Genetic Variations and Physical Activity as Determinants of Limb Bone Morphology: An Experimental Approach Using a Mouse Model

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KEY WORDS bone functional adaptation; experimental evolution; genes; voluntary exercise

ABSTRACT To gain insight into past human physical activity, anthropologists often infer functional loading history from the morphology of limb bone remains. It is assumed that, during life, loading had a positive, dose-dependent effect on bone structure that can be identified despite other effects. Here, we investigate the effects of genetic background and functional loading on limb bones using mice from an artificial selection experiment for high levels of voluntary wheel running. Growing males from four replicate high runner (HR) lines and four replicate nonselected control (C) lines were either allowed or denied wheel access for 2 months. Using μCT, femoral morphology was assessed at two cortical sites (mid-diaphysis, distal metaphysis) and one trabecular site (distal metaphysis). We found that genetic differences between the linetypes (HR vs. C), between the replicate lines within linetype, and between individuals with and without the so-called "mini-muscle" phenotype (caused by a Mendelian recessive gene that halves limb muscle mass) gave rise to significant variation in nearly all morphological indices examined. Wheel access also influenced femoral morphology, although the functional response did not generally result in enhanced structure. Exercise caused moderate periosteal enlargement, but relatively greater endocortical expansion, resulting in significantly thinner cortices and reduced bone area in the metaphysis. The magnitude of the response was independent of distance run. Mid-diaphyseal bone area and area moments, and trabecular morphology, were unaffected by exercise. These results underscore the strong influence of genetics on bone structure and the complexity by which mechanical stimuli may cause alterations in it. Am J Phys Anthropol 148:24–35, 2012.

Investigations of past human activity patterns often proceed from the premise that limb bone morphology reflects functional loading history (e.g., Ruff et al., 1984, 1993; Bridges, 1989; Trinkaus, 1997; Holt, 2003; Marchi et al., 2006, 2011; Shackelford, 2007; Maggiano et al., 2008; Stock et al., 2010). Limb bones are seen by some as mechanically optimized structures of maximal strength and minimal mass, modulated by functional adaptation (*sensu* Roux, 1881). Bones whose size and shape suggest enhanced mechanical strength are assumed to have been loaded vigorously during life, whereas bones that appear more fragile are assumed to have sustained lower levels of loading. The validity of this paradigm has been a source of controversy in anthropology (Demes et al., 1998; Pearson, 2000; Lovejoy et al., 2003; Lieberman et al., 2004; Pearson and Lieberman, 2004; Ruff et al., 2006; Demes, 2007; Schmitt et al., 2010).

That bone cells can respond to changes in the mechanical environment of the skeleton is well documented (Goodship and Cunningham, 2001; Ozcivici et al., 2010). Numerous controlled experiments utilizing animal models have demonstrated the potential for activities involving limb loading—for example, running—to promote bone formation, retard bone loss, and, ultimately, enhance structure and strength (e.g., Woo et al., 1981; Newhall et al., 1991; Biewener and Bertram, 1994; Lieberman, 1996; Judex et al., 1997; Joo et al., 2003; Hamrick et al., 2006a; Plochocki et al., 2008; Barak et al., 2011), providing support for the paradigm within which anthropologists often interpret human limb bone morphology. However, some studies have found running to have little or no effect on limb bones (Judex and Zernicke, 2000; Notomi et al., 2000; Middleton et al., 2008a, 2010), and a few studies have even documented negative effects of "strenuous" running regimes (Matsuda et al., 1986; Li et al., 1991; Bourrin et al., 1994; see also Ma et al., 2010, 2011), highlighting the fact that the relationship between limb loading and bone physiology is complex. In recent decades, knowledge of the complexity of the process of bone mechanotransduction has increased dramatically, largely as a result of biomedical studies seeking to develop effective ways to maintain adequate

Grant sponsor: L.S.B. Leakey Foundation. Grant sponsor: National Institute of Arthritis and Musculoskeletal and Skin Diseases. Grant sponsor: National Science Foundation.

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Received 5 July 2011; accepted 3 January 2012

DOI 10.1002/ajpa.22028
Published online 13 February 2012 in Wiley Online Library (wileyonlinelibrary.com).

bone strength. The responsiveness of bone to altered mechanical signals can depend on an individual's age (Rubin et al., 1992; Pearson and Lieberman, 2004), genetic background (Judex et al., 2002, 2004a; Robling and Turner, 2002), hormonal status (Devlin and Lieberman, 2007; Devlin, 2011), and sex (Squire et al., 2004; Robling et al., 2007). The effects of changes in load bearing can also vary according to the bone's proximodistal location in the limb (Lieberman et al., 2003; Plochocki et al., 2008), the specific site in the bone (Hsieh et al., 2001; Judex et al., 2004a), and the type of bone, i.e., cortical or trabecular (Rubin et al., 2002; Judex et al., 2004a). In addition, several aspects of the mechanical milieu influence responsiveness, including load magnitude (Rubin and Lanyon, 1985), rate (O'Connor et al., 1982), cycle number (Rubin and Lanyon, 1984a), and frequency (Qin et al., 1998; Rubin et al., 2001).

In addition to mechanical signals, limb bone morphology is influenced by many nonmechanical factors, particularly genetics, whose effects alter the degree to which bones reflect loading history. Genetic factors have been estimated to account for 27% to over 50% of the variation in limb bone size and shape among individual humans within a population (Demissie et al., 2007; Havill et al., 2007). Genetic regulation of limb bone morphology involves numerous genes (Liu et al., 2003; Ralston and de Crombrugghe, 2006; Farber et al., 2011), is sex-specific (Ioannidis et al., 2007), and is highly site-specific, even within a given bone (Judex et al., 2004b). The influence of genetics on limb bone morphology is clearly demonstrated by studies of inbred mouse strains, which show that polymorphisms affect major differences in bone structure, despite ostensibly similar mechanical signals engendered by normal cage activity (Beamer et al., 1996; Akhter et al., 2000; Jepsen et al., 2003; Judex et al., 2004b; Wergedal et al., 2005). That interstrain differences in bone morphology are evident during perinatal development, before mice begin functionally loading their limbs, further highlights the impact of genetics on bone phenotypes (Price et al., 2005; Jepsen et al., 2009; see also Wallace et al., 2010). Similarly, in a recent study of the developmental trajectories of limb bone diaphyseal dimensions in seven human samples from geographically and temporally dispersed contexts, Cowgill (2010) found that some populational differences emerge prior to one year of age, before the onset of walking (Stanitski et al., 2000) and when infants are likely carried (Tracer, 2009). Although nongenetic factors (e.g., differences in diet) inevitably contributed to this variation, it is not unreasonable to speculate that genetic differences among populations were the predominant cause for the morphological disparity.

Here, we examine the relative influence of genetics and functional loading on limb bone morphology using mice from a long-term selective breeding experiment for high levels of voluntary wheel running (Swallow et al., 1998; Garland, 2003). The selection protocol (described below) began with a base population of genetically heterogeneous mice from which eight closed lines were established, four replicate selected high runner (HR) lines and four nonselected control (C) lines. By generation 16, selection resulted in a ~2.7-fold increase in daily running distance by the HR lines as compared with the C lines. Mice from these lines are the subject of an extensive body of research (reviewed in Garland, 2003; Rhodes et al., 2005; Middleton et al., 2008b; Swallow et al., 2009), including several studies of limb bone structure and mechanics (Garland and Freeman, 2005; Kelly et al., 2006; Middleton et al., 2008a,b, 2010; Young et al., 2009; Wallace et al., 2010). For this study, to examine the effects of increased functional loading on limb bone morphology, mice from generation 21 (second litters) were either allowed or denied wheel access for 2 months, beginning shortly after weaning. Possible dose relationships between distance run and bone response were assessed using the wheel-access mice. To examine the effects of genetic changes due to selective breeding, comparisons were made between mice from the two linetypes (HR vs. C). Significant linetype effects were presumed to reflect the existence of pleiotropic gene action (i.e., genes that affect both running behavior and bone development) (Garland, 2003). We also tested for differences among the replicate lines within the linetypes to examine the effects of such chance genetic events as random drift, founder effects, and mutations unique to particular lines, as well as "multiple solutions" (Garland, 2003; Garland et al., 2011a). To examine the degree to which genetic changes affected the response of bone to mechanical loading, we tested for statistical interactions between linetype and wheel access, as well as replicate line and wheel access. Finally, we investigated the effects of a naturally occurring Mendelian recessive allele, named "mini muscle" (MM), that causes an ~50% reduction in hind limb muscle mass (Garland et al., 2002; Houle-Leroy et al., 2003). Two of the HR lines exhibit a high frequency of the MM allele.

We asked two general questions. First, do genetic differences between linetypes, among replicate lines, or among individuals (MM vs. non-MM) play a major role in determining limb bone morphology? Second, does limb bone morphology reflect functional loading history, such that bone geometric properties are (a) enhanced in the mice who engaged in voluntary running exercise compared with the sedentary mice and (b) positively correlated with distance run at the level of individual variation for the wheel-access mice? If exercise loading has a positive, dose-dependent effect on bone structure, and genetic variations have little effect, then the results would support the assertion that functional loading history can be inferred from limb bone morphology. However, if bone structure is strongly influenced by genetic background, and/or the effects of exercise loading are negligible or negative, then the results would suggest that prudence is necessary when using limb bone remains to glean information about past levels of physical activity.

MATERIALS AND METHODS

The selection experiment

The complete design of the selection experiment for high voluntary wheel running in mice has been described elsewhere (Swallow et al., 1998; Garland, 2003), and only a summary is provided here. The eight lines involved in the experiment are descended from a base population of outbred mice of the Hsd:ICR stock (Harlan Sprague Dawley, Indianapolis, IN). Each line is maintained with 10 or more breeding pairs per generation. In each successive generation, when the offspring of the pairs are ~6–8 weeks of age, they are housed individually with access to a running wheel for 6 days. Wheel running is monitored with a computer-automated system. The selection criterion is the total number of revolutions on the last 2 days of the 6-day trial. In the

four HR lines, the highest-running male and female from each family are chosen as breeders to propagate the next generation. In the four nonselected C lines, breeders are randomly chosen from each family. Within the lines, chosen breeders are randomly paired, except that sibling mating is not allowed.

The dramatic increase in daily wheel running in the HR lines has been associated with numerous correlated responses to selection (Garland et al., 2011b), including changes in limb bone morphology. Relative to controls, HR mice have larger femoral heads (Garland and Freeman, 2005; Kelly et al., 2006; Middleton et al., 2008a), wider distal femoral condyles (Middleton et al., 2008a), reduced directional asymmetry in hind limb lengths (Garland and Freeman, 2005), and mediolaterally wider femoral and tibial mid-diaphyses (Kelly et al., 2006). Additional changes include increased home-cage activity when housed without wheels (Malisch et al., 2008, 2009), decreased body mass and fat content (Swallow et al., 1999, 2001; Morgan et al., 2003; Nehrenberg et al., 2009), decreased levels of circulating leptin (especially or possibly only in females; Girard et al., 2007; Vaanholt et al., 2007, 2008), and altered signaling in the cannabinoid receptor CB1 pathway (Keeney et al., 2008, and unpublished results), all of which potentially influence skeletal physiology.

The MM allele, which halves hind limb muscle mass, was originally present at low frequency in the base population, and subsequently increased in frequency in two of the HR lines (laboratory designation lines 3 and 6; Garland et al., 2002). The MM allele has been mapped to a 2.6-Mb region of mouse chromosome 11 (Hartmann et al., 2008). Pleiotropic effects include altered muscle contractile physiology (Houle-Leroy et al., 2003; Syme et al., 2005; Guderley et al., 2006, 2008) and reduced hind limb bone diaphyseal diameters (Kelly et al., 2006). Significant effects of the MM allele on limb bone morphology are expected, given that muscle mass and bone structure are strongly correlated throughout development, although the mechanisms responsible for this association remain unresolved (Judex and Carlson, 2009).

Study design

The animals used in this study were from generation 21 (second litters), as described in Kelly et al. (2006). The pups were weaned at 21 days and toe-clipped for identification. At 25–28 days, two males from each of five families in each line ($n = 80$) were housed individually for 8–9 weeks in standard cages, half with and half without access to a running wheel (1.12-m circumference, 10-cm-wide running surface of 10-mm wire mesh; Lafayette Instruments, Lafayette, IN). Thus, four groups were established: HR with wheels, HR without wheels, C with wheels, and C without wheels ($n = 20$ per group) (Fig. 1). Families were dispersed evenly across the four groups. Mice were maintained on a 12:12-h light–dark cycle with access to food (Rodent Diet [W] 8604, Harlan Tekland, Madison, WI) and water ad libitum. A computerized counting system recorded daily wheel revolutions. By the end of the first month of wheel access, HR and C mice were voluntarily running, on average, more than 13 and 5 km/day, respectively (see Kelly et al., 2006, for analyses of wheel-running data). During the second month, average daily running distance declined slightly in HR mice to 11 km, but increased in C mice to 6 km. After the 8–9 week experimental period, mice were sacrificed via CO_2 inhalation and body mass was measured. Mean age at sacrifice was 86 days (range = 80–91). Triceps surae muscles were dissected and weighed to identify the MM mice (line 3: $n = 3$, 2 with wheels; line 6: $n = 6$, 3 with wheels), as described in Garland et al. (2002). Carcasses were skinned and eviscerated, air-dried, and placed in a colony of dermestid beetles. Defleshed limb bones were disarticulated manually as necessary. All procedures were reviewed and approved by the University of Wisconsin-Madison IACUC.

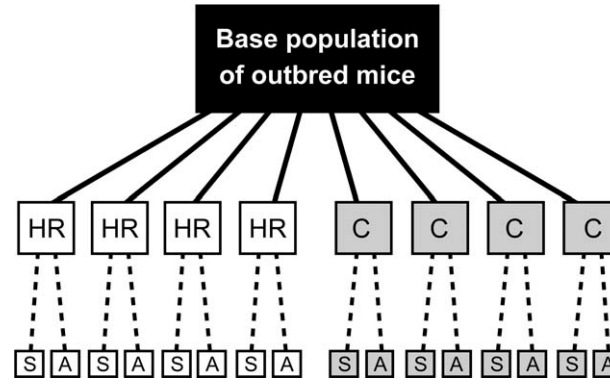

Fig. 1. Schematic representation illustrating how the groups of mice employed in the study were established. Solid lines represent 21 generations of either selective breeding for high levels of voluntary wheel running (four replicate high runner or HR lines) or breeding without regard to amount of wheel running (four replicate control or C lines). Dashed lines represent assignment of individuals within each of the eight lines to either a sedentary (S, no access to a wheel) or active (A, cages attached to wheels) lifestyle.

Microcomputed tomography

Cortical and trabecular bone morphology were assessed in the left femur by microcomputed tomography (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland). A 600-μm-long region of the diaphysis and a 1500-μm-long region of the distal metaphysis were scanned at a resolution of 12 μm (55 kV, 145 μA, and 300-ms integration time). The diaphyseal volume of interest was centered at midspan between the growth plates and encompassed only cortical bone. The metaphyseal volume of interest started 600 μm proximal of the physeal-metaphyseal boundary and encompassed both cortical and trabecular bone (Fig. 2). Micro-CT images were calibrated using hydroxyapatite phantoms (Scanco Medical AG). Volumes were segmented using a constrained 3D Gaussian filter to reduce noise (support = 1, sigma = 0.1 [diaphysis] and 0.5 [metaphysis]) and thresholded to extract the bone phase. The threshold values chosen for cortical and trabecular bone (400 and 295, respectively) were determined empirically to achieve maximal concordance between the raw and thresholded images. Repeatability of this thresholding method is high (Judex et al., 2004b). Importantly, the same thresholds were used for all bones so as to not introduce bias. In the metaphysis, cortical and trabecular bone were separated using an automated algorithm (Lublinsky et al., 2007).

Bone properties were computed using the internal imaging code supplied by the scanner manufacturer.

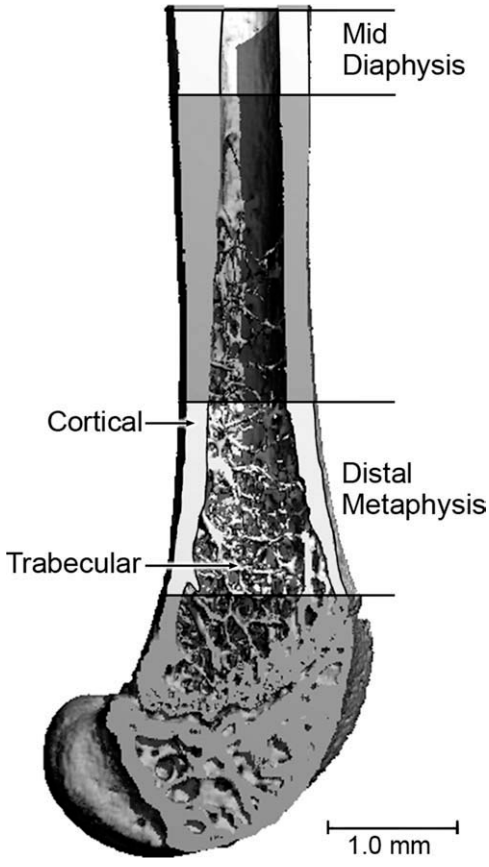

Fig. 2. Three-dimensional reconstruction of the femur of a HR mouse showing the regions in the diaphysis and distal metaphysis that were analyzed using μCT. The diaphyseal region contained only cortical bone and the metaphyseal region contained both cortical and trabecular bone.

Cortical bone morphometric traits included cortical bone area (Ct.Ar) and thickness (Ct.Th), periosteal (Ps.Ar) and endocortical areas (Ec.Ar), and polar moment of area (J). Values were averaged over the length of each region. Trabecular bone traits included bone volume fraction (BV/TV), and trabecular number (Tb.N), thickness (Tb.Th), and spacing (Tb.Sp). Of the morphometric properties measured, the most relevant in terms of the mechanical performance of the bone shaft are cortical bone area and polar moment of area. Cortical bone area approximates a cross section's rigidity in axial loading, and polar moment of area approximates average bending and torsional rigidity, assuming that the material strength of the bone tissue is invariable (Ruff et al., 1993). In the metaphysis, trabecular bone contributes to shaft strength, but its mechanical function is likely limited (Glatt et al., 2007). Although the focus of this study is bone morphology and not tissue composition, we also quantified tissue mineral density (TMD)—the primary determinant of tissue strength (Currey, 2002)—at each site analyzed using hydroxyapatite phantoms.

Statistical analyses

Analysis of covariance (ANCOVA) models with Type III tests of fixed effects were applied using SAS procedure mixed (version 9.2, SAS Institute, Cary, NC). Traits were first analyzed by cross-nested, two-way ANCOVA with linetype (HR vs. C) and activity (wheel vs. no wheel) as the primary grouping factors. Line was nested within linetype, and family was nested within line. Linetype and activity were considered fixed effects, while line and family were considered random effects. The effect of linetype was tested over the effect of line, and the effects of activity and the linetype $\times$ activity interaction were tested over the line $\times$ activity interaction. Presence of the MM allele was also included as a main effect and was tested relative to the residual variance. Body mass was included as a covariate, because it may be associated with cortical bone morphology (e.g., Middleton et al., 2008a, 2010), and because a previous study of this sample found that HR and wheel-access mice were significantly lighter than C and sedentary mice, respectively (Kelly et al., 2006). The effect of body mass was also tested relative to the residual variance.

Variability among the lines was analyzed by two-way ANCOVA with line and activity as the fixed factors and body mass included as a covariate; lines from the two linetypes were analyzed separately (Garland et al., 2011a). For these tests, we did not wish to distinguish between the variance attributable to line and family; therefore, family was not included in the model as a nested variable. Consequently, each effect was tested relative to the residual variance.

To test for dose relationships between distance run and bone response, we used nested, one-way ANCOVA. These analyses involved only the 40 mice given wheel access, none of which were siblings. Linetype was the fixed effect and line was nested within linetype as a random effect. Covariates used in the model included body mass and mean daily running distance during the last 6 days of wheel access. The effect of quantitative wheel running was tested relative to the residual variance. Statistical significance was judged using a 95% criterion ($P < 0.05$), and all tests were two-tailed.

RESULTS

Diaphyseal cortical bone

Body mass was a significant positive predictor of most diaphyseal cortical bone traits (Tables 1 and 2). After controlling statistically for the effects of body mass, bone geometric properties were found to be significantly affected by genetic background. Analyses of the effects of genetic selection history (linetype) on diaphyseal morphology (Table 1) showed that, on average, HR mice have larger periosteal areas ($P < 0.02$), endocortical areas ($P < 0.02$), and polar moments of area ($P < 0.05$). However, this is not true of HR mice harboring the MM allele (Table 1), which were observed to have significantly reduced polar moments of area ($P < 0.05$) and almost significantly smaller periosteal areas ($P = 0.07$) and cortical areas ($P = 0.06$). Analyses of replicate line variation within the linetypes (Table 2) detected among-line differences in endocortical area (HR: $P < 0.02$, C: $P < 0.001$), cortical area (HR: $P < 0.0001$, C: $P = 0.051$), and cortical thickness (HR and C: $P < 0.001$). HR lines, but not C lines, also exhibited significant variation in periosteal area ($P < 0.001$) and polar moment of area ($P < 0.001$).

Two months of voluntary wheel running failed to significantly alter bone quantity (Ct.Ar) but had a significant effect on diaphyseal shape after controlling for body mass (Fig. 3). The most salient response to exercise loading was a significant increase in endocortical expansion,

TABLE 1. Comparison of bone traits across the entire sample

Trait	Linetype		Activity		Linteype × activity	MM		Body mass
DF	1,6		1,6		1,6	1,29		1,29
Diaphyseal cortical								
Ps.Ar	**0.0191**	**HR > C**	0.0590	A > S	0.3606	0.0723	N > MM	**0.0001**
Ec.Ar	**0.0145**	**HR > C**	**0.0017**	**A > S**	0.6729	0.7361	N > MM	**0.0374**
Ct.Ar	0.7855	C > HR	0.2207	S > A	0.4933	0.0557	N > MM	**0.0002**
Ct.Th	0.2503	C > HR	**0.0298**	**S > A**	0.7678	0.1800	N > MM	**0.0416**
J	**0.0477**	**HR > C**	0.5654	A > S	0.2922	**0.0406**	**N > MM**	**<0.0001**
TMD	0.1258	C > HR	0.1942	S > A	0.8588	0.8212	N > MM	0.9226
Metaphyseal cortical								
Ps.Ar	0.0640	HR > C	**0.0498**	**A > S**	0.4537	0.9398	MM = N	**0.0002**
Ec.Ar	**0.0445**	**HR > C**	**0.0088**	**A > S**	0.2253	0.5373	MM > N	**0.001**
Ct.Ar	0.4544	C > HR	**0.0152**	**S > A**	0.2135	**0.0237**	**N > MM**	**0.0001**
Ct.Th	**0.0265**	**C > HR**	**0.0007**	**S > A**	0.2029	0.0519	N > MM	0.4687
J	0.3618	HR > C	0.5660	S > A	0.8502	0.0541	N > MM	**<0.0001**
TMD	0.1085	C > HR	0.0834	S > A	0.3656	0.1037	N > MM	0.9664
Metaphyseal trabecular								
BV/TV	0.5651	HR > C	0.6259	A > S	0.0841	0.1774	N > MM	0.1252
Tb.N	0.6211	HR > C	0.9213	S = A	0.2953	0.6364	N > MM	0.3394
Tb.Th	0.3169	C > HR	0.3742	S > A	0.0660	**0.0159**	**N > MM**	0.1043
Tb.Sp	0.6498	C > HR	0.9944	A = S	0.4323	0.4319	MM > N	0.3349
TMD	0.1915	C > HR	0.3557	A > S	0.9687	**0.0106**	**N > MM**	0.7639

Significance levels are for mixed-model ANCOVAs with body mass as a covariate and linetype, activity, and the linetype × activity interaction, as well as the MM phenotype, as fixed factors. Line was a random effect nested within linetype. Statistically significant differences ($P < 0.05$, unadjusted for multiple comparisons) are indicated in bold. DF, degrees of freedom; HR, mice from lines selected for high wheel running; C, mice from control lines; A, active mice with wheel access; S, sedentary mice without wheel access; MM, MM mice; N, non-MM mice; Ps.Ar, periosteal area; Ec.Ar, endocortical area; Ct.Ar, cortical area; Ct.Th, cortical thickness; J, polar moment of area; TMD, tissue mineral density; BV/TV, bone volume fraction; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing.

TABLE 2. Comparison of bone traits within the two linetypes

Trait	Linetype HR Line	Activity		Line × activity	Body mass	Linetype C Line	Activity		Line × activity	Body mass
DF	3,31	1,31		3,31	1,31	3,31	1,31		3,31	1,31
Diaphyseal cortical										
Ps.Ar	**0.0004**	0.3094	A > S	0.6119	**0.0006**	0.4221	**0.0166**	**A > S**	0.3653	**0.0001**
Ec.Ar	**0.0194**	**0.0263**	**A > S**	0.9925	0.1014	**0.0003**	**<0.0001**	**A > S**	0.1738	**0.0091**
Ct.Ar	**<0.0001**	0.0874	S > A	0.1977	**0.0004**	0.0513	0.6559	S > A	0.0794	**0.0015**
Ct.Th	**0.0003**	**0.0147**	**S > A**	0.3877	0.0506	**0.0008**	**0.0130**	**S > A**	**0.0374**	0.1276
J	**0.0002**	0.8761	S > A	0.3560	**0.0003**	0.5366	0.1110	A > S	0.2884	**<0.0001**
TMD	0.8767	0.4383	S > A	0.9914	0.7842	0.7869	0.3375	S > A	0.4759	0.2433
Metaphyseal cortical										
Ps.Ar	**<0.0001**	0.2845	A > S	0.5966	**0.002**	0.7982	0.0916	A > S	0.9735	**0.0176**
Ec.Ar	**<0.0001**	0.1107	A > S	0.7592	**0.01**	0.3614	**0.0078**	**A > S**	0.9516	**0.0442**
Ct.Ar	**0.0072**	0.1144	S > A	0.2924	**0.0002**	**0.0201**	**0.0052**	**S > A**	0.4166	**0.0143**
Ct.Th	**0.0017**	**0.0029**	**S > A**	0.8814	0.1166	0.1007	**0.0003**	**S > A**	0.5938	0.8118
J	**0.0012**	0.7587	S > A	0.3477	**<0.0001**	0.4874	0.6505	S > A	0.8619	**0.0094**
TMD	0.0675	0.4822	S > A	0.7749	0.6093	0.2491	**0.0410**	**S > A**	0.1327	0.5949
Metaphyseal trabecular										
BV/TV	0.0669	0.0972	A > S	0.4111	0.1334	**<0.0001**	0.3173	S > A	0.348	0.1396
Tb.N	**0.001**	0.3731	A > S	0.3513	0.3543	**<0.0001**	0.2647	S > A	0.0612	0.2479
Tb.Th	0.1703	0.2840	A > S	0.2268	**0.0083**	0.0809	**0.0424**	**S > A**	0.7651	0.9897
Tb.Sp	**0.0006**	0.4230	S > A	0.6771	0.1511	**<0.0001**	0.4219	A > S	**0.0394**	0.4169
TMD	0.3322	0.4022	A > S	0.7561	0.3632	0.5376	0.4924	A > S	0.4520	0.9500

Significance levels are for ANCOVAs with body mass as a covariate, and line, activity, and the line × activity interaction as fixed factors. Significant differences are indicated in bold. DF, degrees of freedom; HR, lines selected for high wheel running; C, control lines; A, active mice with wheel access; S, sedentary mice without wheel access; Ps.Ar, periosteal area; Ec.Ar, endocortical area; Ct.Ar, cortical area; Ct.Th, cortical thickness; J, polar moment of area; TMD, tissue mineral density; BV/TV, bone volume fraction; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing.

resulting in significantly decreased cortical thickness. This pattern was detected when HR and C mice were analyzed together (Ec.Ar: $P < 0.01$, Ct.Th: $P < 0.03$, Table 1) and separately (Ec.Ar: $P < 0.03$ [HR] and 0.0001 [C], Ct.Th: $P < 0.02$ [HR and C], Table 2). Running exercise also had a significant positive effect on periosteal area in C mice ($P < 0.02$, Table 2) but not in HR mice, and a nearly significant effect across the entire sample ($P = 0.06$, Table 1). For no diaphyseal trait was the interaction between linetype and activity significant (Table 1). However, among C lines (Table 2), cortical thickness showed a significant line × activity interaction ($P < 0.04$). Average daily running distance was never a significant (or nearly significant) covariate in the

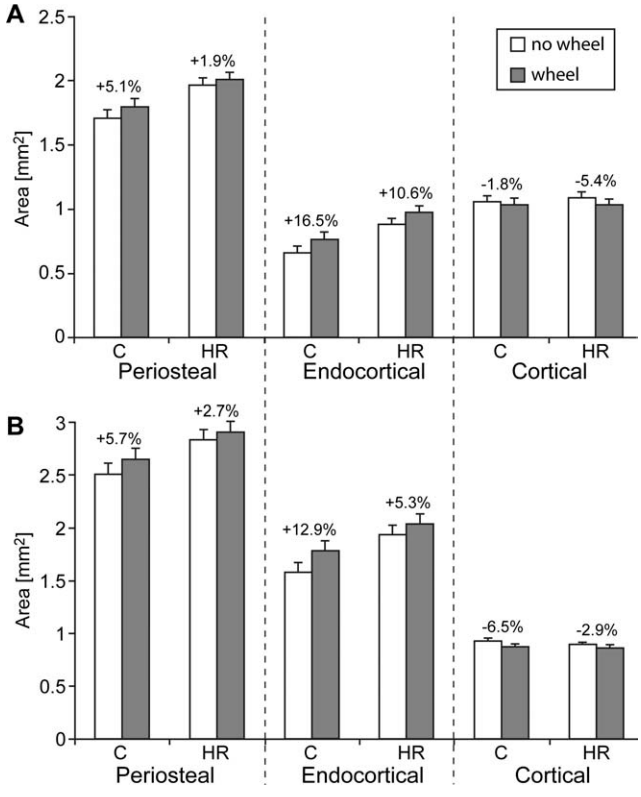

Fig. 3. Least-squares means and standard errors (corresponding to tests presented in Table 1) for periosteal area, endocortical area, and cortical bone area in the (**A**) mid-diaphysis and (**B**) distal metaphysis. Numbers indicate change associated with access to a wheel. In both regions, wheel access induced moderate periosteal enlargement (diaphysis: $P = 0.06$; metaphysis: $P < 0.05$), but relatively greater endocortical enlargement (diaphysis and metaphysis: $P < 0.01$). The imbalance between periosteal and endocortical expansion led to significantly reduced cortical area in the metaphysis ($P < 0.02$), but not in the diaphysis ($P = 0.22$).

ANCOVA models used to test for possible dose responses of exercise loading on diaphyseal morphology ($P > 0.5$ for all traits; results not shown).

Metaphyseal cortical bone

Similar to the diaphysis, most metaphyseal cortical bone traits showed a significant positive correlation with body mass (Tables 1 and 2). Once body mass effects were controlled statistically, genetic background was again found to have a significant effect on cortical bone geometric properties, although the pattern was different from that in the diaphysis. In linetype comparisons (Table 1), HR mice had, on average, significantly larger endocortical areas ($P < 0.05$), significantly thinner cortices ($P < 0.03$), and almost significantly greater periosteal areas ($P = 0.06$). Presence of the MM allele in certain HR mice had a significant negative effect on cortical area ($P < 0.03$, Table 1) and a nearly significant negative effect on cortical thickness ($P = 0.052$) and polar moment of area ($P = 0.054$). Within both linetypes (Table 2), cortical area varied significantly among the replicate lines (HR: $P < 0.01$, C: $P = 0.02$). All other traits varied significantly among the HR lines (Ps.Ar and Ec.Ar: $P < 0.0001$, Ct.Th and J: $P < 0.01$), except tissue mineral density ($P = 0.07$), but not among the C lines.

Voluntary running exercise caused significant changes in metaphyseal cortical bone quantity and shape after controlling for body mass (Fig. 3). Across the entire sample (Table 1), wheel running was associated with significant periosteal expansion ($P < 0.05$), but even greater endocortical expansion ($P < 0.01$), and, ultimately, reduced cortical area ($P < 0.02$) and cortical thickness ($P < 0.001$). When linetypes were analyzed separately, this pattern held for C mice, but less so for HR mice (Table 2). Mice from nonselected lines given wheel access had significantly greater endocortical area ($P < 0.01$), reduced cortical area ($P < 0.01$), and thinner cortices ($P < 0.001$) as well as lower tissue mineral density ($P < 0.05$). In HR mice, the only significant effect of running exercise was decreased cortical thickness ($P < 0.01$). Despite the differences between HR and C mice, no significant linetype $\times$ activity interactions were observed (Table 1), nor were the line $\times$ activity interactions significant (Table 2). Cortical thickness showed a significant negative correlation with daily running distance ($P = 0.0178$). All other metaphyseal cortical traits exhibited no such dose response ($P > 0.5$; results not shown).

Metaphyseal trabecular bone

In contrast to cortical bone structure, body mass was not a statistically significant predictor of metaphyseal trabecular bone traits (Tables 1 and 2), except trabecular thickness in HR mice ($P < 0.01$). Nevertheless, the variance attributable to body mass was accounted for in all analyses. Genetic differences between linetypes did not lead to significant differences in trabecular bone morphology (Table 1). Analyses of the effects of the MM allele (Table 1) showed that MM mice have significantly thinner trabeculae ($P < 0.02$) as well as lower tissue mineral density ($P < 0.02$). Genetic variation among replicate lines had a strong influence on multiple traits (Table 2). Both linetypes exhibited significant among-line differences in trabecular number (HR: $P = 0.001$, C: $P < 0.0001$) and trabecular spacing (HR: $P < 0.001$, C: $P < 0.0001$). Bone volume fraction varied significantly among C lines ($P < 0.0001$) and almost significantly among HR lines ($P = 0.07$). Trabecular thickness also varied among C lines, but not significantly so ($P = 0.08$).

Metaphyseal trabecular bone was generally unresponsive to exercise-induced loads. Only one comparison between mice allowed and denied wheel access showed a statistically significant effect of exercise: among C mice (Table 2), wheel running led to thinner trabeculae ($P < 0.05$). No significant linetype $\times$ activity interactions were observed (Table 1). However, among C lines (Table 2), a significant line $\times$ activity interaction was found for trabecular spacing ($P < 0.04$), and a nearly significant interaction was found for trabecular number ($P = 0.06$). Trabecular number was the only trait that showed a significant positive correlation with daily running distance ($P = 0.039$). Bone volume fraction had an almost significant positive association with distance run ($P = 0.075$). Trabecular spacing and tissue mineral density exhibited nearly significant negative correlations with running distance ($P = 0.057$ and 0.074, respectively). There was no effect of mean daily running distance during the last 6 days of wheel access on trabecular thickness ($P = 0.37$).

MM mice and HR line variation

To examine the degree to which the observed variation among the HR replicate lines (Table 2) was influenced by the MM mice, we performed additional HR line comparisons with these animals excluded (results not shown). Significant among-line differences remained detectable in most cortical bone traits of the diaphysis (Ct.Ar, Ct.Th, J) and metaphysis (Ps.Ar, Ec.Ar, Ct.Th, J). However, trabecular bone morphological differences were no longer detected. These results indicate that the MM mice are responsible for a relatively small fraction of the HR line variation in cortical structure, but for most of the variation in trabecular morphology.

DISCUSSION

The simultaneous effects of genetic background and functional loading on limb bone morphology were investigated using a model system in which mice, half from lines selectively bred for high voluntary wheel running (HR) and half from nonselected control (C) lines, were either allowed or denied wheel access for 2 months, beginning shortly after weaning. At the end of the experiment, femoral morphology was quantified at two cortical sites (mid-diaphysis, distal metaphysis) and one trabecular site (distal metaphysis). Genetics were found to have a strong influence on all morphological indices analyzed. Selectively bred HR mice, on average, had femora with enlarged shafts, expanded marrow areas, and mid-diaphyseal shapes suggesting increased mechanical strength (i.e., higher J values). However, in some HR mice, presence of the MM phenotype (expressed by homozygotes for the recessive MM allele) had a negative effect on cortical bone area, shaft shape, and trabecular thickness. Within the HR and C linetypes, the replicate lines exhibited substantial variation in bone quantity and shape, with the particular traits affected differing between linetypes and regions. Wheel running also influenced femoral morphology, although the exercise-stimulated response did not generally result in enhanced structure. Exercise loading caused moderate periosteal enlargement, but relatively greater endosteal enlargement (Fig. 3). The imbalance between periosteal and endocortical expansion ultimately led to significantly thinner cortices, as well as reduced cortical bone area in the metaphysis. Among individual mice within the wheel-access group, the magnitude of the response was broadly independent of loading dose (distance run). In the mid-diaphysis, where the forces associated with wheel running were likely highest, the two most mechanically important morphometric properties (Ct.Ar, J) were unaffected by exercise. Increased limb loading also failed to alter trabecular bone.

The data also do not indicate an enhancement of bone material properties in response to selective breeding or exercise. Tissue mineral density, a variable of considerable importance for bone strength and stiffness, showed no significant effect of linetype or activity in most cases, and the one significant difference in tissue mineral density of the metaphyseal cortex between active and sedentary C mice favored the sedentary mice. Previous studies of mice as well as humans have demonstrated trade-offs between geometric properties and material properties, with slender bones having greater tissue mineral density (Jepsen et al., 2007; Tommasini et al., 2008). The coupling of tissue mineral density reduction with periosteal expansion in the metaphyseal cortices of the active C mice supports the existence of such a trade-off.

Differences in femoral structure between HR and C mice are presumably pleiotropic effects of genes that regulate both physical activity and bone development, whereas differences among replicate lines are attributable to stochastic genetic events such as random drift, founder effects, mutations in particular lines, and interactions between these factors and selection (Garland, 2003; Swallow et al., 2009; Garland et al., 2011a,b). Molecular links between skeletal physiology and determinants of locomotor activity (e.g., motivation, energy metabolism) are well documented (Bab, 2007; Bab and Zimmer, 2008; Lee and Karsenty, 2008; Confavreux et al., 2009) but have not yet been rigorously investigated in these lines of mice (but see Farber et al., 2011). The genetic mechanisms underlying the among-line differences are also not well understood. However, it has been shown that the high frequency of the MM allele in two of the HR lines, which is clearly responsible for some of the observed variation within that linetype, resulted from chance events during line establishment, followed by random genetic drift and positive selection (Garland et al., 2002). What remains uncertain about the MM mice is the precise etiological pathway for their limb bone phenotype (see also Kelly et al., 2006; Middleton et al., 2008b). Reduced muscle mass and bone structure could represent direct pleiotropic effects of the MM allele if the gene is expressed early in development in related cell populations. Alternatively, the gene might act intrinsically only on the muscle cells and not the bone cells, and the MM bone phenotype could arise via mechanical interactions between bone and the reduced limb musculature. Another possibility is that the MM allele influences a circulating hormone or growth factor that regulates both muscle and bone development. An important goal of future investigations will be to achieve a better genetic and molecular comprehension of the patterns of morphological variation detected in this study.

The variation in femoral morphology observed among the HR mice is interesting, because it suggests that limb bone structure is of relatively little adaptive significance when selection favors high levels of wheel running. The bones need only be rigid enough to sustain normal physiological loads. The relatively gracile bones of the MM mice evidently meet this requirement, given that they did not spontaneously fracture during the experiment or during several similar studies with these lines during later generations (e.g., Meek et al., 2010). In fact, light and slender bones are conceivably advantageous for animals that devote much of their total energy budget to locomotion because such a configuration might save energy by decreasing the moment of inertia of the limbs (Carrano, 1999; Kemp et al., 2005; Kelly et al., 2006). However, Dlugosz et al. (2009) recently measured the energetic cost of running in MM and non-MM HR mice and found that the incremental cost of transport—the energy associated with movement per se—does not differ between the two groups. Moreover, the "postural costs" of wheel running were significantly higher in MM mice (and maximal sprint running speeds were significantly lower). Thus, neither the structurally augmented bones of the non-MM HR mice nor the gracile MM bones provide obvious benefits for wheel running performance; but subtle benefits remain possible.

It is important to note that throughout the experiment, mice were allowed to load their limbs during normal cage activities, thus introducing the possibility that

the variation in femoral morphology detected among linetypes, lines, and individuals was caused, in part, by differences in cage activity and not only alleles directly influencing bone development. Although it is conceivable that variation in cage activity (Malisch et al., 2008, 2009) contributed to the observed morphological variation, its contribution is likely to have been small relative to genetic factors, given that structural differences similar to those reported here have been documented in perinatal mice before the onset of locomotion (Wallace et al., 2010, 2011). For example, at 1 week of age, HR males have femoral diaphyses with expanded periosteal areas and greater polar moments of area (Wallace et al., 2010). Replicate line differences in diaphyseal areas and area moments are also evident at 1 week postnatal (Wallace et al., 2011).

The fact that exercise influenced femoral morphology is not surprising considering that (a) the distances run by the mice in this study well exceeded those completed in many forced exercise studies with mice in which running was found to significantly affect bone structure (Wu et al., 2001, 2004; Hamrick et al., 2006a,b), and (b) the experiment encompassed a period of rapid bone growth in mice (Ferguson et al., 2003; Glatt et al., 2007), and growing bone is generally more responsive to mechanical signals than mature bone (Rubin et al., 1992; Pearson and Lieberman, 2004). What is perhaps surprising is that the functional response did not lead to gains in bone quantity or major improvements in geometry. In a previous analysis of the sample used in this study, Kelly et al. (2006) showed that running had a positive effect on certain external linear dimensions of the femoral shaft. Our results are consistent with those of Kelly et al. as we, too, observed positive effects on shaft perimeter (Ps.Ar). However, external dimensions are inexact estimates of bone quantity and mechanical rigidity. By using μCT to accurately quantify internal and external contours, we were able to derive mechanically more relevant morphometric properties (Ct.Ar, J) and demonstrate that the structural consequences of exercise overall were not very "beneficial" (e.g., no change in J or midshaft Ct.Ar), and were, in fact, somewhat "detrimental" (e.g., decreased metaphyseal Ct.Ar). Recently, Middleton et al. (2010) examined the effects of ~3.5 months of wheel-running exercise on the femora of young female mice from a later generation of the selection experiment. Their results accord with ours in that mid-diaphyseal cortical area and area moments were found to be unaffected by exercise. Also consistent with our findings, Ma et al. (2010, 2011) observed that, in young male mice of the C57BL/6J inbred strain, ~5.25 months of wheel running did not significantly affect femoral mid-diaphyseal cortical area or area moments, but led to significantly thinner cortices and reduced cortical bone quantity in the metaphysis. In contrast, Plochocki et al. (2008) found that growing female C57BL/6J mice given one month of voluntary wheel access displayed enhanced periosteal and endosteal bone formation at the femoral midshaft, which resulted in increased cortical area and area moments. The disparity between the results of Plochocki et al. and other researchers is not easy to explain, but may relate to sex or genetic differences between the animals employed, as well as methodological differences between the studies (e.g., Plochocki et al. did not control for statistically significant effects of body mass).

The agreement between the results of this study and those of Ma et al. (2010, 2011) is important because it implies that the bone functional response observed in our mice was not related to their unique breeding history, nor is it unusual for mice in general. The fact that the unexpected effects of wheel running (e.g., cortical thinning and decreased metaphyseal bone quantity) were found in the C lines—which have not undergone selective breeding—further suggests that our observations concerning the effects of running on bone cannot be explained as a phenomenon related solely to artificial selection.

By using a voluntary running model, we were able to investigate possible dose effects of exercise loading on bone's functional response. Little evidence was found for strong relationships between daily running distance and femoral morphological indices, suggesting that only a small amount of exercise was required to initiate the observed structural changes. Previously, Newhall et al. (1991) examined the dose effects of voluntary wheel running on limb bone structure in growing rats. Like us, they found that the bone response was generally independent of loading dose. (Unlike us, however, they observed that the response led to increased diaphyseal area and area moments.) The lack of significant dose effects in this study and that of Newhall et al. (1991) is plausibly explained by the fact that over the course of the experiments, wheel running engendered surplus loading environments, which may have allowed the bone cells to become accustomed to the habitual loads and less responsive to the routine mechanical signals (Middleton et al., 2008b).

Although one might have expected the bone functional response to have been mediated to some degree by the genetic differences between the linetypes and among the replicate lines, given that polymorphisms among inbred mice cause large differences in mechanoresponsiveness (Judex et al., 2002, 2004a; Robling and Turner, 2002), we found virtually no statistically significant interactions between genetic background and wheel access. However, analysis of variance models have relatively low power to detect interactions (Middleton et al., 2008b, and references therein), and it is possible, likely even, that we have missed important linetype and line differences in responsiveness by relying on this statistical approach. Another way to assess linetype differences in responsiveness might be to compare the activity effects in the separate analyses of the HR and C lines (Table 2). Among HR lines, wheel running was found to significantly affect 3 of the 14 traits analyzed, while among C lines, more than half of the traits were affected by exercise. Therefore, the bones of C mice were seemingly more responsive to exercise loading than those of HR mice, but this may not be a valid comparison considering that the HR and C mice did not receive the same amount of loading. The effect of genetic background on bone's response to mechanical signals in these lines of mice remains an open question.

Mice are widely considered appropriate models for addressing questions about human bone biology (Beamer et al., 2002; Forwood, 2008; Hamrick et al., 2009; Gross et al., 2010), because the genes and molecular pathways affecting the skeleton are highly conserved in the mouse and human (Karsenty, 2003). Nevertheless, major differences exist between mice and humans and the impact of these differences on experimental data is currently unknown. Mouse bones do not undergo Haversian (intracortical) remodeling, which makes them somewhat biologically different from human bones. Perhaps more importantly, skeletal allometry dictates that mouse bones are not subject to the same constraints on functional

capacity as those of larger mammals, such as humans (Biewener, 1990); therefore, they would not necessarily be expected to react in the same way to altered mechanical signals as those of larger mammals. Since limb bone dimensions scale roughly geometrically with body size (Alexander et al., 1979), and bone tissue material properties are similar over a wide range of body size (Currey, 2002), the same proportional forces associated with locomotion should engender greater bone strain in larger mammals than in mice. If strain is the primary physical signal regulating bone's response to loading (Lanyon, 1987), and the "optimal strain environment" for limb bones is generally independent of body size (Rubin and Lanyon, 1984b), then relatively greater increases in bone stress should be required to stimulate osteogenesis in mice compared with larger mammals. However, several studies have found moderate increases in locomotor activity—limb loading at magnitudes well within the normal physiological range—to be anabolic to mouse limb bones (e.g., Kodama et al., 2000; Wu et al., 2001, 2004; Umemura et al., 2002; Mori et al., 2003; Hamrick et al., 2006a; Plochocki et al., 2008), as is often found in studies of larger mammals (e.g., Woo et al., 1981; Lieberman, 1996; Lieberman et al., 2003; Barak et al., 2011), suggesting that the functional response in the mouse skeleton is not atypical for mammals in general, and perhaps indicating that physical signals other than strain are important in modulating bone's functional response (Garman et al., 2007). Nevertheless, extrapolating our results to humans requires caution, but if such extrapolation is warranted, then the results of this study have possible implications for anthropology.

First, patterns of limb bone morphological variation among paleontological and archaeological human samples may reflect, to some extent, genetic differences among populations. With methods now available for ancient DNA retrieval, and a growing knowledge of polymorphisms affecting human skeletal structure, it may soon be feasible to investigate the underlying genetics of morphological variation in the past. Second, the effects of mechanical loading on limb bones can be unpredictable, which limits our ability to infer loading history from bone morphology. Although it is commonly assumed that the purpose of bone's response to increased loading is to enhance its morphology to reduce mechanical stresses and/or strains, structural augmentation is not necessarily what is achieved in every instance. Bone cells react to biophysical forces within a complex environment of genetic and epigenetic signals. Without a doubt, specific combinations of mechanical and nonmechanical inputs will elicit a response which enhances morphology, but other combinations may result in stasis or even diminished structure. In terms of the precise conditions under which bone's response leads to net formation (and/or functional improvement), gaps exist in our understanding. Trade-offs with other systems that are simultaneously stimulated by activities involving limb loading may further obfuscate the links between particular loading regimens and bone structure; e.g., sweating causes calcium loss, which can induce bone resorption (Barry and Kohrt, 2008).

ACKNOWLEDGMENTS

The authors thank Luci Betti-Nash for preparing the figures, Karen Baab for assistance with SAS, and Roberto Fajardo, Mark Hamrick, and Bill Jungers for helpful discussions. Chris Ruff and two anonymous reviewers provided constructive comments on earlier versions of this article.

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