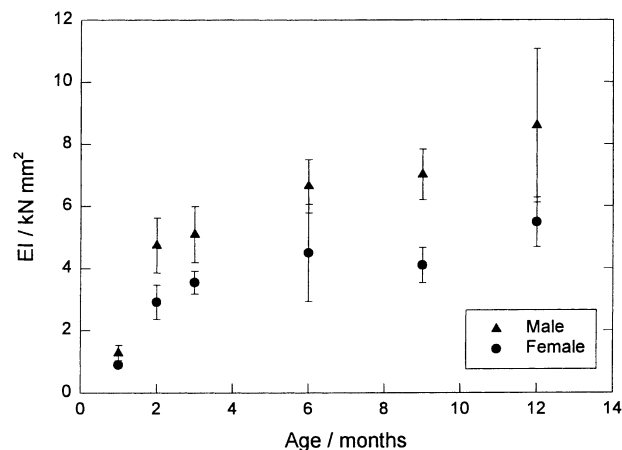
Fig. 4. Estimation of the torsional rigidity (mean and standard deviation), *EI*, of the tibiae derived from the product of the modulus, *E*, and the CSMI, *I*, suggests that it continues to increase throughout the study period, in contrast to other measures, which indicate that maturity has been reached.

the organic fraction and interactions between the matrix components which are not reflected in simple composition and density measurements. These results are qualitatively similar to those found by Brodt et al. [1] who measured marked changes in stiffness and strength of femora over a period of 4 months, while there were no significant changes in ash content or dry density. One possible factor underlying these findings could be changes in collagen cross-linking. Maturation of reducible crosslinks into more stable nonreducible forms is known to increase the stiffness and strength of bone [14] and, in humans, Eyre et al. [15] showed that the content of the mature form of cross-link reaches a maximum by about 10 to 15 years of age before stabilizing at about that value [5]. A reduction in collagen solubility that would correspond to this has been reported in rats during maturation [16]. It is not unreasonable to hypothesize that at least some of the unexplained variance is due to a similar maturation of collagen within the matrix, which was not measured in this study. The source of the greater variability in the mineral and organic fractions, as reflected in the lower correlation coefficients, is not clear. It must be stressed, though, that these curve fits are only approximate models and it may be that there is no real significance in these values.

Growth-like patterns with aging were also shown by all the pQCT measurements; rising sharply over the first 3 to 6 months before reaching a stable value. This pattern is similar to that found for tibial length and shows a general increase in geometrical dimensions during growth to skeletal maturity. The volumetric BMD measurements also follow the same pattern and reflect the increase in mineral content shown in Fig. 1C rather than the material density of the bone. This is not surprising given that it is an absorptiometric measurement governed by mineral content rather than a true density. The cortical cross-sectional area, expressed as a fraction of the total bone cross-sectional area, increases during growth from about 44% to peak at about 55% at 6 months before decreasing slightly, indicating that the cortex occupies an increasing fraction of the total bone cross-section during growth. This is at odds with what is observed in humans in which periosteal expansion and endosteal resorption result in a smaller fraction of cortical bone area, though an increase in strength [17]. This may indicate a different modeling process during growth in these mice and would suggest that this may be a way of rapidly increasing strength during growth.

The torsional rigidity of the bone is given by the product of the modulus and the cross-sectional moment of inertia, *EI*. By using the modulus values measured previously and the corresponding CSMI obtained from the mid-diaphysis from the pQCT measurements this can be estimated. It is of interest to note that although the individual measurements change only slowly once maturity is reached it, can be seen from Fig. 4 that this growth continues to increase throughout the study period, although given that there were only three male animals at the 12-month time-point, caution must be exercised in interpreting this result. These results differ from the pattern of torsional rigidities measured by Brodt et al. in mouse femora that appeared to stop increasing at about 4 months [1]. Because this result is a product of the two values, small increases in each parameter, which are not significant and are stable as far as the curve fitting is concerned, can be seen in Figures 1F and 3D, and these increases are magnified in this parameter. In the female mice this *EI* also increased linearly with body weight, with an R^2 of 0.66, although in the male mice this was only true for the data up to 6 months of age for which R^2 was 0.78. After this, body weight decreased slightly while *EI* continued to increase.

In summary we have shown that skeletal maturity for most factors in C57Bl/6 mice has occurred before the age of 6 months. The female mice grew more slowly than the male mice and were smaller, but there were no differences between modulus or volumetric BMD, although the modulus did not appear to follow the usual growth curve in the females. There was a difference in behavior of bone mineral mass fraction and density, however, between males and females. Because of these differences between the sexes, both in magnitude and in change with age, the use of only one sex in future experiments is advisable to avoid complications arising from this source.

References

1. Brodt MD, Ellis CB, Silva MJ (1999) Growing C57Bl/6 nice increase whole bone mechanical properties by increasing geometric and material properties. J Bone Miner Res 14:2159–2166
2. Halloran BP, Ferguson VL, Simske SJ, Burghardt A, Venton LL, et al. (2002) Changes in bone structure and mass with advancing age in the male C57BL/6 J mouse. J Bone Miner Res 7:1044–1050
3. Beamer WG, Donahue LR, Rosen CJ, Baylink DJ (1996) Genetic variability in adult bone density among inbred strains of mice. Bone 18:397–403
4. Richman C, Kutilek S, Miyakoshi N, Srivastava AK, Beamer WG, et al. (2001) Postnatal and pubertal skeletal changes contribute predominantly to the differences in peak bone density between C3H/HeJ and C57BL/6 J mice. J Bone Miner Res 16:386–397
5. Sheng MH, Baylink DJ, Beamer WG, Donahue LR, Rosen CJ, et al. (1999) Histomorphometric studies show that bone formation and bone mineral apposition rates are greater in C3H/HeJ (high-density) than C57BL/6 J (low-density) mice during growth. Bone 25:421–429
6. Akhter MP, Iwaniec UT, Covey MA, Cullen DM, Kimmel DB, et al. (2000) Genetic variations in bone density, histomorphometry, and strength in mice. Calcif Tissue Int 67:337–344
7. Ashman RB (1989) Experimental techniques. In: Cowin SC (ed) Bone mechanics. CRC Press, Boca Raton, pp 75–95
8. Li B, Aspden RM (1998) A comparison of the stiffness, density and composition of bone from the calcar femorale and the femoral cortex. J Mater Sci Mater Med 9:661–666
9. Lees S, Ahern JM, Leonard M (1983) Parameters influencing the sonic velocity in compact calcified tissues of various species. J Acoust Soc Am. 74:28–33
10. Li B, Aspden RM (1997) Material properties of bone from the femoral neck and the calcar femorale of patients with osteoporosis or osteoarthritis. Osteoporos Int 7:450–456
11. Rho JY, Ashman RB, Turner CH (1993) Young's modulus of trabecular and cortical bone material: ultrasonic and microtensile measurements. J Biomech 26:111–119
12. Lees S, Heeley JD, Cleary PF (1979) A study of some properties of a sample of bovine cortical bone using ultrasound. Calcif Tissue Int 29:107–117
13. Bland JM, Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 8476:307–310
14. Bailey AJ, Paul RG, Knott L (1998) Mechanisms of maturation and ageing of collagen (review). Mech Age Dev 106:1–56
15. Eyre DR, Dickson IR, Van Ness K (1988) Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxypyridinium residues. Biochem J 252:495–500
16. Vogel HG (1979) Influence of maturation and aging on mechanical and biochemical parameters of rat bone. Gerontology 25:16–23
17. Jee WS (2001) Integrated bone tissue physiology: anatomy and physiology. In: Cowin SC (ed) Bone mechanics handbook. CRC Press, Boca Raton, pp 1–68