Experimental Evolution and Phenotypic Plasticity of Hindlimb Bones in High-Activity House Mice

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ABSTRACT Studies of rodents have shown that both forced and voluntary chronic exercise cause increased hindlimb bone diameter, mass, and strength. Among species of mammals, "cursoriality" is generally associated with longer limbs as well as relative lengthening of distal limb segments, resulting in an increased metatarsal/femur (MT/F) ratio. Indeed, we show that phylogenetic analyses of previously published data indicate a positive correlation between body mass-corrected home range area and both hindlimb length and MT/F in a sample of 19 species of Carnivora, although only the former is statistically significant in a multiple regression. Therefore, we used an experimental evolution approach to test for possible adaptive changes (in response to selective breeding and/or chronic exercise) in hindlimb bones of four replicate lines of house mice bred for high voluntary wheel running (S lines) for 21 generations and in four nonselected control (C) lines. We examined femur, tibiafibula, and longest metatarsal of males housed either with or without wheel access for 2 months beginning at 25–28 days of age. As expected from previous studies, mice from S lines ran more than C (primarily because the former ran faster) and were smaller in body size (both mass and length). Wheel access reduced body mass (but not length) of both S and C mice. Analysis of covariance (ANCOVA) revealed that body mass was a statistically significant predictor of all bone measures except MT/F ratio; therefore, all results reported are from ANCOVAs. Bone lengths were not significantly affected by either linetype (S vs. C) or wheel access. However, with body mass as a covariate, S mice had significantly thicker femora and tibiafibulae, and wheel access also significantly increased diameters. Mice from S lines also had heavier feet than C, and wheel access increased both foot and tibiafibula mass. Thus, the directions of evolutionary and phenotypic adaptation are generally consistent. Additionally, S-line individuals with the mini-muscle phenotype (homozygous for a Mendelian recessive allele that halves hindlimb muscle mass [Garland et al., 2002, Evolution 56:1267–1275]) exhibited significantly longer and thinner femora and tibiafibulae, with no difference in bone masses. Two results were considered surprising. First, no differences were found in the MT/F ratio (the classic indicator of cursoriality). Second, we did not find a significant interaction between linetype and wheel access for any trait, despite the higher running rate of S mice. J. Morphol. 267:360–374, 2006. © 2005 Wiley-Liss, Inc.

The primary aim of the present study was to compare the effects of selective breeding (21 generations) for high voluntary wheel running in a 6-day test with the effects of chronic wheel access (8 weeks) on hindlimb bone lengths, diameters, and masses of house mice. In other words, we compare the magnitude of evolutionary changes with the magnitude of phenotypic plasticity, with the realization that either type of change may or may not be "adaptive" in the sense of improving organismal function (e.g., locomotor ability) and/or aspects of Darwinian fitness (i.e., lifetime reproductive success).

A major factor in the acquisition and maintenance of vertebrate skeletal mass and strength is mechanical loading (Newhall et al., 1991; Frost, 1997; Huiskes et al., 2000; Mori et al., 2003). Studies using both forced and voluntary exercise with mice and rats have elucidated relations between loading and skeletal architecture, and have demonstrated increases in bone length, diameter, mass, and strength (Siegel and Jones, 1975; Newhall et al., 1991; Iwamoto et al., 1998, 1999; Westerlind et al., 1998; Kodama et al., 2000; Notomi et al., 2000a,b, 2001; Hart et al., 2001; Mori et al., 2003). Histomorphometric studies have indicated that elevated bone mass and strength are associated with both increased bone formation and decreased bone resorption (Westerlind et al., 1998; Iwamoto et al., 1999; Notomi et al., 2000a,b; Hart et al., 2001; Mori et al., 2003). However, the influence of mechanical stimuli on increased bone mass and local bone formation and resorption remains partially unresolved (Mori et al., 2003). Nonetheless, vertebrate bone is widely considered to be an adaptable and "plastic" tissue

within the lifespan of an individual animal, one that can change in response to varying types and amounts of usage.

Although mechanical loading is undoubtedly an important factor in determining bone properties, we note that its effects may have been overstated. In particular, we agree with Bertram and Swartz (1991) that past studies of putatively adaptive responses of bone to altered loading conditions have failed to acknowledge alternative explanations for bone responses and have often failed to note that the effects are usually quite small (<5%). Therefore, in the Discussion we consider the magnitude of such effects in relation to effects of selective breeding.

We are unaware of any other mouse studies of exactly the present type. However, previous studies of both male and female mice that used voluntary tower climbing (Siegel and Jones, 1975; Mori et al., 2003) and forced jumping exercises (Umemura et al., 2002) for 2–9 weeks revealed increases in femoral length (Siegel and Jones, 1975), femoral diameter (Umemura et al., 2002), and bone mineral density (Umemura et al., 2002; Mori et al., 2003). Additionally, 4–6 weeks of voluntary wheel running in female and male rats resulted in significant increases in hindlimb bone mass (Holy and Zerath, 2000) as well as length, width, and volume (Newhall et al., 1991).

With respect to evolutionary adaptations, mammalian skeletons have also received considerable attention. So-called "cursorial" mammals may include those that can run far, fast, or easily (e.g., see Gregory, 1912; Stein and Casinos, 1977; Hildebrand, 1982) and have parasagittally oriented limbs (Jenkins, 1971; Jenkins and Camazine, 1977; Biewener, 1989, 1990). Apparent evolutionary adaptations associated with cursoriality may include a change in foot posture from the primitive plantigrade position to digitigrade or unguligrade, the reduction, compression, or loss of lateral metapodials and phalanges, and the reduction of the range of limb motion to the sagittal plane (Coombs, 1978; Hildebrand, 1982, 1985; Garland and Janis, 1993; Carrano, 1997). Additional features predicted for the limbs of animals that typically move at relatively high speeds or with relatively low energetic costs are: thinner individual limb segments, longer distal limb segments, more proximal muscle insertions along the limb (Carrano, 1999), and increases in articular surface area of joints (Bramble and Lieberman, 2004; see also Garland and Freeman, 2005).

The metatarsal/femur ratio (MT/F) is also used routinely as an index of cursoriality in mammals. This ratio reflects the degree to which distal limb segments are elongated relative to proximal segments, with larger values suggesting increased locomotor abilities. However, analyzes of the relation between MT/F and actual measures of locomotor performance are rare. In a phylogenetic analysis of maximal sprint running speeds of 30 species of un-gulates and 19 species of Carnivora, Garland and Janis (1993) found a weaker relation with MT/F ratio than might have been expected, and indeed weaker than with hindlimb length (corrected for body size). Skeletal correlates of endurance per se have not been examined in mammals, but Harris and Steudel (1997) found no significant correlation between hindlimb length and either home range area or daily movement distance (or prey size) among 65 species of Carnivora. Those authors did not test for correlations with MT/F, but the data of Garland and Janis (1993) can be combined with the home range data presented in Garland et al. (1993) to do so, and that was a secondary aim of the present study. Interestingly, so far as we are aware, no study of bone phenotypic plasticity has ever reported the effects on MT/F.

To examine phenotypic plasticity of hindlimb bones, we housed groups of house mice either with or without access to running wheels for 2 months, beginning shortly after weaning. To examine evolutionary adaptation in response to selection for high daily running distance, we compared mice from four replicate lines that had been selectively bred for high voluntary wheel running (S lines, at generation 21) with four nonselected lines that serve as their controls (C lines) in the breeding program (Swallow et al., 1998a; Garland, 2003). Thus, we compared a total of four experimental groups: mice from C lines housed without wheels (Sedentary); mice from C lines housed with wheels (Active); mice from S lines housed without wheels; and mice from S lines housed with wheels. We also tested for statistical interactions between these two factors, i.e., possible nonadditive effects of selective breeding and exercise, which would constitute a genotype-by-environment interaction.

Based on existing studies of the effects of mechanical loading (see above), we predicted that wheel access would increase hindlimb bone lengths, diameters, and masses. With respect to genetic selection history, we predicted that hindlimb bone lengths and MT/F ratio would be increased in the S lines, whereas bone diameters would be decreased. The ability of mouse bones to respond to selective breeding regimens that involve other traits has been demonstrated previously, as in Hooper's (1977, 1978) work on lines of mice that had been selected for body mass. (As additional demonstrations of genetic effects on bone properties, inbred strains of mice are known to differ in bone properties and in the responsiveness of bone to mechanical loading [e.g., Kodama et al., 2000; Amblard et al., 2003], and strain crosses have been used to identify quantitative trait loci that affect bone properties [e.g., Bouxsein et al., 2004; Lang et al., 2005].) Finally, because mice from the selected lines exhibit substantially elevated levels of wheel running as compared with those from the control lines (next paragraph), they might be expected to exhibit a greater training response, as

has indeed been observed for both muscle enzyme activities and blood hematocrit and hemoglobin content in studies of mice from generation 14 (Houle-Leroy et al., 2000; Swallow et al., 2005).

The experimental evolution protocol began in 1993 from an outbred (genetically variable) base population (Swallow et al., 1998a; Garland, 2003). After 16 generations of selection, high-activity mice ran, on average, ~170% more revolutions per day as compared with mice from the C lines, primarily by running faster, although males also show a significant increase in amount of time spent running per day (Koteja and Garland, 2001). The factorial differential in revolutions/day was maintained through generation 21 and beyond (Garland, 2003). Mice from these lines have been the subject of a variety of physiological and behavioral investigations, as reviewed elsewhere (Garland, 2003; Rhodes et al., 2005; Rezende et al., 2005, 2006a,b; Swallow et al., 2005).

A final aim of the present study was to investigate the effects of a Mendelian recessive allele that halves hindlimb muscle mass (Garland et al., 2002). Two of the S lines have exhibited a dramatic increase in the frequency of this allele. The so-called "mighty mini-muscle" phenotype (representing the double-recessive genotype) shows a variety of pleiotropic effects, including effects on: organ masses, muscle enzyme activities, muscle contractile properties, glycogen depots, and HSP72 expression (Garland et al., 2002; Houle-Leroy et al., 2003; Gomes et al., 2003, 2004; Belter et al., 2004; Swallow et al., 2005; Syme et al., 2005). Mice expressing the mini-muscle phenotype may also run faster on wheels as compared with other mice from the S lines (Syme et al., 2005; present results). The dramatic alteration of muscle mass in conjunction with differences in running speeds (and presumably stride frequencies) might be expected to affect hindlimb bone dimensions and/or masses. Also, this alteration in hindlimb muscle mass may be affecting skeletal architecture throughout ontogeny, especially given that blood vessels and muscles mold the surface of the bones they contact (Lanyon and Rubin, 1985).

MATERIALS AND METHODS
Statistical Analysis of Comparative Data from the Literature

We reanalyzed data presented in Garland and Janis (1993; hindlimb length and MT/F ratio) as well as Garland et al. (1993; home range area) using the phylogenetic tree presented in the latter article. Analyses of these same data, presented in Blomberg et al. (2003), show that all of these traits show highly significant phylogenetic signal (all $P < 0.001$), which is one justification for the use of phylogenetically based statistical analyses. We used both conventional least-squares linear regression analyses and the equivalent with Felsenstein's (1985) method of phylogenetically independent contrasts (see also Garland et al., 1992, 2005). We used the PDTREE program to compute phylogenetically independent contrasts, then imported those contrasts to SPSS (Chicago, IL) for analyses. PDTREE is a part of the PDAP package, available from T.G. on request, and is discussed in various articles, including Garland and Ives (2000).

For the conventional analysis, we first $\log_{10}$-transformed all traits except MT/F. We then regressed each trait on body mass and computed residuals. Finally, we tested whether residual hindlimb length and/or residual MT/F ratio was a significant predictor of residual home range area. In both cases we predicted that they would be positive predictors, so we used one-tailed tests. We also analyzed predictive ability in a multiple regression. For the phylogenetic analysis the same procedure was followed, except that standardized contrasts of log-transformed values were used, and all regressions were computed through the origin, as is required by the contrasts algorithm (Felsenstein, 1985; Garland et al., 1992, 2005).

Subjects From the Selectively-Bred Lines

High voluntary wheel-running mice (generation 21) were studied. The original progenitors were outbred, genetically variable house mice (*Mus domesticus*) of the HSD:ICR strain (Harlan-Sprague-Dawley, Indianapolis, IN). Mice were randomly mated for two generations, paired, and assigned to eight closed lines (10 pairs in each). In each successive generation, when the offspring of these pairs were 6–8 weeks old, they were housed individually with Wahman-type running wheels (circumference = 1.12 m) for 6 days. Daily wheel-running activity was monitored with a computer-automated system. Wheel running was quantified as the total number of revolutions on days 5 and 6 of the 6-day test.

In the four "selected" lines (S) the highest running male and female were chosen from each family as breeders. In the four "control" lines (C), a male and a female were randomly chosen from each family. Within all lines the chosen breeders were randomly paired, except that sibling matings were disallowed. Full details of the selection experiment are provided elsewhere (Swallow et al., 1998a).

To supply animals for the current experiment, generation 21 parents were allowed to produce a second litter (generation 21b) that was not included in the routine selection protocol. At 21 days of age, two male pups from within each of five families within each of the eight lines (n = 80) were weaned, toe-clipped for identification, and housed four/cage. Males were chosen for study because they are substantially larger than females, hence reducing error when measuring bone dimensions via calipers. At 25–28 days of age, mice were housed individually for

8–9 weeks in standard cages, half of which were attached to a running wheel (Wahman-type, 1.12 m circumference), as used in the routine selective breeding protocol. Cages with and without wheels were placed alternately on racks as shown in this photograph: http://www.biology.ucr.edu/people/faculty/Garland/WheelsNoWheels.jpg

Thus, four groups were to be compared: mice from C lines housed without wheels (Sedentary, n = 20); mice from C lines housed with wheels (Active, n = 20); mice from S lines housed without wheels (n = 20); and mice from S lines housed with wheels (n = 20) (families were distributed evenly across treatment groups). Food (Harlan Teklad, Madison, WI, Laboratory Rodent Diet [W] 8604) and water were always available ad libitum. Rooms were controlled for temperature (~22°C) and photoperiod 12/12-h light/dark cycle (lights on at 0700).

The automated counting system recorded revolutions in 1-min bins. From these records, total revolutions/day was computed. As in previous studies (e.g., Koteja and Garland, 2001), we also recorded the number of 1-min bins that included at least one full revolution as an indicator of the amount of time spent running. We divided total revolutions by number of intervals active to estimate average running speed (i.e., rev/min). Finally, we noted the single-highest 1-min interval as an indicator of maximum voluntary running speed. We then computed mean values for the above-mentioned wheel-running traits for the first 6 days of wheel access, for 6 days in the middle of the experiment (days 24–29), and for the last 6 days for which data were available for all individuals (days 47–52).

After 8–9 weeks of wheel access (or not for the Sedentary group), mice were sacrificed by CO_2 a few animals per day to allow harvesting of organs for other purposes. Mice had wheel access up to and including the day of sacrifice. Mean age at sacrifice was 85.6 ± 2.82 (±SD) days (range, 80–91 days). Crown–rump length and mass were measured immediately after sacrifice, and mice were then frozen and stored at –20°C.

At a later date, mice were defrosted, skins and internal organs removed, carcasses dried in an oven, and then placed with a colony of dermestid beetles maintained by the University of Wisconsin Zoological Museum. All procedures used in this study adhered to the guidelines established by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

Bone Measurements

Sixteen hindlimb (femur, tibiafibula, and metatarsal) morphometric traits putatively relevant to locomotion were measured to the nearest 0.01 mm with digital calipers (Mitsutoyo, Kangagawa, Japan) for both left and right bones. The traits were: 1) femoral length, dorsal tip of acetabulum to distal-most end of the medial condyle; 2) tibiafibula length, greatest articular length of tibia, from the medial, proximal articular surface (the cup rather than the edge of the medial condyle to the cup not tip of the medial malleolus of the tibia); 3) metatarsal length, greatest length on the dorsal surface of the metatarsal while still articulated with the proximal end of the digit; 4) anterior-posterior (A-P) depth of femoral acetabulum (see also Garland and Freeman, 2005); 5) femoral least width, at its least constriction and distal to the trochanter muscle scar; 6) femoral least depth, at its least constriction and perpendicular to width; 7) femoral distal width, greatest distal width of the femur at the medial and lateral epicondyles; 8) A-P femoral depth, diameter at midpoint of bone shaft (see also Hull, 2000); 9) transverse femoral width, diameter at midpoint of bone shaft (see also Hull, 2000); 10) tibial proximal width, greatest mediolateral distance across the proximal end of the tibia and parallel to tibial groove, includes little spike on fibular side; 11) tibial proximal depth, greatest A-P depth, perpendicular to width and to tibial groove; 12) tibiafibula least width, least width (mediolateral) across tibia and fibula; 13) tibiafibula least depth, A-P depth across tibia and fibula and perpendicular to width; 14) tibiafibula distal width, greatest width at the distal end of the tibiafibula; 15) A-P tibia depth, diameter at midpoint of bone shaft (see also Hull, 2000); and 16) transverse tibia width, diameter at midpoint of bone shaft (see also Hull, 2000). All values were measured for all 80 skeletons and then measured a second time at a later date. These values were averaged to obtain left and right measures, and those were averaged for statistical analyses. (Left–right symmetry will be the subject of a separate report.) Total hindlimb length was computed as the sum of femur, tibiafibula, and metatarsal lengths. Metatarsal/femur (MT/F) ratio was also analyzed.

Air-dried femora, tibiafibulae, and whole feet were weighed to the nearest 0.0001 gram on an electronic balance (XE-100, Denver Instrument, Denver, CO). Again, all bones were weighed twice for both left and right sides, and values were averaged for analyses.

Statistical Analysis of Bone Measurements

The MIXED procedure in SAS (SAS Institute, Cary, NC) was used to apply nested analysis of covariance (ANCOVA) models (e.g., see Swallow et al., 1999, 2005; Houle-Leroy et al., 2000, 2003). A cross-nested, two-way ANCOVA was used to simultaneously test the effects of linetype (Selected vs. Control lines) and activity (Sedentary vs. Active) on hindlimb bone properties.

In the analysis of bone morphological traits, replicate line (n = 8 total), nested within linetype, was always considered a random effect, and the effect of linetype was tested over the mean squares of line

TABLE 1. *Analysis of wheel-running traits during first 6 days, middle 6 days, and last 6 days*

Trait		P days 1–6	Control	Selected normal	Selected mini	
Revolutions	Linetype	0.1140	1,206	2,078	1,868	LSM
	Mini	0.6782	323.6	343.0	513.4	SE
	Age	**0.0226**				
Minutes	Linetype	0.4506	235	278	270	LSM
	Mini	0.8480	37.4	38.6	50.6	SE
	Age	0.2288				
Mean RPM	Linetype	**0.0140**	5.35	7.95	6.71	LSM
	Mini	0.3304	0.496	0.572	1.116	SE
	Age	0.1406				
Max RPM	Linetype	**0.0202**	12.6	17.5	15.3	LSM
	Mini	0.3792	1.02	1.17	2.23	SE
	Age	0.2821				
Trait		P days 24–29	Control	Selected normal	Selected mini	
Revolutions	Linetype	**0.0004**	4,796	11,618	13,239	LSM
	Mini	0.2650	640.2	733.1	1,247.8	SE
Minutes	Linetype	0.0660	363	487	452	LSM
	Mini	0.4392	38.2	39.8	50.9	SE
Mean RPM	Linetype	**0.0021**	12.4	23.7	30.7	LSM
	Mini	**0.0184**	1.48	1.64	2.56	SE
Max RPM	Linetype	**0.0017**	24.3	40.8	51.9	LSM
	Mini	**0.0051**	2.07	2.27	3.46	SE
Trait		P days 47–52	Control	Selected normal	Selected mini	
Revolutions	Linetype	**0.0042**	5,483	9,667	10,580	LSM
	Mini	0.4828	620.8	698.9	1,146.2	SE
Minutes	Linetype	**0.0220**	378	466	442	LSM
	Mini	0.5032	19.3	21.3	33.6	SE
Mean RPM	Linetype	**0.0063**	14.0	20.8	24.9	LSM
	Mini	0.0744	1.10	1.24	2.00	SE
Max RPM	Linetype	**0.0106**	25.0	34.5	43.1	LSM
	Mini	**0.0147**	1.76	1.95	3.03	SE

Significance levels (*P* values; bold indicates *P* < 0.05) for the effects of both linetype and mini-muscle are presented, as well as least squares adjusted means and standard errors from nested analysis of covariance models implemented in SAS PROC MIXED (n = 38−40 individual mice).

with 1 and 6 degrees of freedom (df). (Family was included as an additional random factor, nested within line.) The two main grouping factors, linetype and activity, were considered fixed effects. Effects of activity and the activity × linetype interaction were tested over the means squares of the activity × line interaction, again with 1 and 6 df. A main effect of the mini-muscle phenotype (see Introduction and Garland et al., 2002; Houle-Leroy et al., 2003) was also included and was tested over the mean square error with 1 and 19 df (or fewer in the case of missing values). Body mass was included as a covariate, also tested with 1 and 19 df. When analyzing body mass, various models were used, some including crown–rump length and age as covariates. Replicate line variation was not investigated but will be the focus of future studies (e.g., Rutledge et al., 1974).

For the wheel-running data, analyses involved only 40 mice (or slightly fewer in the case of wheel malfunctions on some days), none of them siblings. SAS PROC MIXED was again used to compare linetypes (1 and 6 df) and to examine the effect of the mini-muscle phenotype (again tested over the error term with 1 and 31 df). For the first 6 days of wheel running, age (range, 25–28 days) was included as a covariate because it had an effect on revolutions/day.

Because multiple statistical tests were performed on the same set of 80 skeletons, it is appropriate to attempt some adjustment of individual *P* values (significance levels) in order to control the "experiment-wise" type I error rate (probability of rejecting a null hypothesis when it is in fact true). However, the best method for performing such adjustments is controversial (e.g., see review in Curran-Everett, 2000). In addition, the results presented include a mixture of traits about which we had clear a priori directional hypotheses, thus warranting one-tailed tests, and others for which we did not have directional hypotheses. Moreover, some are merely composites of others (e.g., leg length). Therefore, we adopted the expedient approach of presenting two-tailed *P* values, while emphasizing general
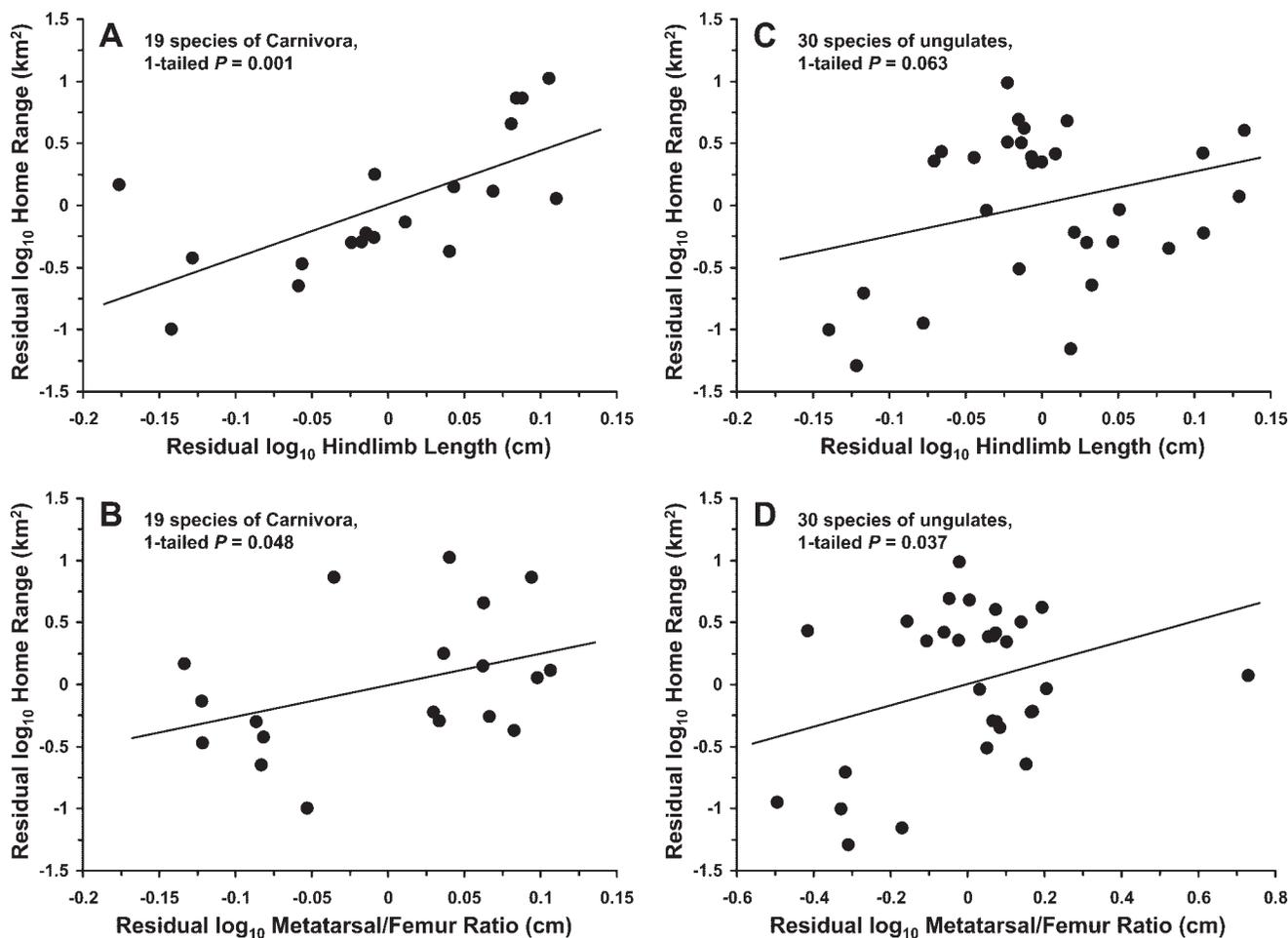
Fig. 1. Conventional least-squares regression of residual $\log_{10}$ home range area (km^2) on residual $\log_{10}$ hindlimb length (cm) and residual metatarsal/femur ratio (cm) for 19 species of Carnivora (**A,B**) and 30 species of ungulates (**C,D**). As described in Materials and Methods, data were complied and analyzed from earlier reports (Garland and Janis, 1993; Garland et al., 1993). See text for results of analyses with phylogenetically independent contrasts.

patterns in the results that seem clear and hence do not depend on details of how P values might be adjusted for multiple comparisons.

RESULTS
Interspecific Covariation of Hindlimb Dimensions and Home Range Area

As shown in Figure 1, the conventional (nonphylogenetic) statistical analysis indicated that residual $\log_{10}$ hindlimb length was a highly significant positive predictor of residual $\log_{10}$ home range area within the Carnivora (one-tailed $P = 0.001$, $r^2 = 0.456$, Fig. 1A) but not within the ungulates (one-tailed $P = 0.063$, $r^2 = 0.082$, Fig. 1C). Residual MT/F ratio was a significant positive predictor of residual $\log_{10}$ home range area within both the Carnivora (one-tailed $P = 0.048$, $r^2 = 0.155$, Fig. 1B) and the ungulates (one-tailed $P = 0.037$, $r^2 = 0.110$, Fig. 1D). To determine whether hindlimb length or MT/F ratio was the better predictor, we performed multiple

regressions. Within the Carnivora, only residual hindlimb length was a significant predictor of residual home range (one-tailed $P = 0.0035$), with the sign of the partial regression for residual MT/F becoming negative (two-tailed $P = 0.517$). Within the ungulates, both partial regression coefficients remained positive, but neither was a significant predictor of residual home range (one-tailed $P = 0.342$ for hindlimb, 0.159 for MT/F).

Analyses with phylogenetically independent contrasts also revealed residual $\log_{10}$ hindlimb length as a significant positive predictor of residual $\log_{10}$ home range area within the Carnivora (one-tailed $P = 0.010$, $r^2 = 0.279$) but not within the ungulates (one-tailed $P = 0.099$, $r^2 = 0.058$). Similarly, residual MT/F ratio was also a significant predictor of residual $\log_{10}$ home range area within the Carnivora (one-tailed $P = 0.037$, $r^2 = 0.176$) but not in the ungulates (the relation was actually negative: two-tailed $P = 0.909$, $r^2 = 0.000$). (Note that r^2 values for conventional and independent contrasts regressions

TABLE 2. Significance levels (P values; bold indicates P <0.05, unadjusted for multiple comparisons) from two-way nested analysis of covariance models implemented in SAS PROC MIXED

Trait	N	Linetype	Activity	Activity × linetype	Mini-muscle	Body mass
Degrees of freedom		1, 6	1, 6	1, 6	1, ~19	1, ~19
Body mass	80	**0.0029**−	**0.0124**−	0.9539	0.0512−	
Body mass	80	**0.0030**−	**0.0106**−	0.9243	0.0766−	0.1695[a]
Body mass	80	**0.0032**−	**0.0178**−	0.4046	**0.0106**−	**<.0001**[b]
Crown-rump length	80	**0.0409**−	0.2562−	0.3414	0.9897−	
Crown-rump length	80	**0.0430**−	0.2566−	0.3323	0.9885+	0.7138[a]
Triceps surae mass	80	0.5255+	0.9344−	0.8422	**<.0001**−	**<.0001**
Lengths						
Femoral length	78	0.7627+	0.7968−	0.9995	0.0519+	**<.0001**
Tibiafibula length	72	0.5170−	0.7989−	0.5305	**0.0074**+	**<.0001**
Metatarsal length	80	0.6075−	0.7722−	0.4405	0.0606+	**<.0001**
Leg length (sum of 3 above)	70	0.9268−	0.9844−	0.7804	**0.0168**+	**<.0001**
Metatarsal/Femur ratio	78	0.8719+	0.7299+	0.5304	0.9588−	0.2036
Diameters						
A-P depth femoral condyle	78	**0.0119**+	**0.0349**+	0.2785	0.6667+	**<.0001**
Femoral least width	80	0.3978+	**0.0059**+	0.4660	**0.0009**−	**<.0001**
Femoral least depth	80	0.1430+	**0.0080**+	0.2941	0.9664−	**<.0001**
Femoral distal width	79	0.2068+	0.0590+	0.7702	0.2569+	**0.0001**
A-P femoral depth	80	0.0807+	**0.0020**+	0.1352	0.8856+	**<.0001**
Transverse femoral width	80	**0.0203**+	0.1742+	0.9423	**0.0052**−	**0.0001**
Tibial proximal width	68	0.1479+	**0.0464**+	0.7496	0.5529−	**0.0002**
Tibial proximal depth	79	**0.0431**+	**0.0028**+	0.5136	0.7387−	**<.0001**
Tibiafibula least width	72	0.1078+	**0.0195**+	0.5870	0.8023+	**0.0024**
Tibiafibula least depth	72	0.7933−	**0.0243**+	0.7443	0.1080−	**0.0144**
Tibia/Fibia distal width	78	0.2440+	**0.0054**+	0.4284	0.0709+	**<.0001**
A-P tibia depth	80	**0.0176**+	**0.0129**+	0.3767	0.1614−	**0.0098**
Transverse tibia width	80	**0.0419**+	0.0552+	0.2255	**0.0021**−	**0.0002**
Bone masses						
Mass of femur	79	0.3999+	0.6674+	0.6823	0.1241−	**<.0001**
Mass of tibiafibula	78	0.0635+	**0.0068**+	0.3666	0.1693−	**<.0001**
Mass of foot (all bones)	80	**0.0488**+	**0.0004**+	0.0709	0.7358+	**<.0001**
Mass of all leg bones	77	0.0615+	**0.0086**+	0.2780	0.2177−	**<.0001**

Signs following *P* values indicate direction of effect: + indicates S lines > C or mice housed with wheels > those housed without.
[a]Age as the covariate for analysis of body mass and crown-rump length.
[b]Crown–rump length as the covariate for analysis of body mass.

are not directly comparable.) In a multiple regression within the Carnivora, only residual hindlimb length was a significant predictor of residual home range (one-tailed $P = 0.0102$), with the sign of the partial regression for residual MT/F becoming negative (two-tailed $P = 0.0685$). In a multiple regression within the ungulates, neither trait was a significant predictor of residual home range (one-tailed $P = 0.0785$ for hindlimb; two-tailed $P = 0.516$ for MT/F with negative sign).

Wheel Running

As expected from numerous previous studies (e.g., Swallow et al., 1998a; Koteja and Garland, 2001; Garland, 2003), S lines ran more than C, although the difference was not statistically significant during the first 6 days of wheel access (Table 1). The increased running of S lines was mainly attributable to their running faster, rather than for more min/day, but a significant increase in time spent running was apparent for days 47–52.

Mice with the mini-muscle phenotype ran significantly faster than S-line mice with normal-sized muscles. No statistically significant effects of the mini-muscle phenotype on rev/day were observed; however, an apparent trend was that the mini-muscle individuals ran fewer revolutions on days 1–6, but more on days 24–29 and on days 47–52.

Body Size and Bone Measurements

For clarity, we first present results comparing the effects of genetic selection history (linetype) and wheel access and then separately consider the effects of the mini-muscle phenotype (Tables 2, 3). All significance levels are presented in Table 2, whereas least-squares adjusted means and standard errors are presented in Table 3.

In our experimental design, interactions between linetype and access to running wheels would constitute genotype-by-environment interactions. No trait measured in this study showed statistically significant interactions (Table 2). Body mass was a highly significant positive predictor of all bone measures (Table 2) except MT/F ratio ($P = 0.2036$).

TABLE 3. *Least-squares means and standard errors from SAS PROC MIXED, corresponding to tests presented in Table 2*

| | Control lines | | | | High-runner lines | | | | Mini-muscle | | | |
| | Sedentary | | Active | | Sedentary | | Active | | Normal | | Mini | |
Trait	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body mass (g)	33.8	0.82	32.5	0.82	29.6	0.71	28.3	0.69	32.1	0.44	30.0	0.98
Body mass (g)	33.8	0.81	32.6	0.82	29.7	0.71	28.4	0.68	32.0	0.44	30.2	0.98
Body mass (g)	33.1	0.63	31.9	0.62	29.8	0.52	29.1	0.51	32.1	0.31	29.9	0.77
Crown-rump length	105.4	1.18	105.2	1.18	102.8	1.06	101.3	1.04	103.7	0.63	103.7	1.32
Crown-rump length	105.4	1.19	105.3	1.19	102.9	1.07	101.3	1.04	103.7	0.63	103.7	1.33
Triceps surae mass (g)	0.1305	0.00428	0.1299	0.00418	0.1337	0.00403	0.1339	0.00418	0.1633	0.00243	0.1007	0.00442
Lengths (mm)												
Femoral length	15.37	0.174	15.35	0.172	15.44	0.166	15.42	0.171	15.24	0.104	15.55	0.172
Tibiafibula length	17.67	0.109	17.69	0.107	17.62	0.100	17.56	0.103	17.45	0.054	17.82	0.121
Metatarsal length	7.38	0.065	7.33	0.063	7.39	0.059	7.40	0.061	7.30	0.033	7.45	0.072
Leg length	40.40	0.310	40.44	0.309	40.40	0.290	40.36	0.298	39.98	0.176	40.82	0.328
Metatarsal/Femur ratio	0.4797	0.00590	0.4789	0.00583	0.4791	0.00559	0.4820	0.00577	0.4801	0.00342	0.4798	0.00591
Diameters (mm)												
A-P depth femoral	1.444	0.0138	1.473	0.0135	1.511	0.0128	1.523	0.0136	1.485	0.0074	1.491	0.0150
Femoral least width	1.630	0.0311	1.687	0.0306	1.675	0.0294	1.714	0.0303	1.730	0.0197	1.623	0.0314
Femoral least depth	1.355	0.0236	1.410	0.0234	1.419	0.0230	1.449	0.0234	1.409	0.0148	1.408	0.0202
Femoral distal width	2.698	0.0445	2.731	0.0439	2.774	0.0425	2.817	0.0437	2.733	0.0273	2.777	0.0436
A-P femoral depth	1.265	0.0259	1.322	0.0256	1.349	0.0246	1.380	0.0252	1.327	0.0161	1.331	0.0253
Transverse femoral	1.646	0.0289	1.667	0.0280	1.751	0.0260	1.774	0.0275	1.761	0.0147	1.657	0.0335
Tibial proximal width	3.355	0.0397	3.433	0.0390	3.435	0.0354	3.495	0.0376	3.444	0.0178	3.415	0.0453
Tibial proximal depth	3.016	0.0356	3.113	0.0350	3.139	0.0334	3.214	0.0347	3.126	0.0201	3.114	0.0371
T/F least width	1.282	0.0184	1.324	0.0180	1.328	0.0160	1.357	0.0170	1.320	0.0080	1.326	0.0219
T/F least depth	0.920	0.0180	0.962	0.0179	0.919	0.0165	0.952	0.0169	0.953	0.0091	0.923	0.0181
T/F distal width	1.385	0.0163	1.432	0.0157	1.417	0.0150	1.449	0.0159	1.404	0.0084	1.437	0.0179
A-P tibia depth	1.053	0.0226	1.111	0.0219	1.147	0.0200	1.181	0.0212	1.143	0.0110	1.104	0.0261
Transverse tibia	1.361	0.0240	1.422	0.0233	1.452	0.0221	1.469	0.0233	1.471	0.0115	1.381	0.0261
Bone masses (g)												
Mass of femur	0.0449	0.00136	0.0456	0.00132	0.0467	0.00127	0.0467	0.00133	0.0471	0.00071	0.0449	0.00144
Mass of tibiafibula	0.0365	0.00103	0.0402	0.00100	0.0397	0.00093	0.0420	0.00098	0.0404	0.00047	0.0388	0.00111
Mass of foot	0.0379	0.00092	0.0433	0.00088	0.0416	0.00081	0.0445	0.00087	0.0416	0.00040	0.0420	0.00110
Mass of all leg bones	0.1187	0.00276	0.1286	0.00266	0.1277	0.00247	0.1330	0.00265	0.1291	0.00119	0.1249	0.00312

Effects of Selective Breeding and of Wheel Access. As expected from previous studies: (e.g., Swallow et al., 1999, 2001), mice from S lines were lighter than C, and wheel access reduced body mass of both groups, with no effect of age (Table 2). When crown–rump length was used as a covariate, both effects on body mass were still significant. Crown–rump length itself was significantly shorter in the S lines but was unaffected by wheel access.

Once the effects of body mass were controlled statistically, no bone length was significantly affected by either selection history or wheel access, nor did any length measure even show a trend to be affected (Table 2). The classic indicator of cursoriality in mammals, MT/F ratio, also was unaffected by either factor (and did not correlate with body mass). However, both selection history and wheel access increased bone diameters and masses, with the effects of wheel access being generally more pervasive (most *P* values smaller for effect of wheel access), especially for masses. Additionally, the interaction between linetype and activity was never statistically significant (Table 2).

Figures 2–7 illustrate different patterns for different measures. As shown in Figures 2 and 5, femoral mass and tibiafibula length did not vary in relation to linetype or wheel access. Figures 3 and 6, respectively, illustrate that wheel access significantly increased tibiafibula mass (two-tailed *P* = 0.0068) and femoral least depth (two-tailed *P* = 0.0080). Finally,

selection history (two-tailed *P* = 0.0119) and wheel access (two-tailed *P* = 0.0349) both affected A-P femoral condyle depth (Fig. 7). Interestingly, mice from selected lines had significantly heavier feet (Fig. 4) (two-tailed *P* = 0.0488), while wheel access also had a strong positive effect on foot mass (two-tailed *P* = 0.0004).
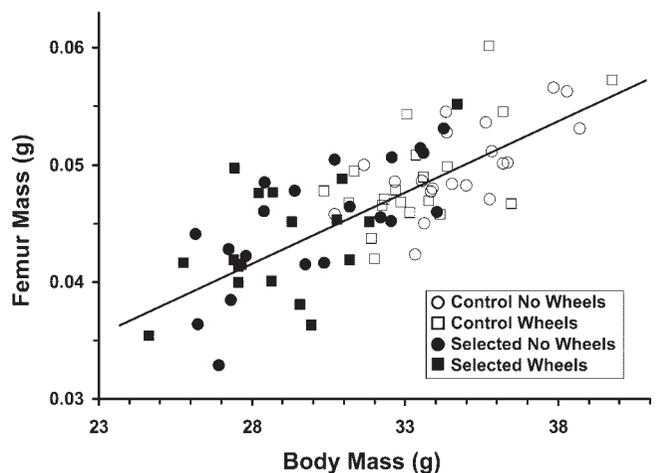

Fig. 2. Relation between femur mass (mean of left and right legs) and body mass for four experimental groups of mice (see text). ANCOVA (Table 2) revealed that body mass was a highly significant predictor of femur mass, with no significant effects of either linetype (S vs. C) or wheel access. Solid line is simple least-squares linear regression.
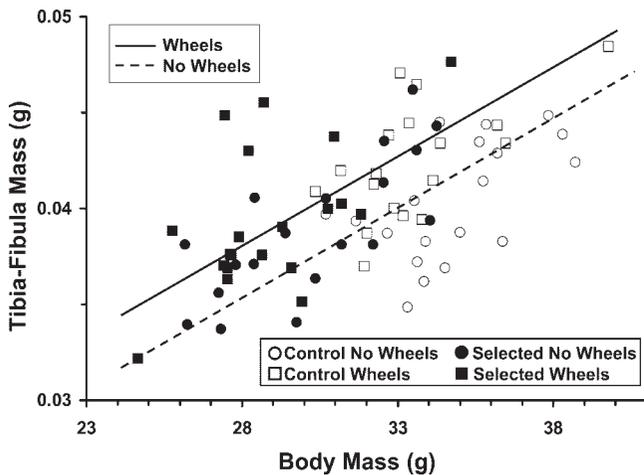
Fig. 3. Tibiafibula mass in relation to body mass. ANCOVA revealed that activity (wheels vs. no wheels) had a significant positive effect on tibulafibula mass, with body mass being a significant covariate (Table 2). Linetype (S vs. C) and the mini-muscle phenotype, however, had no significant effect. Lines shown correspond to a nested ANCOVA similar to those reported in Table 2, but after removing the mini-muscle and linetype variables.
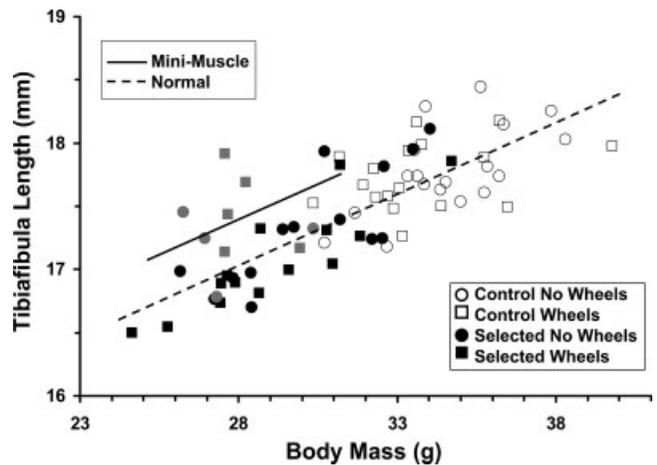

Fig. 5. Tibiafibula length in relation to body mass. ANCOVA revealed that body mass was a highly significant predictor of tibiafibula mass with no significant effects of either linetype (S vs. C) or activity (wheels vs. no wheels) (Table 2). The mini-muscle phenotype, however, had a significant positive effect on tibiafibula length. Lines shown correspond to a nested ANCOVA similar to those reported in Table 2, but after removing the linetype and activity variables.

Effects of the Mini-Muscle Phenotype. Of the 80 mice examined, a total of nine were classified as having the mini-muscle phenotype (three in selected line 3 [lab designation] and the others in selected line 6). This result is consistent with the known line-specific occurrence of the phenotype as described previously (Garland et al., 2002). Two of the mini-muscle mice in line 3 and three of them in line 6 were housed with wheels. Thus, although few mice overall had the mini-muscle phenotype, it was possible to test for its effects.

Mice with the mini-muscle phenotype were significantly lighter in body mass: a similar difference has been reported in some other samples from these lines (Garland et al., 2002), although not in all samples (Houle-Leroy et al., 2003). Mini-muscle mice did not, however, show reduced crown–rump length. By definition, the "triceps surae" (soleus + medial and

Fig. 4. Foot mass in relation to body mass. ANCOVA revealed that both linetype (S vs. C) and activity (wheels vs. no wheels) had significant positive effects on foot mass, with body mass also being a significant predictor (Table 2). The mini-muscle phenotype, however, had no significant effect. The significance level for the interaction between linetype and activity was $P = 0.0709$. Lines shown correspond to a nested ANCOVA similar to those reported in Table 2, but after removing the mini-muscle variable.
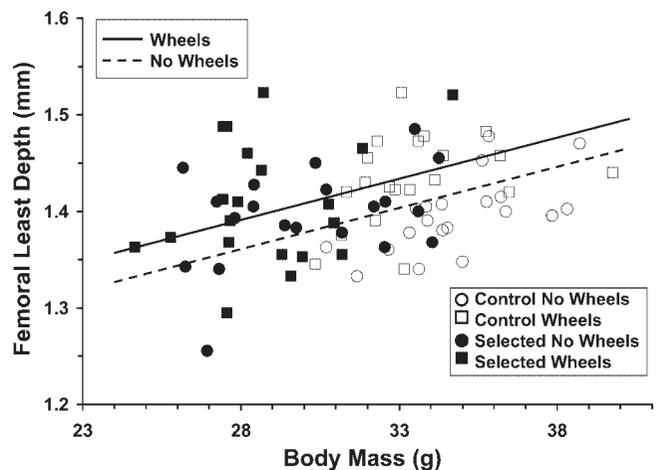

Fig. 6. Femoral least depth (and perpendicular to width) in relation to body mass. ANCOVA revealed that activity (wheels vs. no wheels) had a significant positive effect, with body mass also being a significant predictor (Table 2). Linetype (S vs. C) and the mini-muscle phenotype, however, had no significant effect. Lines shown correspond to a nested ANCOVA similar to those reported in Table 2, but after removing the mini-muscle and linetype variables.
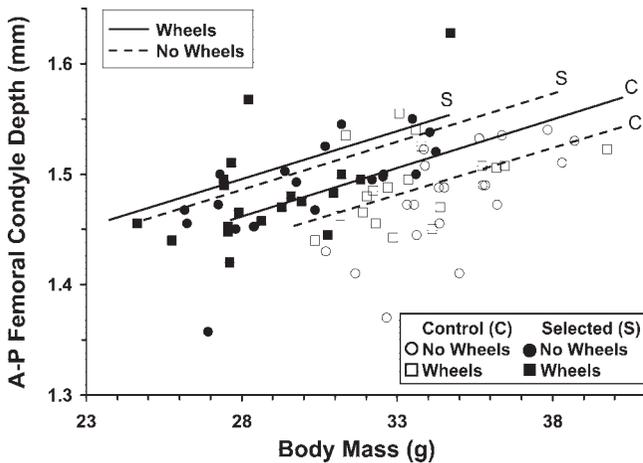
Fig. 7. Anterior-posterior (A-P) femoral condyle depth in relation to body mass (see also Garland and Freeman, 2005). ANCOVA revealed that both linetype (S vs. C) and activity (wheels vs. no wheels) had significant positive effects on A-P femoral condyle depth, with body mass also being a significant predictor (Table 2). The mini-muscle phenotype, however, had no significant effect. The interaction between linetype and activity was not significant ($P = 0.2785$). Lines shown correspond to a nested ANCOVA similar to those reported in Table 2, but after removing the mini-muscle variable.

decline in total revolutions as the study continued, whereas C mice did not exhibit the decline. Similar patterns have been reported previously (Swallow et al., 1998b; Koteja et al., 1999; Dumke et al., 2001; Morgan et al., 2003). The greater differential in total running distance of S and C lines observed in the middle of the study would be expected because it corresponds approximately with the age at which mice are tested on wheels during the routine selection protocol.

Effects of Wheel Access

For both S and C lines, access to running wheels for 8 weeks caused a significant decrease in body mass but not body length. Wheel access also caused an increase in body mass-adjusted diameters of the femur and tibiafibula, and masses of the tibiafibula and foot, although it did not affect femur mass (Table 2). However, it is noteworthy that the activity-related increases in the above traits (femur and tibiafibula diameters) were quite small (Fig. 8), which is consistent with previous literature on bone modeling adaptation (Biewener and Bertram, 1993, 1994). Conversely, some of the effects of wheel access on bone masses were larger (femur +0.8%, tibi-
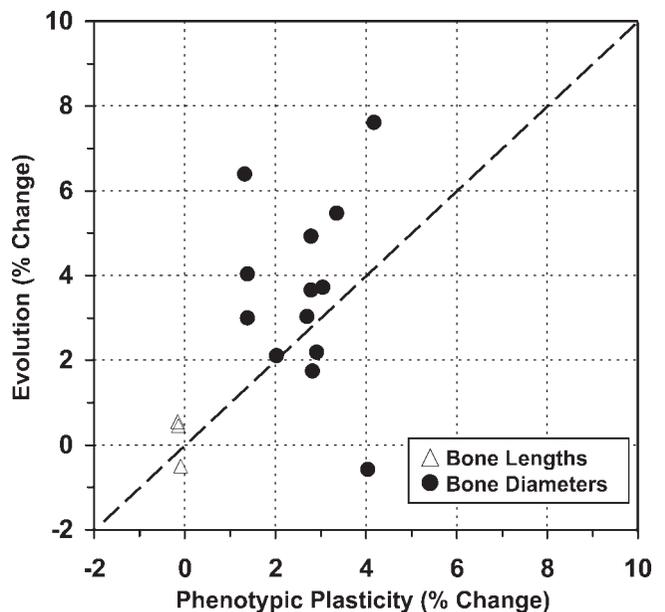
lateral gastrocnemius + plantaris) was significantly reduced in mass in the mini-muscle individuals, resulting in a nearly halving of the mass of this skeletal muscle group (Tables 2, 3). Mice with mini-muscles had longer and thinner hindlimb bones (Fig. 5), but showed no difference in bone masses as compared with unaffected individuals.

DISCUSSION

This study reveals two major findings. First, both voluntary physical activity and selective breeding for high activity levels alter hindlimb bone dimensions, and in similar ways: increases in diameters and masses, but no effect on lengths or MT/F ratio. Second, a Mendelian recessive allele that halves hindlimb muscle mass—but has been favored by the selection protocol—also affects hindlimb bones, making them longer and thinner, with no significant change in mass. We discuss these effects in turn.

Wheel Running

During 8 weeks of wheel access, mice from S lines always ran more than did C lines, as expected from numerous previous studies (e.g., Swallow et al., 1998a; Koteja and Garland, 2001; Garland, 2003). As in previous studies, the difference was primarily achieved by an increase in average speed, but a significant difference in amount of time spent running was also apparent, especially during the last week. There was a general trend for S mice to increase in total revolutions from days 1–29, and then

Fig. 8. Comparison of the effects of 21 generations of selective breeding for high voluntary wheel running ("Evolution") with the effects of eight weeks of wheel access ("Phenotypic Plasticity"), based on least-squares (adjusted) means presented in Table 3. Dashed line represents a 1:1 relation. Bone lengths were unaffected by either factor (significance levels are in Table 2). However, both selective breeding (one exception) and wheel access had positive effects on bone diameters, with the former having somewhat larger effects, on average. As noted in the Discussion, the magnitude of training effects is similar to what has been reported previously for mammalian bone in response to either forced or voluntary exercise.

afibula +7.9%, foot +10.5%). The effects on bone diameter may possibly be attributable to a decreased resorption and an increase in bone formation, as has been reported as a result of voluntary tower climbing in male mice (Mori et al., 2003). Moreover, the effects on mass and diameter are generally consistent with findings of previous studies of rodents (see Introduction; Siegel and Jones, 1975; Newhall et al., 1991; Iwamoto et al., 1998, 1999; Westerlind et al., 1998; Kodama et al., 2000; Notomi et al., 2000a,b, 2001; Hart et al., 2001; Mori et al., 2003). Newhall et al. (1991) demonstrated increases in length, width, mass, and mineral content (femur and tibiafibula) as a result of 6 weeks of voluntary wheel running in adult male rats. Additionally, 2–4 weeks of voluntary tower climbing caused increases in mineral content, cross sectional area, and moments of inertia in the femora of male mice (Mori et al., 2003). Although Mori et al. (2003) found significant differences in climbing distance among experimental groups, body mass, femoral length, and femoral volume were not affected.

Although we might have expected mice from S lines to experience greater training effects as compared with C mice, given that they ran significantly more revolutions per day (Table 1), we found no evidence for a genotype–environment interaction. This may possibly be explained using the three fundamental "rules" (according to Turner, 1998) that govern bone adaptation by mechanical loading: 1) it is driven by dynamic loading (as opposed to static); 2) only a short duration of mechanical loading is needed to initiate an adaptive response; and 3) bone cells become accustomed to a routine loading environment which makes them less responsive to habitual mechanical stimuli. Throughout the 8 weeks of wheel access, both S and C mice presumably experienced a redundant loading environment, which may have made bone cells less responsive to the routine mechanical stimuli, regardless of how much S lines ran versus C lines. Furthermore, neither S or C lines experienced distributed loading "bouts" with significant rest periods inserted within each loading cycle, which as been previously reported to enhance the osteogenic potential by restoring mechanosensitivity (Robling et al., 2001, 2002a,b; Srinivasan et al., 2002). This restoration of mechanosensitivity was demonstrated by Mori et al. (2003, p. 2008) as male mice voluntarily climbed for ~12 min/day, which accordingly "…seem to provide sufficient regulation of bone marrow osteogenic and osteoclastogenic cells in mice, allowed the recovery of their responses during the climbing 'bouts' for the initial 2 weeks after the start of exercise." Mice from the S lines do run more intermittently that C (only females have been studied), but differences in the average length of pauses within peak wheel-running bouts are only about 5 sec in length (Girard et al., 2001).

Ours is apparently the first study of the effects of voluntary wheel running on mouse bone. Wheel access for 8 weeks did not significantly alter any bone length. These results are consistent with previous studies of femurs and tibias of both male and female mice that used voluntary tower climbing (Mori et al., 2003) and forced jumping exercises (Umemura et al., 2002) for 2–4 weeks. However, 6 weeks of voluntary wheel access in male rats (Newhall et al., 1991) and 9 weeks of voluntary tower climbing in mice (Siegel and Jones, 1975) both significantly increased femoral length.

Effects of Selective Breeding

After 21 generations, replicated selection for high voluntary locomotor activity led to a significant reduction in body size (mass and length), an increase in the body mass-adjusted diameters of the femur and tibiafibula, and an increase in the mass of the foot and possibly of the tibiafibula (Table 2). These results of experimental evolution are only partly consistent with what would be expected based on comparative studies of interspecific variation in mammals. With respect to body size, most studies of mammalian home range area and of daily movement distance show positive relations with body size (e.g., Garland, 1983; Goszczynski, 1986; Carbone et al., 2005). However, as reviewed by McLoughlin and Ferguson (2000), home range area can sometimes exhibit a negative relation with body mass among populations within a species (and among individuals within a population). Thus, our finding that body size has decreased as activity level increased is not unprecedented in the comparative literature (see also Dewsbury, 1980; Swallow et al., 1999; Garland, 2003).

As discussed in the Introduction, the only previous comparative study found no correlation between (size-corrected) hindlimb length and either home range area or daily movement distance among species of Carnivora (Harris and Steudel, 1997). In contrast (Fig. 1), we found a significant positive association between residual hindlimb length and MT/F ratio in a sample of 19 species of Carnivora, but not within a sample of 30 species of ungulates (data from Garland and Janis, 1993; Garland et al., 1993). The discrepancy between the two studies may be a result of the analysis of different datasets (including differing numbers of species and differing sources regarding home range size and limb lengths). In any case, our positive results bolster the hypothesis that increases in both hindlimb length and MT/F ratio should occur as a correlated response to selective breeding for high locomotor distances. Finally, although MT/F would be predicted to evolve in conjunction with increased running speed and distance (e.g., Garland and Janis, 1993; Carrano, 1999; present comparative analyses), including in rodents

(Steudel and Beattie, 1993), we found no evolutionary change.

Given that the S lines run for long distances, long periods of time, and at relatively high speeds, they could reasonably be considered "cursors" on behavioral or performance grounds, according to some previous definitions (e.g., see Gregory, 1912; Stein and Casinos, 1977; Hildebrand, 1982). However, considering the traits measured in the present study, S lines display few of the putative hindlimb adaptations associated with "cursoriality." According to Carrano (1999), cursorial (moving at greater speeds with less energetic costs) animals should have thinner individual limb segments, longer distal leg segments, and more proximal muscle insertions along the limb. All else being equal, a reduction in limb mass should reduce the cost of locomotion, and more energetically efficient locomotion should generally be favored by selection. Moreover, lightening of distal limb segments would increase the relative proportion of total proximal muscle mass, resulting in a more efficient rotation of the foot during the support phase of the stride (Carrano, 1999). However, lighter bones will also be less strong, all else being equal. Opposite of those predictions, S lines generally displayed thicker and shorter hindlimbs, and a trend for heavier hindlimbs with significantly heavier feet (Table 2). Heavier feet may be a result of the advantage that an increase in grip strength would yield in running on wheels constructed of wire mesh. Future experiments will examine muscle insertion location. Finally, in *Homo* (compared with *Pan* and *Australopithecus*), articular surface areas relative to body mass in most joints of the lower body (e.g., femoral head and knee, the sacroiliac joint, and the lumbar centra) have increased, presumably because they lower joint stress and facilitate endurance running (Rose, 1984; Jungers, 1988; Ruff et al., 1998; Sanders, 1998) (defined as running many kilometers over extended time periods using aerobic metabolism [Bramble and Lieberman, 2004]). Mice from our S lines do display thicker anterior-posterior depth of the femoral condyle (Table 2), and this presumably decreases joint stress during locomotion (see also Garland and Freeman, 2005).

Given that at least some dimensions of mouse hindlimb bones clearly can respond to selection on other traits (Rutledge et al., 1974; Hooper, 1977, 1978; present study), it seems surprising that several expected responses were not observed in our selection experiment. Several factors might explain this (see also Garland and Freeman, 2005). First, mice are small, and some have suggested that cursorial adaptations are only likely to be present in mammalian species with large body sizes (see Carrano, 1999). Steudel and Beattie (1993) also point out that the reduction, compression, or loss of lateral metapodials and phalanges and the reduction in joint mobility occur (often in conjunction with other putative indicators of "cursoriality") only at large body sizes. However, Steudel and Beattie (1993) argue that total limb length and MT/F show little size-specific proportional differences across a wide range of body sizes in mammals. Second, selection for high levels of straight-line running on a virtually smooth wire mesh surface does not require the use of lateral movements to avoid impediments, as would be the case in the natural world. Third, our mice are always provided food ad libitum, so they never face energy shortages that might occur in nature and impose selection related to locomotor efficiency (see also Rezende et al., 2006b). Another possible explanation for a lack of "cursorial adaptations" in the hindlimbs of S mice is that they have instead evolved differences in running behavior and/or posture (e.g., see Steudel and Beattie, 1993). Indeed, Girard et al. (2001) found that the S lines run more intermittently (see also Rezende et al., 2006b), which could have effects on endurance and/or energetic costs of locomotion, as well as bone growth and remodeling.

The finding that our Selected lines of mice have evolved thicker hindlimb bones is clearly counter to expectations for cursorial animals. One possible explanation is that S mice may exhibit an elevated home-cage activity (Rhodes et al., 2001, 2005), which causes increases in bone thickness and mass, and that this phenotypic plasticity overrides any genetic tendency of S mice to have thinner bones. This hypothesis could be addressed by detailed monitoring of home cage activity in conjunction with longitudinal sampling of bone.

Effects of the Mini-Muscle Phenotype

Individuals exhibiting the "mini-muscle" phenotype are homozygous for a Mendelian recessive that causes an ~50% reduction in hindlimb muscle mass and cross-generational data indicate that this allele has been favored by the selective breeding protocol (Garland et al., 2002). When homozygous (heterozygotes cannot presently be identified), the mini-muscle allele has a variety of pleiotropic effects, in addition to its general effects on hindlimb muscle mass, many of which could be advantageous in the context of selective breeding for high-endurance running. These effects include higher activities of aerobic enzymes per gram of mixed hindlimb muscle, more glycogen per gram of soleus and gastrocnemius muscle, increased fatigue resistance of medial gastrocnemius, larger soleus muscles (unlike most other muscles in the hindlimb), larger hearts, and reduced body mass (Garland et al., 2002; Houle-Leroy et al., 2003; Gomes et al., 2003, 2004; Belter et al., 2004; Swallow et al., 2005; Syme et al., 2005). The spectrum of pleiotropic effects might be further conceptualized as "direct" vs. "indirect" effects, with a possible continuum in between. To illustrate what we mean, consider the effect on heart mass. It could be considered a "direct" pleiotropic effect if the allele

S.A. KELLY ET AL.

is actually causing changes in expression of genes that directly control growth and maintenance of heart muscle. It could be indirect if, for example, the mini-muscles offer greater resistance to blood flow, which necessitates increased blood pressure that leads to cardiac hypertrophy.

In the present study, we found that individuals exhibiting the "mini-muscle" phenotype had longer and thinner hindlimb bones (femur and tibiafibula: Table 2). Interestingly, these alterations correspond with two of the classic indicators of cursoriality as discussed above, although MT/F ratio and bone masses were unaltered. Thus, it is possible that the mini-muscle allele has been favored in part because of its effects on bone shape. However, we also found that mini-muscle mice ran significantly faster on wheels (Table 1; see also Syme et al., 2005), and this alone or in combination with the differences in muscle mass (Lanyon and Rubin, 1985) might affect bone shape during ontogeny. Because of small sample sizes, we were unable to test for possible interactive effects of wheel access and the mini-muscle phenotype. We also do not know if mini-muscle mice might be more active (e.g., move faster) when housed in cages without wheels, although we have some evidence that Selected mice in general exhibit more intense home-cage activity (see Rhodes et al., 2001, 2005). Another possibility is that the longer, thinner (although not significantly lighter) bones of mini-muscle individuals are conducive to higher running speeds. In any case, the manifold effects of the mini-muscle allele on whole-animal performance, muscle properties, and bone shape deserve further study.

ACKNOWLEDGMENTS

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