Available online at www.sciencedirect.com
ScienceDirect
Zoology 111 (2008) 135–147
ZOOLOGY
www.elsevier.de/zool

The relative importance of genetics and phenotypic plasticity in dictating bone morphology and mechanics in aged mice: Evidence from an artificial selection experiment

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Received 8 February 2007; received in revised form 11 June 2007; accepted 13 June 2007

Abstract

Both genetic and environmental factors are known to influence the structure of bone, contributing to its mechanical behavior during, and adaptive response to, loading. We introduce a novel approach to simultaneously address the genetically mediated, exercise-related effects on bone morphometrics and strength, using mice that had been selectively bred for high levels of voluntary wheel running (16 generations). Female mice from high running and control lines were either allowed ($n = 12$, 12, respectively) or denied ($n = 11$, 12, respectively) access to wheels for 20 months. Femoral shaft, neck, and head were measured with calipers and via micro-computed tomography. Fracture characteristics of the femoral head were assessed in cantilever bending. After adjusting for variation in body mass by two-way analysis of covariance, distal width of the femur increased as a result of selective breeding, and mediolateral femoral diameter was reduced by wheel access. Cross-sectional area of the femoral mid-shaft showed a significant linetype × activity effect, increasing with wheel access in high-running lines but decreasing in control lines. Body mass was significantly positively correlated with many of the morphometric traits studied. Fracture load of the femoral neck was strongly positively predicted by morphometric traits of the femoral neck ($r^2 > 0.30$), but no significant effects of selective breeding or wheel access were found. The significant correlations of body mass with femoral morphometric traits underscore the importance of controlling for body size when analyzing the response of bone size and shape to experimental treatments. After controlling for body mass, measures of the femoral neck remain significant predictors of femoral neck strength.

Keywords: Artificial selection; Body mass; Bone morphometrics; Exercise; Mechanical loading

Introduction

Genetics and exercise are foremost among many factors that act individually and in concert to determine

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doi:10.1016/j.zool.2007.06.003

the geometry and material properties of a bone. The shape and constituent materials of the bone, in turn, determine how it will respond to a given load. In mammals, genetic factors are thought to determine 50–80% of the variation in mineral content of adult bone, with most of the balance dictated by amount, frequency, and/or intensity of exercise (Eisman, 1999; Ferrari et al., 1999). However, the interaction between genetic and environmental factors is complex, and the effects of loading may be modulated by the physiological response of bone, which itself is genetically determined to an important extent (Robling and Turner, 2002; Koller et al., 2003; Robling et al., 2003).

Although genetic factors are known to mediate exercise effects, the two are often addressed separately in experimental settings. For example, bone mineral content and activity effects have frequently been studied in rodents, without attention to possible interactions (Gordon et al., 1989; Umemura et al., 1995; Iwamoto et al., 1999; Klein et al., 2001; Turner and Burr, 2001; Bennell et al., 2002; Akhter et al., 2004a, b; Niehoff et al., 2004). Recently, however, more attention has been directed at variation among different strains of mice.

Several aspects of femoral geometry and strength have been shown to vary widely among different genetic strains of mice (Koller et al., 2003; Wergedal et al., 2005), as have responses to hind limb unloading (Amblard et al., 2003; Judex et al., 2004; Squire et al., 2004) and ovariectomy (Bouxsein et al., 2005). Expanded understanding of the role of mechanotransduction in bone has resulted from comparisons of different genetic strains of mice under artificial loading regimens (e.g., Robling and Turner, 2002; Robling et al., 2003; Kesavan et al., 2005, 2006; Li et al., 2005; Lau et al., 2006; Sawakami et al., 2006).

Although jump training (i.e., impact loading) has been used to study the effects of naturalistic loading on bone (Honda et al., 2001; Umemura et al., 2002; Welch et al., 2004), relatively rarely have the *combined effects* of genetic background and exercise been addressed under natural, musculoskeletal loading. One exception is a study by Kodama et al. (2000), who compared the response of bone to 4 weeks of jump training in C57BL/6J and C3H/HeJ mice. These authors found that both strains of mice responded to loading, but that the relative response differed between strains and concluded that bone of the C3H/HeJ mice were less responsive to mechanical loading.

In addition to the complex interaction of genetics and loading history in forming bone, these two factors play an important role in the changes that bone exhibits as an animal ages. The effects of aging on bone properties have been studied extensively in rodents (Yamamoto et al., 1995; Mosley and Lanyon, 1998, 2002; Halloran et al., 2002; Hamrick et al., 2006; McNamara et al., 2006) and in humans (Smith and Walker, 1964; Mosekilde, 1989; Russo et al., 2006). Similarly, exercise effects on age-related changes to bone have received a great deal of attention, both in humans (Weinstein and Hutson, 1987; Heaney et al., 1997; Kaptoge et al., 2006) and in rodent models (Raab et al., 1990; Søgaard et al., 1994; Silva and Gibson, 1997).

One way to effectively probe relations between genetics and external influences, such as exercise, is to take advantage of artificial selection experiments. Recently, a few research programs have overcome the technical challenges of applying this labor- and time-intensive approach to mammals and have successfully used rodents in selection experiments targeting exercise-related phenotypes (Hussain et al., 2001; Koch and Britton, 2001; Henderson et al., 2002; Garland, 2003; Wisløff et al., 2005). Although diverse mammalian species vary considerably in bone structure and physiology (Bagi et al., 1997), and extrapolations from rodents to humans and other species must be made only with caution, it is now possible to add artificial selection to the range of techniques used to address questions in bone biology. Furthermore, selection experiments provide a valuable tool for assessing the relative magnitudes of evolutionary and phenotypic plasticity in skeletal biology (Garland and Kelly, 2006).

Here, we employ mice selectively bred for high levels of voluntary wheel running to explore the combined effects of genetic background and voluntary exercise on bone morphometrics and mechanics. We focus on whole-bone morphometrics and mechanical performance during loading, and do not consider variations in tissue-level properties such as bone mineral density and collagen fiber orientation that may affect mechanical behavior under load. For our study population we chose mice that were allowed or denied access to a running wheel for 20 months to explore whether presumptive changes in bone morphometrics and mechanical properties resulting from exercise would be retained as the animals aged.

We ask: (1) Does the size, shape, and mechanical performance of the femur differ between animals from lines that have been artificially selected for high-voluntary exercise in comparison with unselected control lines (genetic effect)? (2) Do the same characteristics differ between individuals allowed free access to running wheels for 20 months in comparison with mice denied wheels (exercise effect)? (3) Are there interactions between these two factors such that the effect of exercise depends on genetic background?

Materials and methods

Model system

We employed mice (*Mus domesticus*) in this study because they were part of a long-term artificial selection

experiment on high levels of exercise (voluntary wheel-running behavior; for additional information, see Garland, 2003; Rhodes et al., 2005). Among the goals of this selection study are understanding heritable aspects of behavior and physiology in mammals, including neurobiological and physiological processes relevant to human health. The complete experimental design is described elsewhere (Swallow et al., 1998; Garland, 2003; Morgan et al., 2003), and we provide only a brief summary here.

From a base population of outbred Hsd:ICR mice, 8 closed lines were established in which the parents of each subsequent generation are those which exhibit the highest (4 selected lines) or typical (4 control lines) levels of voluntary wheel running. Mice from the ICR strain were chosen because they exhibit high levels of genetic variation and had previously been selected for large litter sizes and high-weaning success (Swallow et al., 1998). Because genetic heterogeneity is a prerequisite for evolutionary change in response to selective breeding, the use of an inbred strain would have been impossible. Furthermore, we employed within-family selection to decrease the possible deleterious effects of inbreeding and potentially confounding maternal effects. Mean adjusted heritability of wheel running was 0.28 for the first 10 generations of selection (Swallow et al., 1998).

Running was scored as total number of exercise wheel revolutions run on day 5 plus day 6 of a 6-day exposure to wheels. Total revolutions per day in the lines selected for high running increased rapidly for the first 16 generations and then reached an apparent plateau, at which point the selected lines ran about 170–200% more revolutions per day than controls (Swallow et al., 1998; Garland, 2003). Summed revolutions on days 5 and 6 is the sole criterion for selection, but selection for high levels of voluntary exercise has the potential to lead to evolution in many diverse features because of pleiotropic effects of alleles that affect wheel running. The selected lines have diverged genetically and phenotypically from the control lines and are distinct morphologically, physiologically, and behaviorally (Swallow et al., 1999; Garland, 2003; Garland and Kelly, 2006; Kelly et al., 2006; Malisch et al., 2007).

Study population

Our sample consisted of femora of 47 female mice from generation 16 of the selection experiment. Mice were weaned at 21 days of age, housed individually, and either allowed or denied access to an exercise wheel (0.73 m circumference) for 80 weeks, beginning at 28 ± 3 days of age (for additional details see Morgan et al., 2003; Bronikowski et al., 2006). Mean age at sacrifice was 596 days (range = 590–600). Approximately half of the mice were from the selected lines ($n = 23$) and half from the control lines ($n = 24$). Within each of these groups, half were raised with access to a wheel ($n = 12$ and 12, from selected and control lines, respectively) to allow for voluntary wheel running and half were sedentary (no wheel; $n = 11$ and 12, from selected and control lines, respectively). All mouse cages were standard size, and mice were permitted food and water *ad libitum*. All protocols were approved by and in compliance with guidelines set by the Washington State University IACUC.

Specimen preparation

After sacrifice, the mice were weighed and frozen until dissection. The mice were later thawed and both hind limbs dissected; the femora were separated from the distal limb elements and defleshed. Right femora were used for morphometric measurements and mechanical testing, and left femora were scanned using microcomputed tomography (μCT). The femur was chosen for detailed study because it is subject to the most direct loading from the axial skeleton, and the proximal femur is the site of most femoral fractures (Crawford and Fretz, 1985; Embertson et al., 1986; Zhang et al., 2000; Harasen, 2003).

Whole-bone morphometrics

Measuring with digital calipers to the nearest 0.01 mm, eight morphometric traits of the femur were quantified: length from the superior (articular) surface of the femoral head to the distal femoral condyles, proximal width (greater trochanter to medial surface of femoral head), distal width (mediolateral distance across distal femoral condyles), anteroposterior and mediolateral mid-diaphyseal diameter, proximodistal height and anteroposterior depth of the femoral neck, and height (proximodistal) and depth (anteroposterior) of the femoral head (Fig. 1). Many of these measurements (e.g., femur length, mid-shaft diameters, distal width, and head depth) overlap with morphometric data reported for exercise-selected mice from different generations (Garland and Freeman, 2005; Kelly et al., 2006) allowing comparisons among data sets. Note that our use of femoral head here equates to "femoral condyle" in prior studies (Garland and Freeman, 2005; Kelly et al., 2006). The angle of the femoral neck was measured from digital photographs as the angle formed between the femoral shaft and a line drawn parallel to the long axis of the femoral neck.

Microcomputed tomography

Left femora were scanned using a high-resolution, fan-beam μCT scanner (μCT 20; Scanco Medical AG,

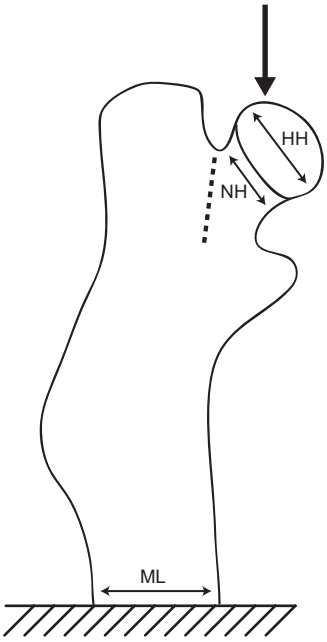

Fig. 1. Mouse proximal femur in anterior view. With the distal femur fixed, load is applied to the femoral head to cause cantilever bending at the femoral neck. The arrow indicates the point of load application. The most common fracture orientation is marked with a dashed line. A subset of the morphometric traits which were measured are shown, including mediolateral femoral diameter (ML), femoral head height (HH), and height of the femoral neck (NH). Anteroposterior diameter, femoral head depth, and depth of the femoral neck were measured perpendicular to ML, HH, and NH, respectively.

Bassersdorf, Switzerland). For the mid-diaphysis, 10 slices with a voxel size of 9 μm^3 were acquired midway between the superior surface of the femoral head and the distal end of the femur. For the femoral neck, 16 slices with a voxel size of 17 μm^3 were acquired midway along the femoral neck. Mid-diaphyseal slices were acquired perpendicular to the long axis of the bone, and neck slices were acquired perpendicular to the long axis of the femoral neck. Cross-sectional areas, maximum and minimum second moments of inertia (I_{max}, I_{min}), and maximum section moduli (I_{max}/C_{min}) were calculated for each slice using the scanner's built-in software routines and averaged over all slices for the femoral diaphysis and neck for each individual.

Mechanical testing

To compare maximum load at failure among groups, we loaded the femoral neck to fracture in cantilever bending using an Instron 4222 materials testing machine (Instron Corporation; Norwood, MA). To approximate loading experienced by the femur during locomotion, a compressive load was applied to the femoral head with the distal end of the femur fixed in solid bismuth alloy (LMA-117; Small Parts, Inc., Miami Lakes, FL). This method of fixing the distal end of the bone facilitated later release of the entire bone specimen from the mounting medium by melting the potting material in a hot water bath (Fig. 1). Our methodology is similar to that used to test femoral neck strength in mice (Akhter et al., 2004b) and rats (Bagi et al., 1996). Tests were conducted under displacement control, with a constant loading velocity of 0.5 mm/min, and data were collected at a rate of 20 samples/s. Load and displacement were recorded by computer and load at fracture was calculated.

Statistical analysis

Traits were analyzed by two-way analysis of variance (ANOVA) and analysis of covariance (ANCOVA) using SAS Procedure Mixed (version 9.1; SAS Institute; Cary, NC) with Type 3 tests of fixed effects with or without body mass as a covariate. Using ANOVA and ANCOVA, differences between control and selected lines (effects of linetype), wheel and no wheel treatments (effects of activity), and the interaction between these two main effects were tested for statistical significance (Swallow and Garland, 2005; Kelly et al., 2006). In the analyses of predictors of fracture load, we included both body mass and femoral morphometric traits as covariates. Pearson correlations were calculated between body mass and the morphometric traits to check for potential multicollinearity. Correlations were always <0.7, indicating that multicollinearity was not a concern in these data (Slinker and Glantz, 1985).

To address concerns related to performing multiple statistical tests on the same set of data, we carried out a FDR analysis using the *qvalue* software package (Storey, 2002) for R (version 2.4.1; R Development Core Team, 2007). The total number of hypotheses under test is 89: 60 in Table 1 (see Results), 19 in Table 2 (body mass covariate column only, as all others are redundant with Table 1), and 10 in Table 4 (only *p*-values for the covariate correlation are included). Due to the relatively low number of *p*-values, we used the "bootstrap" option of *qvalue* in estimating the proportion of true null hypotheses. Results of the FDR analysis indicate that, rather than the typical $a = 0.05$ for judging statistical significance, a more conservative level of $a = 0.0374$ (corresponding to a positive false discovery rate of 5%; Storey, 2002) is appropriate given the number of hypotheses tested and the distribution of *p*-values we obtained. In the tables, we present nominal *p*-values for two-tailed tests, unless specifically noted for traits for which we had *a priori* predictions about the direction of main effect or

covariate (e.g., smaller body mass in wheel access and in selected-line mice).

Results

Morphometrics

Results of the two-way ANOVAs with activity level and linetype (but *not* body mass as a covariate) show significant effects of activity on several traits (Table 1). Results of the two-way ANCOVAs show that activity level has a significant negative effect on body mass ($p = 0.0374$, one-tailed) and linetype has a nearly significant effect ($p = 0.0698$, one-tailed), with both active and selected mice having lower body mass than sedentary or control mice, respectively (Tables 2 and 3). In ANCOVAs including body mass as a covariate, most femoral morphometric traits showed a significant positive correlation with body mass even after controlling statistically for main effects (Table 2). Mice bred for high levels of wheel running had wider distal femora than controls, regardless of wheel access ($p = 0.0349$), and wheel access led to mediolaterally narrower femoral shafts in both selected and control lines ($p = 0.0166$; Tables 2 and 3). Only one interaction was statistically significant: wheel access decreased midshaft cross-sectional area in control mice but increased it in selected mice (Tables 2 and 3).

Table 1. Results (p-values) of two-way analysis of variance models

Trait	n	Linetype	Activity	Linetype × activity
Body mass	47	0.0698−[a]	**0.0374**−[a]	0.7223
Femur length	47	0.8242+	0.5369+	0.5037
ML diameter	47	0.8165−	**0.0098**−	0.2964
AP diameter	47	0.5789+	0.1136−	0.8003
Distal width	47	0.0625+	0.3782−	0.3273
Proximal width	47	0.7746−	0.5931+	0.7464
Head height	47	0.5121+	0.7136+	0.5410
Head depth	47	0.1527+	0.5452+	0.7597
Neck height	47	0.8106−	0.9132−	0.6362
Neck depth	47	0.6324−	0.5504−	0.1981
Femur neck angle	47	0.5101−	0.7497+	0.9099
Fracture load	46	0.2559	0.8492	0.3802
Shaft XS area	46	0.1290−	0.0834−	**0.0206**
Shaft I_{max}	46	0.3027−	**0.0488**−	0.1814
Shaft I_{min}	46	0.3002−	0.1017−	0.1876
Shaft I_{max}/C_{min}	46	0.2488−	**0.0349**−	0.1033
Neck XS area	45	0.5184−	0.6842−	0.6168
Neck I_{max}	45	0.6292−	0.6932−	0.4577
Neck I_{min}	45	0.6778−	0.8358−	0.8329
Neck I_{max}/C_{min}	45	0.6232−	0.7110−	0.4183

p-Values < 0.05 are noted in bold. In the columns *linetype* and *activity*, + indicates selected or wheel access is larger than control or sedentary, respectively; − indicates smaller. Note that head depth equates to "condyle depth" in previous studies (Garland and Freeman, 2005; Kelly et al., 2006). Degrees of freedom for testing the effects of linetype, activity, and the linetype × activity interaction were always 1 and 6; d.f. for testing the body mass effect depended on sample size and ranged between 1 and 28 and 1 and 30.

[a]One-tailed p-values corresponding to *a priori* directional predictions (see text).

Mechanical properties

When the distal femur was fixed and loads were applied to the femoral head, the femur always fractured near the base of the neck, not distally along the shaft (Fig. 1). Table 4 summarizes results of analyses to determine the best morphometric predictors (i.e., correlates) of femoral neck fracture load, while controlling for all main effects and correlations with body mass. There was a significant positive correlation between load at fracture and both height and depth of the femoral neck, as well as between load at fracture and all femoral neck traits measured via μCT ($r^2 > 0.3$; $p < 0.01$; Fig. 2; Table 4). In these analyses, the effects of linetype and of activity were never statistically significant. None of the femoral shaft traits were significant predictors of fracture load (results not shown).

Discussion

In this study, we explored the combined effects of genetic background and voluntary exercise on bone morphology and femoral neck strength using mice from lines that had been selectively bred for high levels of voluntary wheel running. We sought to determine whether the femora of mice selected for high levels of exercise would differ from controls (genetic effect), whether those with access to a running wheel would differ from those without (exercise effect), and whether the exercise effect, if any, was mediated by genetic background (interaction effect).

Wheel running and body mass

The mice analyzed in this study represent a subset of the female mice for which wheel-running ontogeny was previously described (Morgan et al., 2003). Wheel running increased rapidly during the first 8 weeks of ontogeny, peaking at means of 76 km/week in mice selected for high levels of wheel running and 52 km/week in control mice (Fig. 3). From 8 weeks of age until the end of the experiment, wheel running declined roughly linearly, with means of 36 and 21 km/week at 84 weeks (selected and control lines, respectively). Both the position and the shape of the wheel-running ontogeny were different between selected and control mice; selected mice ran significantly

Table 2. Results (p-values) of two-way analysis of covariance models including body mass as a covariate

Trait	n	Linetype	Activity	Linetype × activity	Body mass
Femur length	47	0.4892 +	0.1732 +	0.3837	**0.0055 +** [a]
ML diameter	47	0.9540 −	0.0166 −	0.3172	0.2497 + [a]
AP diameter	47	0.1693 +	0.2199 −	0.8520	**0.0019 +** [a]
Distal width	47	**0.0349 +**	0.6114 −	0.2686	0.0636 + [a]
Proximal width	47	0.9640 +	0.3439 +	0.8039	0.0687 + [a]
Head height	47	0.2265 +	0.3803 +	0.5896	**0.0073 +** [a]
Head depth	47	**0.0342 +** [a]	0.2859 +	0.6956	**0.0091 +** [a]
Neck height	47	0.8093 +	0.7590 +	0.5804	**0.0320 +** [a]
Neck depth	47	0.8455 −	0.9617 −	0.1638	0.0465 + [a]
Femur neck angle	47	0.8235 −	0.5103 +	0.8607	0.1059 + [a]
Fracture load	46	0.4739 −	0.4604 +	0.3773	**0.0239 +** [a]
Shaft XS area	46	0.2335 −	0.1755 −	**0.0120**	**0.0086 +** [a]
Shaft I_{max}	46	0.6908 −	0.1147 −	0.1492	**0.0008 +** [a]
Shaft I_{min}	46	0.7143 −	0.3116 −	0.1336	**0.0002 +** [a]
Shaft I_{max}/C_{min}	46	0.4961 −	0.0905 −	0.1078	**0.0150 +**
Neck XS area	45	0.7010 −	0.8868 +	0.6935	**0.0491 +** [a]
Neck I_{max}	45	0.8727 −	0.8639 +	0.5249	**0.0430 +** [a]
Neck I_{min}	45	0.8874 −	0.7676 +	0.9126	0.0618 + [a]
Neck I_{max}/C_{min}	45	0.8150 −	0.9331 +	0.4724	0.1696 +

p-Values < 0.05 are noted in bold. In the columns *linetype* and *activity*, + indicates selected or wheel access is larger than control or sedentary, respectively; − indicates smaller. + in body mass column indicates positive correlation; − indicates negative correlation. Note that head depth equates to "condyle depth" in previous studies (Garland and Freeman, 2005; Kelly et al., 2006). Degrees of freedom for testing the effects of linetype, activity, and the line type × activity interaction were always 1 and 6; d.f. for testing the body mass effect depended on sample size and ranged between 1 and 28 and 1 and 30.

[a]One-tailed p-values corresponding to *a priori* directional predictions (see text).

Table 3. Least squares means and standard errors for the four experimental groups (two-way analyses of covariance for which p-values are presented in Table 2)

	Control lines				Selected lines			
	Sedentary $n = 12$		Active $n = 12$		Sedentary $n = 11$		Active $n = 12$	
Trait	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body mass (g)[a]	36.02	1.33	33.41	1.33	32.94	1.37	31.10	1.33
Femur length (mm)	15.94	0.227	16.28	0.220	16.28	0.225	16.37	0.225
ML diameter (mm)	1.74	0.029	1.64	0.028	1.71	0.029	1.66	0.029
AP diameter (mm)	1.56	0.030	1.51	0.029	1.60	0.029	1.56	0.029
Distal width (mm)	3.10	0.088	3.16	0.084	3.45	0.087	3.31	0.087
Proximal width (mm)	3.53	0.059	3.56	0.057	3.52	0.058	3.57	0.058
Head height (mm)	1.53	0.029	1.54	0.028	1.55	0.028	1.60	0.028
Head depth (mm)	1.49	0.024	1.53	0.024	1.56	0.024	1.57	0.024
Neck height (mm)	1.14	0.041	1.18	0.039	1.18	0.040	1.17	0.040
Neck depth (AP) (mm)	0.90	0.048	0.94	0.047	0.93	0.047	0.89	0.047
Femur neck angle (°)	37.99	1.38	39.16	1.30	37.89	1.36	38.59	1.36
Fracture load (N)	18.95	1.33	18.84	1.29	16.87	1.31	18.39	1.34
Shaft XS area (mm^2)	1.4613	0.0835	1.2568	0.0829	1.1737	0.0829	1.2507	0.0830
Shaft I_{max} (mm^4)	0.3989	0.0250	0.3297	0.0246	0.3546	0.0265	0.3495	0.0247
Shaft I_{min} (mm^4)	0.3261	0.0222	0.2859	0.0219	0.2913	0.0220	0.2993	0.0220
Shaft I_{max}/C_{min} (mm^4)	0.4622	0.0234	0.3898	0.0230	0.4082	0.0230	0.4041	0.0231
Neck XS area (mm^2)	0.5629	0.0401	0.5561	0.0404	0.5318	0.0395	0.5469	0.0396
Neck I_{max} (mm^4)	0.0413	0.0062	0.0391	0.0062	0.0370	0.0060	0.0408	0.0061
Neck I_{min} (mm^4)	0.0223	0.0038	0.0238	0.0039	0.0223	0.0038	0.0234	0.0038
Neck I_{max}/C_{min} (mm^4)	0.1031	0.0116	0.0975	0.0117	0.0933	0.0114	0.1004	0.0114

[a]Least squares means and standard errors for body mass are calculated for models with no additional covariates.

Table 4. Results (*p*-values) of two-way analysis of covariance models to determine best predictors of fracture load of the femoral neck including body mass as a covariate

Trait	*n*	Covariate correlation	Linetype	Activity	Linetype × activity	Body mass
Proximal width	46	0.9599 +	0.4789 −	0.4747 +	0.3835	0.0589 +
Head height	46	0.2787 −	0.6047 −	0.3564 +	0.3456	**0.0244** +
Head depth	46	0.7414 +	0.4358 −	0.5258 +	0.3656	0.1002 +
Neck height	46	**0.0013** + [a]	0.2626 −	0.5795 +	0.2050	0.4580 +
Neck depth	46	< **0.0001** + [a]	0.2699 −	0.3464 +	0.0696	0.3631 +
Femur neck angle	46	0.9597 +	0.4778 −	0.4742 +	0.3830	0.0547 +
Neck XS area	44	**0.0003** + [a]	0.6421 −	0.5889 +	0.2657	0.3497 +
Neck I_{max}	44	**0.0049** + [a]	0.5373 −	0.6215 +	0.3389	0.2984 +
Neck I_{min}	44	**0.0099** +	0.5265 −	0.6794 +	0.2572	0.2463 +
Neck I_{max}/C_{min}	44	**0.0104** +	0.5689 −	0.5862 +	0.3475	0.2323 +

p-Values < 0.05 are noted in bold. In the columns *linetype* and *activity*, + indicates selected or wheel access is larger than control or sedentary, respectively; − indicates smaller. The column *covariate correlation* contains *p*-values for the relation of the trait with fracture load while adjusting for all other main effects and body mass. Note that head depth equates to "condyle depth" in previous studies (Garland and Freeman, 2005; Kelly et al., 2006). Degrees of freedom for testing the effects of linetype, activity, and the linetype × activity interaction were always 1 and 6; d.f. for testing the body mass effect depended on sample size and ranged between 1 and 28 and 1 and 30.

[a]One-tailed *p*-values corresponding to *a priori* directional predictions (see text).

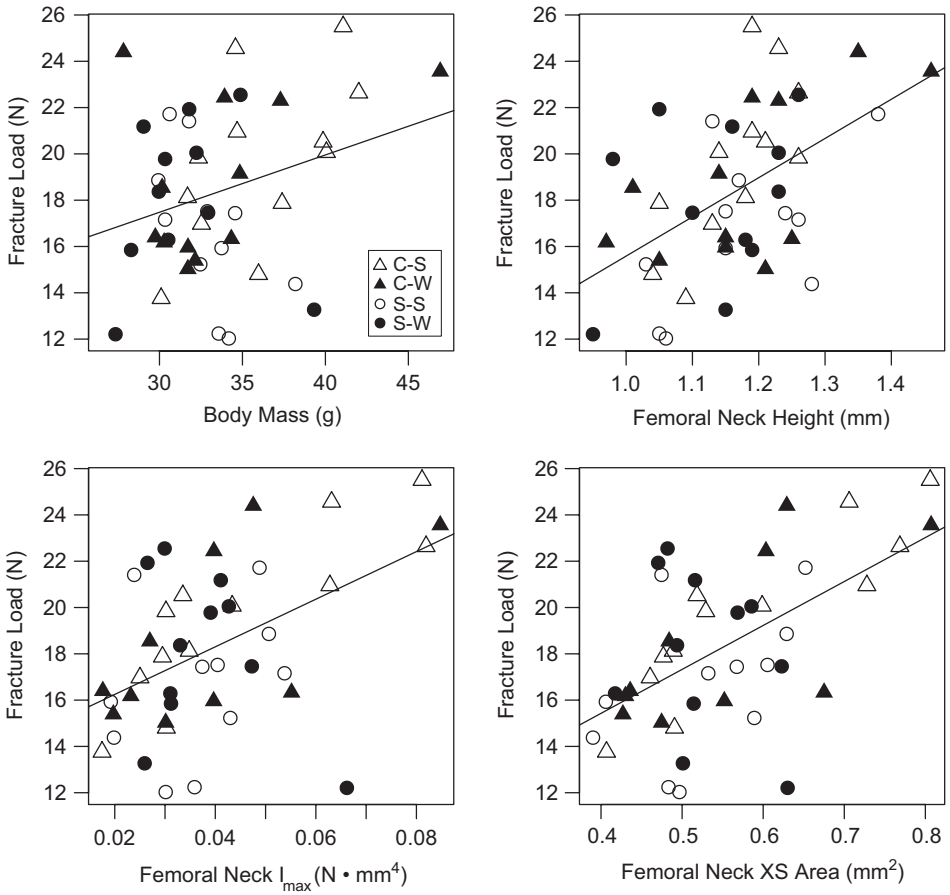

Fig. 2. Relationship between femoral neck fracture load, body mass, and morphometric traits of the femoral neck (height, I_{max}, cross-sectional (XS) area). A significant positive relationship was found with each trait. Points are coded according to ANCOVA grouping by linetype and activity level: control-sedentary (C-S), control-wheel access (C-W), selected-sedentary (S-S), and selected-wheel access (S-W). In each panel, a least squares regression line is drawn through all points. Note that the slope of this line is slightly different from that estimated from the mixed-model ANCOVA; however, because linetype or wheel access effects were not significant, the least squares regression slope is a good approximation.

more across ontogeny and showed a steeper decline than control mice (Fig. 3; Morgan et al., 2003). In all mice, body mass was characterized by rapid early growth during weeks 4 through 20, followed by slower growth to week 84. Mice from selected lines were significantly smaller than controls, and those with wheel access were significantly smaller than those without (Morgan et al., 2003).

Combined effects of genetic background and of exercise on skeletal traits

Of the 19 skeletal traits examined, only two (distal width of the femur, femoral head depth) showed significant effects of linetype, one showed effects of exercise (mediolateral femoral diameter), and one (femoral shaft cross-sectional area) showed a linetype-by-exercise interaction (Tables 2 and 4). These results suggest that neither selection over 16 generations for high levels of voluntary wheel running nor constant physical activity is associated with broad-scale modifications of the mouse femur at the whole-bone level, despite peak activity levels of 76 km/week in exercise-selected mice and 52 km/week in control mice (Fig. 3). However, these results differ substantially from those of previous training studies on mice from different generations of the same selection experiment (Garland and Freeman, 2005; Kelly et al., 2006) as well as twin and family studies in humans (Seeman et al., 1994; Cummings et al., 1995; Arden et al., 1996), which have demonstrated significant genetic underpinnings to the structure and function of bone.

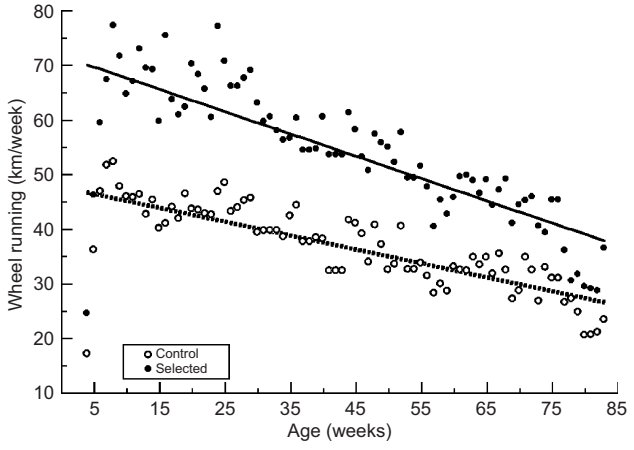

Fig. 3. Ontogeny of wheel running in lines of mice selectively bred for high levels of voluntary wheel running (closed symbols) and in unselected control lines (open symbols). Mean wheel running (km/week) is shown for each week of the 84-week duration. After peaking at 8 weeks of age, wheel running declined linearly in both selected (solid line) and control (dashed line) mice. Selected mice were significantly different from control mice in amount of running and in rate of decline in wheel running (Morgan et al., 2003).

In our study of musculoskeletal loading described here, the lack of significant differences between selected and control lines or between active and sedentary mice is potentially a consequence of the subjects' age. At 84 weeks of age, these animals were almost certainly post-reproductive (Gosden et al., 1983) and had shown a gradual decrease in the level of exercise associated with aging in this study population (Fig. 3). Given the likelihood of post-reproductive status, they may have experienced a decrease in bone strength similar to that observed in ovariectomized mice (Peng et al., 1994; Mosekilde et al., 1998) and in post-menopausal women (e.g., Riggs et al., 1986; Garnero et al., 1996; Ahlborg et al., 2001, 2003).

Additionally, the beneficial effects to bone of repetitive loading are lost after loading ceases (i.e., detraining; Snow et al., 2001; Järvinen et al., 2003). As our study population aged, the number of wheel revolutions per day in the selected mice decreased to levels that may have been below the threshold for exercise-related benefits. Differences in bone morphometrics or strength that may have been present earlier in ontogeny could have been lost.

Musculoskeletal vs. artificial loading

Most studies of bone loading in rodents involve artificial loads exerted by mechanical bending apparatuses, either in axial or four-point bending (Robling and Turner, 2002; Robling et al., 2003; Kesavan et al., 2005; Li et al., 2005; Lau et al., 2006; Sawakami et al., 2006). While this technique does allow for controlled experimental conditions, loading patterns, and very high strains, the loads typically applied (often 10 N or more) are not biologically realistic. Jump-induced impact loading does provide a viable alternative to artificial loading (e.g., Honda et al., 2001; Umemura et al., 2002; Welch et al., 2004), but wheel running is perhaps the most realistic activity behaviorally. An additional drawback of three- or four-point bending is that, under natural conditions, bones are rarely loaded in pure bending but rather in a combination of axial compression and bending (Lovejoy et al., 1976; Biewener et al., 1983; Alexander, 2003; Lieberman et al., 2003, 2004).

The smaller body size of mice relative to other mammals may also be a factor in the apparent absence of large-scale effects of locomotion on bone morphometrics. Whereas, a large body of literature exists regarding skeletal change due to loading in large mammals and birds (e.g., Rubin and Lanyon, 1984; Burr et al., 1998; Rubin et al., 2001; Lieberman et al., 2003, 2004), relatively fewer studies address skeletal mechanics of small mammals (exceptions include Biewener, 1983; Fischer, 1994; Fischer et al., 2002; Witte et al., 2002). These studies have shown that small

mammals generally employ more crouched limb postures than large mammals, which have relatively more erect limb postures that limit moment arms around joints. More crouched postures in small mammals may cause higher joint moments which may be resisted by relatively more massive limb bones, muscular forces, or a combination of both. Thus, in small mammals, such as mice, bone strains resulting from muscular forces may be relatively more important than in large mammals.

Morphometrics – importance of body mass

Previous studies of these mice in similar experimental designs have shown that body mass is lower in exercise-selected lines and is reduced in mice that are given constant wheel access (both selected and control lines; Swallow et al., 1999; Morgan et al., 2003; Swallow and Garland, 2005; Kelly et al., 2006). Our results here are consistent, although the linetype effect did not reach statistical significance (Tables 2 and 3). This discrepancy may be attributable to the greater age of the mice in this study, and/or the relatively small sample sizes. In the previous study (Morgan et al., 2003), with a larger sample of mice, weighed at multiple points across ontogeny, the linetype effect is statistically significant.

Body mass was the major determinant of variation in femoral size, shape, and strength in all of the mice in this study. All traits were positively correlated with body mass, even after accounting for effects of selective breeding and exercise, and 12 of 19 correlations were statistically significant ($p<0.05$, not adjusted for multiple comparisons; Table 2). Of those traits that were not significantly correlated with body mass, two showed significant linetype or activity effects (Table 2).

Although body mass effects were included in the mixed-model ANCOVA analysis (Tables 2 and 4), morphometric traits that are related to load-bearing, such as cross-sectional diameters or areas, are often also normalized to femoral length (Ruff et al., 1993; Trinkaus, 1997). We performed an additional ANCOVA with both body mass and femur length as covariates (results not shown). Because of the high correlation between femur length and body mass, including both yielded little additional information at the expense of a more complicated statistical model, because femur length was rarely a significant covariate.

The widespread correlation of body mass with femoral morphometrics underscores the importance of accounting for body size in studies of the effects of exercise or other environmental factors on the skeleton. Without adequate control for the effects of body size, skeletal traits may appear to vary significantly among groups that experience different exercise regimens when the groups differ primarily in body size alone. Some studies on the effect of exercise or loading on bone do include body mass in statistical analyses (e.g., Gordon et al., 1989; Kannus et al., 1995; Järvinen et al., 2003; Binkley and Specker, 2004), but many do not, even when body mass differs significantly between control and treatment groups (e.g., Shaw et al., 1987; Niehoff et al., 2004; Wu et al., 2004).

Fracture strength and naturalistic loading

Three- or four-point bending tests are frequently employed to assess the mechanical strength of bones and can uncover differences in strength related to genetics, physiology, or experimental treatments (Kodama et al., 2000; Turner and Burr, 2001; Bennell et al., 2002; Akhter et al., 2004a; Silva et al., 2004). Although this methodology has the advantage of lending itself to calculations of the bone's Young's modulus, loading of the shafts of long bones *in vivo* is typically a combination of axial loading and a relatively small amount of bending, transmitted through the joints (Lovejoy et al., 1976; Biewener et al., 1983; Alexander, 2003; Biewener, 2003; Lieberman et al., 2003, 2004).

By loading the head in compression with the distal femur fixed, we mimicked natural loading, and thereby tested the strength of the femoral neck in cantilever bending (Fig. 1). Similar techniques have been used in mice (Akhter et al., 2004b), rabbits (Ohnishi et al., 2003), and humans (Augat et al., 1996). Because of its lower cross-sectional area and moments of inertia, coupled to large bending moments, the femoral neck could be expected to be the most likely site of fracture. This prediction is supported by studies in humans and other mammals (Crawford and Fretz, 1985; Embertson et al., 1986; Zhang et al., 2000; Harasen, 2003) and our experimental results: all femora fractured proximally at the neck, rather than in the shaft.

Some caution is warranted in the functional interpretation of differences in moments of inertia and section moduli. Loading in the femoral shafts and necks consists of a combination of axial and bending forces which shift the neutral axis away from the centroid of the bone (Lovejoy et al., 1976; Biewener et al., 1983; Lieberman et al., 2003, 2004). Without experimental measurement of the neural axis during loading (e.g., Judex et al., 1997; Demes et al., 1998, 2001; Lieberman et al., 2004) it is impossible to know the exact location of the neutral axis of the mouse femur at peak strain.

Although some aspects of gross bone geometry that can influence fracture strength, such as mineralization and collagen fiber orientation, were not quantified in this study, we were able to assess the net effect of variations in bone geometry on load bearing in a manner that is comparable to that experienced by intact organisms. Traits related to the structural geometry of the femoral neck are significantly correlated with

fracture load even after accounting for the effect of body mass (Table 4). These traits include cross-sectional area of the femoral neck, maximum and minimum second moments of inertia, and maximum section modulus. In humans, femoral neck angle is correlated with fracture strength (Gnudi et al., 1999, 2002; Ciarelli et al., 2000; Center et al., 2004); however, in the mice studied here fracture load was not correlated with neck angle. We suggest that these differences in part reflect the differences in the anatomy of human and rodent femora; the human femoral neck is far longer than that of other mammals and is more highly angled with respect to the shaft (Bagi et al., 1997).

Conclusions

We introduce a unique model system to investigate the combined effects of exercise and genetics on femoral structure and performance of mice that have been selectively bred for high levels of voluntary wheel running. We found that body mass was a pervasive correlate of femoral structure, underscoring the importance of statistically controlling for body mass in studies of bone morphometrics. However, we did not find widespread effects of long-term wheel access. This general result differs from many previous studies and may result from differences in loading patterns (natural vs. artificial; postural differences) and allometric effects of body mass in small mammals vs. large ones.

Acknowledgments

The authors thank Scanco USA, Inc. for loan of the μCT system. We also thank E. Mathiowitz for allowing us access to equipment in her lab and E. Edwards for technical assistance. This manuscript benefited from the helpful suggestions of two anonymous reviewers. The project described was supported by NIH Grant no. 1F32AR053008-01 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases to K.M. Middleton, National Science Foundation Grant nos. DEB-0083638, DEB-0105079, and EF-0328594 to P.A. Carter, and IOB-0543429 to T. Garland, Jr. Additional support was provided by the Rhode Island Hospital Orthopedic Foundation and University Orthopedics, Inc.

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