

RESEARCH ARTICLE

WILEY

JOURNAL OF
morphology

Evolution of hindlimb bone dimensions and muscle masses in house mice selectively bred for high voluntary wheel-running behavior

Alberto A. Castro | Theodore Garland, Jr. 

Department of Evolution, Ecology, and Organismal Biology, University of California, Riverside, Riverside, California 92521

Correspondence

Theodore Garland, Department of Evolution, Ecology, and Organismal Biology, University of California, Riverside, Riverside, CA 92521.
Email: tgarland@ucr.edu

Funding information

N.S.F., Grant/Award Number: DEB-1655362

Abstract

We have used selective breeding with house mice to study coadaptation of morphology and physiology with the evolution of high daily levels of voluntary exercise. Here, we compared hindlimb bones and muscle masses from the 11th generation of four replicate High Runner (HR) lines of house mice bred for high levels of voluntary wheel running with four non-selected control (C) lines. Mass, length, diameter, and depth of the femur, tibia-fibula, and metatarsal bones, as well as masses of gastrocnemius and quadriceps muscles, were compared by analysis of covariance with body mass or body length as the covariate. Mice from HR lines had relatively wider distal femora and deeper proximal tibiae, suggesting larger knee surface areas, and larger femoral heads. Sex differences in bone dimensions were also evident, with males having thicker and shorter hindlimb bones when compared with females. Several interactions between sex, linetype, and/or body mass were observed, and analyses split by sex revealed several cases of sex-specific responses to selection. A subset of the HR mice in two of the four HR lines expressed the mini-muscle phenotype, characterized mainly by an ~50% reduction in hindlimb muscle mass, caused by a Mendelian recessive mutation, and known to have been under positive selection in the HR lines. Mini-muscle individuals had elongated distal elements, lighter and thinner hindlimb bones, altered 3rd trochanter muscle insertion positions, and thicker tibia-fibula distal widths. Finally, several differences in levels of directional or fluctuating asymmetry in bone dimensions were observed between HR and C, mini- and normal-muscled mice, and the sexes. This study demonstrates that skeletal dimensions and muscle masses can evolve rapidly in response to directional selection on locomotor behavior.

KEYWORDS

artificial selection, exercise, experimental evolution, locomotion, skeleton

1 | INTRODUCTION

Locomotion places more demands on the limbs than does any other behavior (Biewener, 1990). Limb bones transmit muscular and propulsive forces, support the axial skeleton, and exhibit phenotypic plasticity in response to loading during locomotion (Gosnell, Butcher, Maie, & Blob, 2011; Kelly, Czech, Wight, Blank, & Garland, 2006; Middleton, Kelly, & Garland, 2008). Therefore, limb bones may be expected to show evidence of evolutionary coadaptation with locomotor behavior and ecology.

In mammals, numerous studies have provided evidence of coadaptation between the skeleton and locomotor behavior or performance

ability. For example, animals that run fast and/or for long distances are often considered "cursorial" (Gregory, 1912; Stein & Casinos, 1997). Some of the most emblematic cursorial mammalian lineages, such as Carnivora, Perissodactyla, and Artiodactyla, have evolved a high metatarsal-femur (MT/F) ratio that is postulated to increase locomotor speed and/or efficiency, and a high MT/F ratio has therefore often been used as a proxy to identify cursorial species (Carrano, 1999; Coombs, 1978; Gambaryan, 1974; Garland & Janis, 1993; Hildebrand, 1974; Howell, 1944; Lovegrove & Mowoe, 2014; Smith & Savage, 1956). Other aspects of limb morphology have also been associated with cursoriality, including elongated distal limb bones, elevated foot posture, more proximally located muscle masses, more proximal muscle

insertions relative to bone length (closer to hip or shoulder joint), and thinner and lighter limb bone elements (Carrano, 1999; Samuels, Meachen, & Sakai, 2013; Van Valkenburgh, 1987).

Some of the putative indicators of cursoriality seem to be associated with body size (Carrano, 1999; Middleton, Kelly, et al., 2008) and/or phylogeny (Garland & Janis, 1993), rather than only with high locomotor performance, and some workers suggest that cursorial adaptations are present only in larger-bodied animals (e.g., see Carrano, 1999). However, others have argued that small-bodied mammals do sometimes exhibit cursorial adaptations (Steudel & Beattie, 1993). For example, elephant shrews and cursorial lagomorphs have evolved elongated distal limb bones and more gracile limb elements (Lovegrove & Mowoe, 2014; Young, Danczak, Russo, & Fellmann, 2014). Furthermore, "cursorial" lagomorphs have lower limb joint mechanical advantages, which may allow increased limb output velocity and faster cycling of limbs (Young et al., 2014). However, distal limb bone robusticity did not differ between "cursorial" and non-cursorial lagomorphs, which suggests the greater importance of bone strength versus locomotor economy at the distal end of long bones (Young et al., 2014). In any case, the rich literature associated with studies of cursoriality provides many sources for hypotheses regarding coadaptation of the skeleton with locomotor behavior.

In addition to the traits typically associated with cursoriality in mammals, increased articular surface areas around joints may be good indicators of high locomotor performance (Bramble & Lieberman, 2004; Garland & Freeman, 2005). Also, in paleo-anthropological studies, increased limb bone robusticity has been associated with increased physical activity which may co-occur in populations that have a history of heightened physical activity (Wallace et al., 2010 and references therein).

Beyond variation in limb bone and muscle sizes and proportions, asymmetry of the appendicular skeleton may impact locomotion. Fluctuating asymmetry (FA) involves small, non-directional deviations from perfect bilateral symmetry, which can be caused by environmental stress and random developmental noise (Pelabon, Hansen, Carter, & Houle, 2006; Valen, 1962). Directional asymmetry (DA) is the consistent deviation of bilateral structures such that one side is larger than the other (Carter, Osborne, & Houle, 2009). FA in limb lengths is negatively correlated with racing ability in horses (Manning & Ockenden, 1994), and in lizards hindlimb and femoral asymmetry was associated with reduced escape performance (Martín & López, 2001).

Another aspect of limb bone morphology that may influence skeletal evolution is sexual dimorphism, which sometimes results from sexual selection. In mammals, sexual size dimorphism in body mass is common, with males usually being the larger sex (Lindenfors, Gittleman, & Jones, 2007). In a study analyzing skeletal shape and size in Carnivora from the perspective of sexual selection, males generally had more robust limb elements and higher mechanical advantages, which may increase functional advantages during male competition and/or prey capture (Morris & Carrier, 2016 and references therein). In many species of mammals, including laboratory house mice as used in the present study, the pelvis is sexually dimorphic, which likely has significance for locomotion (Schutz, Donovan, & Hayes, 2009; but see Warriner, Lewton, Pontzer, & Lieberman, 2015).

One way to study the coadaptation and microevolution of the skeleton with behavior, and of genetically correlated traits in general, is via experiments in which selectively bred lines are compared with non-selected control lines (Garland & Rose, 2009; Sparrow et al., 2017). In the present study, we compared replicate lines of High Runner (HR) mice that had been selectively bred over 11 generations for voluntary wheel-running behavior with those of four non-selected Control (C) lines (Swallow, Carter, & Garland, 1998). Daily wheel-running distances of the HR lines reached ~75% greater than the C lines by generation 10, mainly by increased average speed (Koteja, Garland, Sax, Swallow, & Carter, 1999; Swallow, Carter, and Garland, 1998). A subset of the mice had the mini-muscle phenotype, caused by a Mendelian recessive allele that was present at a low frequency (~7%) in the original base population. Mini-muscle mice exhibit a 50% reduction in the triceps surae and total hindlimb muscle mass, caused by a significant reduction of type IIb muscle fibers (Guderley, Houle-Leroy, Diffie, Camp, & Garland, 2006; Talmadge, Acosta, & Garland, 2014). Population-genetic modeling indicates that the allele was under (unintentional) positive selection in the HR lines (Garland et al., 2002), and so the mini-muscle phenotype is viewed as one aspect of adaptive morphological evolution in the HR lines.

The first study of skeletal materials from the HR lines was a brief communication regarding mice from generation 11, which represents the earliest available set of specimens from this selection experiment. With body mass as a covariate, HR mice had larger femoral heads and reduced directional and fluctuating asymmetry of hindlimb bone lengths (femur + tibiafibula + metatarsal), with no statistical difference in hindlimb lengths or the MT/F ratio, as compared with mice from C lines (Garland & Freeman, 2005). Additionally, males had relatively shorter hindlimb lengths and larger MT/F ratios.

Here, we extended these comparisons to body mass and length, hindlimb bone dimensions, standard mammalian measurements (ear, tail, and hindfoot lengths), and hindlimb bone and muscle masses. Additionally, we computed ratios that index the relative size of distal versus proximal limb bones, including the MT/F ratio (Garland & Freeman, 2005; Garland Jr. & Janis, 1993). We also computed various hindlimb bone morphological indices used to examine limb bone robusticity, bone density, and anatomical advantage (in-lever/out-lever lengths of hindlimb muscles) (Samuels et al., 2013; Samuels & Van Valkenburgh, 2008; Van Valkenburgh, 1987).

We formulated several hypotheses regarding limb bones and muscles of HR mice, based on basic biomechanical principles, numerous previous empirical studies of mammals (many of which focus on cursoriality: see above), paleo-anthropological studies, and previous studies of these lines of mice (see Discussion). We hypothesized that mice from the HR lines would have relatively long and gracile limb bones, more proximally located muscle insertions, and potentially reduced muscle masses (see above). Alternatively, we might expect more robust (wider or thicker) limbs in HR mice, which would serve to increase bone strength and increased bone diameters, bone depth, and bone widths at or near surface areas to reduce joint stress (Bramble & Lieberman, 2004). Female HR mice have evolved by increased running speed, whereas males have evolved mainly by increased running speeds, but

also time spent running (Garland, Schutz, et al., 2011), and males are larger than females, which might lead to sex-specific evolutionary pathways in the skeleton. Therefore, another aim of our study was to examine sex differences in bone dimensions, muscle mass, and morphology, reasoning that sex-specific responses may have occurred (Garland, Kelly, et al., 2011; Keeney, Meek, Middleton, Holness, & Garland, 2012).

2 | MATERIALS AND METHODS

2.1 | Artificial selection model for high voluntary wheel-running

Specimens were drawn from lab generation 11 of four replicate lines of a mouse colony selectively bred for high voluntary wheel-running behavior. The founding population consisted of 224 outbred, genetically variable laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain (Harlan-Sprague-Dawley, Indianapolis, Indiana). Four lines of mice were selected for high voluntary wheel-running (HR) and compared with four randomly bred control (C) lines (Swallow et al., 1998). Briefly, mice are weaned at 21 days of age, and then housed in same-sex groups of four per cage until age 6–8 weeks. At that point, mice are housed individually in cages with attached computer-monitored wheels (1.12 m circumference) that record revolutions in 1-min bins over 6 days of wheel access. For HR lines, the highest-running male and female from each family are used as breeders. The selection criterion is total revolutions on days 5 and 6. In the C lines, a male and a female are randomly chosen from each family. Each line comprises 10 breeding pairs per generation, with no sibling pairs. Mice have food and water ad lib. All experimental procedures were approved by the University of Wisconsin-Madison institutional animal care and use committee.

2.2 | Body, bone, and muscle measurements

After routine wheel testing of all mice from generation 11 (i.e., each individual was given wheel access for 6 days), a random sample of males and females was chosen for study ($n = 142$). As sampling was random, some individuals needed to be used as breeders for the ongoing selection experiment. Mice were paired for breeding at approximately 10 weeks of age. After breeding, all individuals were housed individually without wheel access until sacrifice by carbon dioxide inhalation at a mean of 232 days of age, weighed (to nearest 0.01 g), then frozen for subsequent measurements.

After thawing, mice were again weighed and we took the following standard mammalian body measurements (Hall, 1981): body length (tip of the nostril to the end of bone in tail, to nearest 1 mm), tail length (base of tail to end bone of tail, to nearest 1 mm), hindfoot (heel to tip of nails, to nearest 0.1 mm), and ear length (notch in ear to tip, to nearest 0.1 mm). The gastrocnemius and quadriceps muscles were dissected, weighed to the nearest 0.0001 g, and averaged for subsequent statistical analyses. Mice were skinned and eviscerated, and then air dried. Dried carcasses were placed in a colony of dermestid beetles,

and bones were further cleaned manually under a dissecting scope as necessary (Garland & Freeman, 2005).

Bone measurements were taken to the nearest 0.01 mm with Fowler calipers (Fowler Sylvac, ultra-cal mark III) linked to a foot pedal and a small printer. All measurements were taken by Dr. Patricia A. Freeman, blind with respect to linetype. The caliper set-up ensured that the instrument was not put down between measurements, and allowed for rapid re-measurement when needed. To reduce measurement error, three measurements taken in quick succession were averaged and recorded. Both the right and left sides were measured to allow for analysis of asymmetry (Garland & Freeman, 2005).

Eight measurements were recorded for the femur. (a) Femur articular length: length from dorsal tip of head to distalmost end of the medial head. (b) Length of head to third trochanter scar: length from dorsal tip of head to distal end of trochanter muscle scar. (c) depth of femoral head: anterior–posterior diameter of the head. (d) Femoral proximal width: greatest medio-lateral width of the femur at the proximal end, from the medial side of the head to the lateral side of the greater trochanter. (e) Femoral width at 3rd trochanter: medial-lateral width across the femoral shaft at the third trochanter. (f) Femoral least width: medial-lateral width taken on femoral shaft at its least constriction and distal to the trochanter muscle scar; measurement is similar to mid-shaft diameter. (g) Femoral least depth: depth taken on femoral shaft at its least constriction and perpendicular to width. (h) Femoral distal width: greatest distal width of the femur at the medial and lateral epicondyles. Six measurements were recorded for the tibia-fibula. (a) Tibial length: greatest articular length of tibia, from the medial, proximal articular surface; the cup rather than the edge of the medial head to the cup, not tip of the medial malleolus of the tibia. The fibula is not part of this measurement. (b) Tibial proximal width: greatest medio-lateral distance across the proximal end of the tibia. (c) Tibial proximal depth: greatest antero-posterior depth, perpendicular to width. (d) Tibia-fibula least width: medial-lateral least width across tibia and fibula; measurement is similar to mid-shaft diameter. (e) Tibia-fibula least depth: least depth across tibia and fibula and generally perpendicular to width. (f) Tibia-fibula distal width: greatest medial-lateral width at the distal end of the tibia-fibula. In addition, we recorded the 3rd metatarsal length, measured on the dorsal surface of the metatarsal while still articulated with the proximal end of the digit (greatest length was taken because articular length was too small for calipers to grip).

We computed the ratio of metatarsal/femur length (MT/F), which shows the relative proportions of proximal and distal bone elements of the hindlimb, and relative size of the hindfoot (Garland & Freeman, 2005; Garland & Janis, 1993; Samuels et al., 2013; Samuels & Van Valkenburgh, 2008), tibia/femur ratio (T/F; also known as crural index), which indicates relative proportions of proximal and distal elements of the hind limb (Biancardi & Minetti, 2012; Samuels et al., 2013; Samuels & Van Valkenburgh, 2008; Vanhooydonck & Van Damme, 2001), and of the length from the femoral head to the 3rd trochanter scar divided by femur length (3rd/F), which indicates changes in quadratus femoris muscle insertion site position (“in-lever”) relative to bone length (“out-lever”), which would likely affect mechanical advantage of the muscle when rotating the hip joint (Charles, Cappellari, Spence, Hutchinson, &

Wells, 2016). We also computed the ratio of femoral least width divided by femoral length (FLW/F), which indicates robusticity of femur and ability to resist shearing and bending stresses and the ratio of tibia–fibula least width divided by tibial length (TFLW/T) which indicates robustness of tibia and ability to resist shearing and bending stresses (Samuels et al., 2013; Samuels & Van Valkenburgh, 2008). In addition, we computed the ratio of the tibia–fibula distal width divided by tibial length (TFDW/T) which infers relative distal hindlimb bone robustness (Morris & Carrier, 2016). Finally, because preliminary analysis revealed varying results of bone mass in males and females when either body mass or body length was used a covariate, we computed the ratio of femoral mass divided by (femoral length * [FLW²]) and the ratio of tibia–fibula mass divided by (tibial length * [TFLW²]) (e.g., see Marchini et al., 2014).

2.3 | Symmetry computations

Directional asymmetry (DA) and fluctuating asymmetry (FA) were previously reported for leg length (Garland & Freeman, 2005) computed as the sum of the lengths of the femur, tibia–fibula, and metatarsal bones, but not for the separate bone lengths and bone widths as in other studies (Auerbach & Ruff, 2006; Sarringhaus, Stock, Marchant, & McGrew, 2005). DA was computed as the right minus the left value of a trait, and FA was computed as the absolute value of the right–left difference. FA/DA was also computed since FA can be affected by DA (Palmer & Strobeck, 2003).

2.4 | Statistical analysis

As in numerous previous studies of these lines of mice, the MIXED procedure in SAS (SAS Institute, Cary, NC) was used to apply nested analysis of covariance (ANCOVA) models (Garland & Freeman, 2005; Houle-Leroy, Garland, Swallow, & Guderley, 2000; Houle-Leroy, Guderley, Swallow, & Garland, 2003; Swallow, Koteja, Carter, & Garland, 1999). Body mass was included as a covariate, except for symmetry measures and functional ratios, and results sometimes differed when body length was used instead (see Result section). We also included bone length as a covariate for bone width, mass, and depth measurements. Results for models using body length or bone length as a covariate are not shown in the tables, but are mentioned in the text when results differed from analyses using body mass as a covariate.

A cross-nested, two-way ANCOVA was used to simultaneously test the effects of linetype (HR vs. C lines) and sex. Replicate line nested within linetype was a random effect, and the effect of linetype, sex, and the sex * linetype interaction were tested with 1 and 6 degrees of freedom. A main effect of the mini-muscle phenotype (Garland et al., 2002; Houle-Leroy, Guderley, Swallow, & Garland, 2003; Kelly, Czech, Wight, Blank, & Garland, 2006) was also included and tested relative to the residual variance with 1 and ~119 d.f. (or fewer in the case of missing values). In the present sample of 142 mice (not all of which had data for all traits), the number of mini-muscle individuals was 6 in HR line 3 (2 females, 4 males), 2 in HR line 6 (1 female, 1 male).

To analyze interactions, we used a cross-nested, two-way ANCOVA to simultaneously test the main effects of linetype (HR vs. C lines) and sex, their interaction, and the linetype * body mass, sex * body mass, and linetype * sex * body mass interactions. Random effects included replicate line nested within linetype, sex * line(linetype), body mass * line(linetype), and body mass * sex * line(linetype). In these “full” models, the effect of linetype, sex, sex * linetype, body mass, body mass * sex, body mass * linetype, and body mass * sex * linetype were tested with 1 and 6 degrees of freedom, whereas the effect of mini-muscle phenotype was tested with 1 and the residual d.f. (~100 for combined analyses of males and females). In addition, a main effect of the mini-muscle phenotype was included (Garland et al., 2002; Houle-Leroy et al., 2003; Kelly et al., 2006).

In these full models with all of the indicated fixed and random effects, we often obtained covariance parameter estimates of zero or near-zero for some of the interactive random effects, in which case we removed them from the model, but we always retained the line(linetype) and sex * line(linetype) random effects, given the nature of the experimental design. When the higher-order random effects were removed, then d.f. for testing the main effects and their interactions were increased, as can be seen (supporting information Table S1). In the full models, we often found statistically significant ($p < .05$) or suggestive ($p < .1$) interactions involving sex and/or linetype and/or body mass, and so we then redid analyses split by sex, as our focus here is comparisons of the HR and C lines of mice. In these sex-specific models, when we did not find an interaction between linetype and body mass, we removed that interaction term and reran the analyses. For all models, outliers were removed when the standardized residual exceeded ~3. We used an α of ≤ 0.05 for statistical significance. For simplicity, all p values reported in tables and in the Results section, are 2-tailed.

To address the issues of inflated experiment-wise Type I error rates when making multiple comparisons, we applied the positive False Discovery Rate (pFDR Q-value) procedure, as implemented in SAS Procedure Multtest. We applied this to the 156 p values reported in Tables 1, 2, and 3. Nominally, 68 of the 160 p values were $< .05$. The Q-values indicated that only one of these should not be considered significant, a value of $p = .0481$, which is reported in Table 2, but not discussed.

3 | RESULTS

Tables 1–3 present significance levels from results of ANOVAs and ANOVAs (using body mass as a covariate), whereas Table 4 presents Least Squares Means (group means adjusted for variation in body mass) for all of the analyses.

3.1 | Body size

Preliminary analyses indicated that a female from Control line 4 was the heaviest mouse in the data set (48.82 g, ID = 14085), and was also a high outlier in analyses of body mass with body length as a covariate. Therefore, we concluded that this individual probably had a large amount of body fat, and we decided to exclude her measurements

TABLE 1 Analyses of body size, standard mammalian measurements, and muscle masses with use of either body mass or body length as a covariate

Trait Degrees of Freedom	N	Linetype 1, 6	Sex 1, 6	Sex*Linetype 1, 6	Mini-Muscle 1, ~19	Body Size 1, ~19	Covariate 1, ~19
<i>Body Size (g)</i>							
Body Mass	136	0.2326–	<.0001+	0.4205	0.0297–		
Body Mass	135	0.2871–	<.0001+	0.7300	0.0148–	<.0001	BodyL
<i>Standard Mammalian (mm)</i>							
Hindfoot	134	0.4272–	0.0249–	0.3119	0.0583+	<.0001	Mass
Ear Length	135	0.1793–	0.6902–	0.3951	0.0889+	<.0001	Mass
Tail Length	136	0.3751+	0.0335–	0.6709	0.1838+	<.0001	Mass
<i>Muscle Mass (g)</i>							
Quadriceps	136	0.8939–	0.0531+	0.5929	<.0001–	<.0001	Mass
Gastrocnemius	133	0.5265+	0.0041+	0.6123	<.0001–	<.0001	Mass

Note. Significance levels (*p* values; bold indicates $p < .05$) are from two-way nested analysis of covariance models implemented in SAS PROC MIXED. Signs after *p* values indicate direction of effect: + indicates HR > C, Male > Female, or Mini-muscle > normal muscle.

from all subsequent analyses that involved body mass, including when it was used as a covariate.

Although HR mice tended to be smaller than C mice, body mass and body length differences were not statistically significant (Table 1). Body mass also did not differ between linetypes when body length was included as a covariate. Mini-muscle mice had significantly reduced body mass, including when body length was a covariate, but not a reduced body length (see Table 4 for Least Squares Means). Males were significantly heavier than females, with or without body length as a covariate, but the sexes did not differ in body length (Table 1).

3.2 | Standard mammalian measurements

Linetype differences were never significant for ear, tail or hindfoot lengths, regardless of the body-size covariate used (Table 1). Mini-muscle mice had significantly longer hindfoot lengths when body length was used as a covariate (results now shown). Males had significantly shorter tails with body mass as a covariate. Males had significantly shorter hindfeet with body mass as a covariate, but significantly longer hindfeet with body length as a covariate. The linetype * sex interactions were not significant for any trait (Table 1).

TABLE 2 Analyses of bone dimensions and masses

Trait Degrees of Freedom	N	Linetype 1, 6	Sex 1, 6	Sex*Linetype 1, 6	Mini-Muscle 1, ~19	Body Mass 1, ~19
<i>Bone lengths</i>						
Leg length	130	0.9967+	<.0001–	0.1568	0.2087+	<.0001
Femur	134	0.9303–	<.0001–	0.0919	0.6813–	<.0001
Tibia–fibula	134	0.7818–	<.0001–	0.3514	0.0274+	<.0001
3rd Metatarsal	130	0.3056+	0.0309–	0.6962	0.1758+	<.0001
<i>Femur</i>						
A–P depth femoral head	133	0.0366+	0.7301+	0.8728	0.7816–	<.0001
Femoral distal width	134	0.0176+	0.2685+	0.8537	0.0087–	0.0001
Femoral proximal width	132	0.0760+	0.4130+	0.5038	0.2793+	<.0001
Femoral width 3rd trochanter	133	0.4579–	<.0001+	0.1022	<.0001–	<.0001
Femoral least width	133	0.1560+	0.0024+	0.6184	<.0001–	0.0093
Femoral least depth	134	0.4576+	0.0209–	0.8432	0.3656–	<.0001
Femoral head to 3rd trochanter	134	0.5368+	0.0046–	0.2522	0.1068+	0.0080
<i>Tibia–Fibula</i>						
Tibial proximal depth ^a	134	0.0351+	0.2562+	0.6720	0.9993–	0.0001
Tibial proximal width ^a	134	0.1480+	0.2856+	0.5342	0.9973–	<.0001
Tibia–fibula least width	132	0.1946+	0.8511+	0.8987	<.0001–	<.0001
Tibia–fibula least depth	133	0.9432+	^{&} 0.0481+	0.3615	0.1344–	0.0020
Tibia–fibula distal width	133	0.6212+	0.0105–	0.3128	0.0381+	<.0001
<i>Bone masses</i>						
Femur	134	0.6365+	0.0010–	0.7405	0.0038–	<.0001
Tibia–fibula	134	0.4927+	0.0090–	0.4863	0.0055–	<.0001

Note. Significance levels (*p* values; bold indicates $p < .05$) are from two-way nested analysis of covariance models implemented in SAS PROC MIXED. Signs after *p* values indicate direction of effect: + indicates HR > C, Male > Female, or Mini-muscle > normal muscle. The value marked with & is not significant after correcting for multiple comparisons with the pFDR Q-Value procedure (see Methods).

^aVariable was rank-transformed for statistical analyses.

TABLE 3 Analyses of functional ratios and indicators of bone density

Trait Degrees of Freedom	N	Linetype 1, 6	Sex 1, 6	Sex*Linetype 1, 6	Mini-Muscle 1, ~19
MT/F	135	0.6636+	0.0001+	0.5765	0.0674+
T/F	139	0.7975–	0.0023+	0.3722	0.0069+
3rd/F	139	0.4257+	0.5833+	0.6812	0.0239+
FMW/F	139	0.3371+	<.0001+	0.5329	<.0001–
TFW/T	138	0.3150+	0.0007+	0.9425	<.0001–
FDW/F	138	0.1435+	0.0002+	0.8563	0.0837–
TFDW/T	138	0.5418+	0.0004+	0.7574	0.9530+
FM/(FL * FLW ²)	139	0.1885–	<.0001–	0.6714	0.0061+
TM/(TL * TFLW ²)	138	0.1804–	0.7141–	0.4622	<.0001+

Note. Significance levels (p values; bold indicates $p < .05$ or $p < .1$ for interaction terms) are from two-way nested analysis of variance models implemented in SAS PROC MIXED. Body mass was not used as a covariate in these analyses. Signs after p values indicate direction of effect: + indicates HR > C, Male > Female, or Mini-muscle > normal muscle.

In the models testing for interactions with body mass, the body mass * linetype * sex interaction was marginally significant for hindfoot length ($p = .0717$: supporting information Table S1). Analyses split by sex indicated that, for females, the body mass * linetype interaction was significant ($p = .0075$) as was the linetype effect ($p = .0434$). For males, only the body mass effect was significant ($p < .0001$). Inspection of scatterplots showed that female HR mice had longer feet at larger body masses, as compared with female C mice.

3.3 | Muscle masses

Adjusting for body mass, quadriceps and gastrocnemius muscle mass did not differ statistically between HR and C mice, but mini-muscle individuals had significantly reduced quadriceps and gastrocnemius masses (all $p < .0001$). Males had heavier quadriceps and gastrocnemius when using body mass ($p = .0531$ and $p < .0001$, respectively or body length ($p < .0001$ and $p = .0318$, respectively) as a covariate (Table 1).

The interaction model for quadriceps showed a strong body mass * linetype interaction ($p = .0066$: supporting information Table S1), with a steeper slope for HR mice. Analyses split by sex also showed this interaction ($p = .0618$ for females, $p = .0202$ for males). Inspection of scatterplots showed that HR mice tended to have lighter quadriceps at lower body mass but heavier quads at a higher mass. For gastrocnemius, the body mass * linetype interaction was also significant ($p = .0178$: supporting information Table S1), again with HR mice having a higher slope. Analyses split by sex showed that this interaction was significant for males only ($p = .0164$), with their regression lines crossing at intermediate masses. For females, the effect of mass was significant ($p < .0001$), but linetype was not.

3.4 | Bone dimensions and masses

Linetype differences were not significant for femur length, tibia-fibula length, 3rd metatarsal length, or leg lengths, regardless of the body size

covariate used. Additionally, linetype differences were not significant for femur and tibia-fibula masses (Table 2). Mice from HR lines had increased anterior-posterior depth of the femoral head (Figure 1: $p = .0366$ with body mass as covariate; $p = .0640$ with body length), increased femur distal width (Figure 2: $p = .0176$ with body mass; $p = .0567$ with body length), and increased tibia proximal depth (rank $p = 0.0351$ for body mass; $p = .0497$ for tibia-fibula length).

Mini-muscle mice had thinner hind limb bones for many measurements in the femur and tibia-fibula (see Table 2). Femoral distal width (Figure 2 all covariates), femoral width at 3rd trochanter (Figure 3, all covariates), femoral least width (all covariates), and the tibia-fibula least width (all covariates) all had reduced medial-lateral width measurements. Femoral (Figure 4) and tibia-fibula mass were significantly reduced in mini-muscle mice, regardless of covariate used (see Table 2). Mini-muscle mice also had significantly longer tibia-fibula lengths with body mass as a covariate (Figure 5). Tibia-fibula least depth was significantly reduced when tibia-fibula length was used as a covariate. In contrast, tibia-fibula distal width was larger in mini-muscle individuals with body mass as a covariate ($p = .0381$).

Males had significantly shorter hindlimb bones (femur + tibia-fibula + metatarsal), as compared with females, whether body mass or length was used as a covariate (Table 2). Males had significantly greater anterior posterior depth of femoral heads ($p = .0002$ with body length; $p = .0001$ with femur length), femoral width at 3rd trochanter muscle scar (Figure 3, all covariates), and femoral least width (all covariates: Table 2). In addition, femoral proximal and distal widths were increased when using body length and femur length as covariates. Males had reduced femoral least depths with body mass as a covariate. Femur mass was reduced in males when using body mass as a covariate (Figure 4), but increased with femur length as a covariate. For the tibia-fibula, males had significantly increased tibial proximal width, tibial proximal depth, and tibia-fibula least width measurements for body length and tibia-fibula bone lengths as covariates (Table 2). Tibia-fibula distal width was reduced in males with body mass as the covariate, but

TABLE 4 Least squares means and associated standard errors from SAS procedure Mixed, corresponding to statistical results presented in Tables 1–3

Trait	Units	Control		Control		High Runner		High Runner		Normal		Mini-	
		Female Mean	SE	Male Mean	SE	Female Mean	SE	Male Mean	SE	Muscle Mean	SE	Muscle Mean	SE
Body mass	g	33.94	1.49	44.51	1.45	32.44	1.41	41.80	1.35	39.99	0.79	36.35	1.75
Body mass/w body length	g	33.88	1.10	44.33	1.08	32.84	1.06	42.79	1.01	39.93	0.55	36.99	1.25
Hindfoot	mm	19.66	0.14	19.41	0.13	19.84	0.14	19.43	0.11	19.42	0.06	19.74	0.17
Ear length	mm	16.62	0.12	16.51	0.12	16.43	0.12	16.45	0.09	16.37	0.04	16.64	0.16
Tail length	mm	105.72	1.21	102.32	1.15	106.14	1.17	103.40	0.94	103.31	0.38	105.48	1.58
Quadriceps	g	0.1458	0.0047	0.1568	0.0044	0.1467	0.0046	0.1548	0.0037	0.1994	0.0018	0.1027	0.0057
Gastrocnemius	g	0.1292	0.0048	0.1451	0.0045	0.1337	0.0047	0.1474	0.0041	0.1823	0.0025	0.0954	0.0052
Leg length	mm	43.61	0.33	41.44	0.32	43.42	0.33	41.63	0.30	42.34	0.19	42.70	0.33
Femur length	mm	16.65	0.19	15.55	0.18	16.52	0.19	15.65	0.17	16.12	0.11	16.06	0.18
Tibia–fibula length	mm	19.29	0.14	18.38	0.13	19.19	0.14	18.40	0.12	18.66	0.07	18.97	0.15
3rd metatarsal length	mm	7.66	0.05	7.54	0.04	7.69	0.05	7.60	0.04	7.58	0.02	7.66	0.06
A–P depth femoral head	mm	1.57	0.02	1.57	0.02	1.62	0.02	1.62	0.02	1.60	0.01	1.59	0.02
Femoral distal width	mm	2.95	0.04	2.98	0.03	3.05	0.04	3.09	0.03	3.07	0.02	2.96	0.04
Femoral proximal width	mm	3.62	0.03	3.61	0.02	3.68	0.03	3.65	0.02	3.62	0.01	3.66	0.03
Femoral width 3rd trochanter	mm	2.16	0.04	2.55	0.04	2.18	0.04	2.48	0.03	2.46	0.01	2.23	0.05
Femoral least width	mm	1.69	0.04	1.84	0.03	1.75	0.03	1.88	0.03	1.89	0.02	1.69	0.04
Femoral least depth	mm	1.50	0.03	1.42	0.03	1.52	0.03	1.45	0.02	1.49	0.01	1.46	0.03
Femoral head to 3rd trochanter	mm	6.86	0.10	6.47	0.09	6.85	0.09	6.60	0.08	6.61	0.04	6.78	0.11
Tibial proximal depth (rank)	rank	53.12	11.34	62.36	10.51	74.39	11.19	90.50	9.37	70.10	4.51	70.09	12.72
Tibial proximal width (rank)	rank	54.18	13.19	60.59	12.43	72.75	13.11	88.04	11.58	68.91	6.90	68.87	13.49
Tibia–fibula least width	mm	0.98	0.02	0.97	0.02	1.00	0.02	1.00	0.01	1.04	0.01	0.94	0.02
Tibia–fibula least depth	mm	1.20	0.02	1.25	0.02	1.21	0.02	1.24	0.02	1.24	0.01	1.21	0.02
Tibia–fibula distal width	mm	2.68	0.03	2.62	0.02	2.69	0.03	2.64	0.02	2.63	0.01	2.68	0.03
Femur mass	g	0.0642	0.0016	0.0567	0.0015	0.0652	0.0016	0.0571	0.0013	0.0637	0.0007	0.0579	0.0020
Tibia–fibula mass	g	0.0511	0.0015	0.0479	0.0014	0.0527	0.0015	0.0487	0.0013	0.0521	0.0008	0.0481	0.0016
MT/F	ratio	0.4639	0.0055	0.4838	0.0054	0.4682	0.0053	0.4856	0.0052	0.4711	0.0033	0.4797	0.0055

(Continues)

TABLE 4 (Continued)

Trait	Units	Control		Control		High Runner		High Runner		Normal		Mini-	
		Female Mean	SE	Male Mean	SE	Female Mean	SE	Male Mean	SE	Muscle Mean	SE	Muscle Mean	SE
T/F	ratio	1.1599	0.0087	1.1824	0.0086	1.1608	0.0084	1.1761	0.0081	1.1575	0.0049	1.1821	0.0098
3rd/F	ratio	0.4149	0.0053	0.4154	0.0052	0.4179	0.0051	0.4214	0.0049	0.4110	0.0026	0.4238	0.0059
FMW/F	ratio	0.1017	0.0025	0.1185	0.0024	0.1054	0.0024	0.1203	0.0023	0.1173	0.0013	0.1056	0.0028
TFW/T	ratio	0.0506	0.0010	0.0532	0.0010	0.0518	0.0010	0.0545	0.0009	0.0558	0.0006	0.0493	0.0011
TFDW/T	ratio	0.1381	0.0015	0.1425	0.0015	0.1395	0.0014	0.1436	0.0014	0.1409	0.0009	0.1410	0.0016
FM/(FL * FLW ²)	ratio	0.001288	0.000037	0.001122	0.000037	0.001235	0.000036	0.001053	0.000034	0.001118	0.000021	0.001231	0.000043
TM/(TL * TFLW ²)	ratio	0.002744	0.000058	0.002759	0.000057	0.002690	0.000055	0.002647	0.000052	0.002569	0.000027	0.002850	0.000069

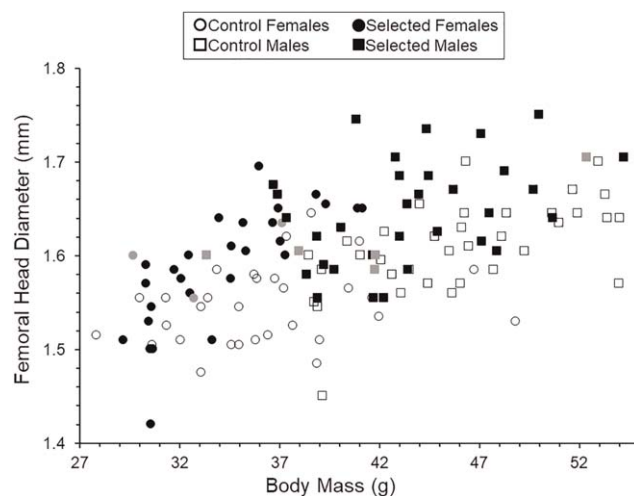


FIGURE 1 Mean anterior-posterior depth of femoral head in relation to body mass. Larger mice had larger femoral heads, and mice from the selectively bred High Runner lines had significantly larger femoral heads for a given body size (see Table 2 for statistical analyses)

increased with body length or tibia-fibula length as the covariate. Tibia-fibula mass was reduced in males with body mass as a covariate, but increased with body length or tibia-fibula length as a covariate (Table 2).

Several bone dimensions showed significant interactions with body mass, and full analyses are presented in supporting information Table S1. Here, we discuss a few of the stronger interaction effects. For example, in the femoral head interaction models, the body mass * linetype * sex interaction was marginally significant ($p = .0515$) and so was the body mass * sex interaction ($p = .0838$, supporting information Table S1). Analyses split by sex indicated that, for females (Figure 6a),

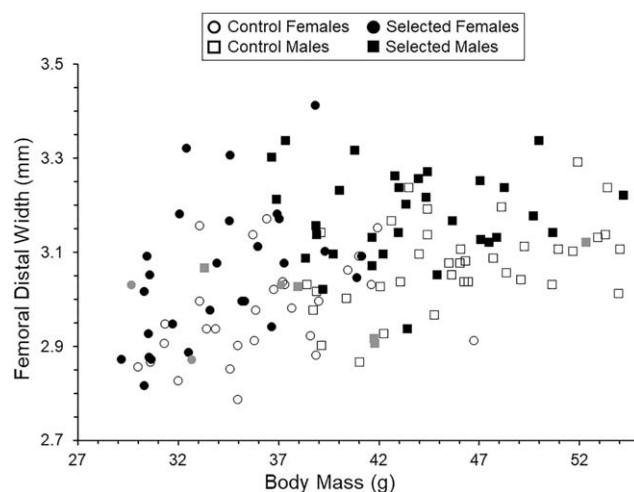


FIGURE 2 Mean femoral distal width in relation to body mass. Mice from the selectively bred High Runner lines had significantly broader femoral distal widths for a given body mass, suggesting increased muscle attachment area and increased articular surface area around the knee joint. Mini-muscle mice had reduced femoral distal widths (see Table 2 for statistical analyses)

TABLE 4 Least squares means and associated standard errors from SAS procedure Mixed, corresponding to statistical results presented in Tables 1–3

Trait	Units	Control		Control		High Runner		High Runner		Normal		Mini-	
		Female Mean	SE	Male Mean	SE	Female Mean	SE	Male Mean	SE	Muscle Mean	SE	Muscle Mean	SE
Body mass	g	33.94	1.49	44.51	1.45	32.44	1.41	41.80	1.35	39.99	0.79	36.35	1.75
Body mass/w body length	g	33.88	1.10	44.33	1.08	32.84	1.06	42.79	1.01	39.93	0.55	36.99	1.25
Hindfoot	mm	19.66	0.14	19.41	0.13	19.84	0.14	19.43	0.11	19.42	0.06	19.74	0.17
Ear length	mm	16.62	0.12	16.51	0.12	16.43	0.12	16.45	0.09	16.37	0.04	16.64	0.16
Tail length	mm	105.72	1.21	102.32	1.15	106.14	1.17	103.40	0.94	103.31	0.38	105.48	1.58
Quadriceps	g	0.1458	0.0047	0.1568	0.0044	0.1467	0.0046	0.1548	0.0037	0.1994	0.0018	0.1027	0.0057
Gastrocnemius	g	0.1292	0.0048	0.1451	0.0045	0.1337	0.0047	0.1474	0.0041	0.1823	0.0025	0.0954	0.0052
Leg length	mm	43.61	0.33	41.44	0.32	43.42	0.33	41.63	0.30	42.34	0.19	42.70	0.33
Femur length	mm	16.65	0.19	15.55	0.18	16.52	0.19	15.65	0.17	16.12	0.11	16.06	0.18
Tibia–fibula length	mm	19.29	0.14	18.38	0.13	19.19	0.14	18.40	0.12	18.66	0.07	18.97	0.15
3rd metatarsal length	mm	7.66	0.05	7.54	0.04	7.69	0.05	7.60	0.04	7.58	0.02	7.66	0.06
A–P depth femoral head	mm	1.57	0.02	1.57	0.02	1.62	0.02	1.62	0.02	1.60	0.01	1.59	0.02
Femoral distal width	mm	2.95	0.04	2.98	0.03	3.05	0.04	3.09	0.03	3.07	0.02	2.96	0.04
Femoral proximal width	mm	3.62	0.03	3.61	0.02	3.68	0.03	3.65	0.02	3.62	0.01	3.66	0.03
Femoral width 3rd trochanter	mm	2.16	0.04	2.55	0.04	2.18	0.04	2.48	0.03	2.46	0.01	2.23	0.05
Femoral least width	mm	1.69	0.04	1.84	0.03	1.75	0.03	1.88	0.03	1.89	0.02	1.69	0.04
Femoral least depth	mm	1.50	0.03	1.42	0.03	1.52	0.03	1.45	0.02	1.49	0.01	1.46	0.03
Femoral head to 3rd trochanter	mm	6.86	0.10	6.47	0.09	6.85	0.09	6.60	0.08	6.61	0.04	6.78	0.11
Tibial proximal depth (rank)	rank	53.12	11.34	62.36	10.51	74.39	11.19	90.50	9.37	70.10	4.51	70.09	12.72
Tibial proximal width (rank)	rank	54.18	13.19	60.59	12.43	72.75	13.11	88.04	11.58	68.91	6.90	68.87	13.49
Tibia–fibula least width	mm	0.98	0.02	0.97	0.02	1.00	0.02	1.00	0.01	1.04	0.01	0.94	0.02
Tibia–fibula least depth	mm	1.20	0.02	1.25	0.02	1.21	0.02	1.24	0.02	1.24	0.01	1.21	0.02
Tibia–fibula distal width	mm	2.68	0.03	2.62	0.02	2.69	0.03	2.64	0.02	2.63	0.01	2.68	0.03
Femur mass	g	0.0642	0.0016	0.0567	0.0015	0.0652	0.0016	0.0571	0.0013	0.0637	0.0007	0.0579	0.0020
Tibia–fibula mass	g	0.0511	0.0015	0.0479	0.0014	0.0527	0.0015	0.0487	0.0013	0.0521	0.0008	0.0481	0.0016
MT/F	ratio	0.4639	0.0055	0.4838	0.0054	0.4682	0.0053	0.4856	0.0052	0.4711	0.0033	0.4797	0.0055

(Continues)

TABLE 4 (Continued)

Trait	Units	Control		Control		High Runner		High Runner		Normal		Mini-	
		Female Mean	SE	Male Mean	SE	Female Mean	SE	Male Mean	SE	Muscle Mean	SE	Muscle Mean	SE
T/F	ratio	1.1599	0.0087	1.1824	0.0086	1.1608	0.0084	1.1761	0.0081	1.1575	0.0049	1.1821	0.0098
3rd/F	ratio	0.4149	0.0053	0.4154	0.0052	0.4179	0.0051	0.4214	0.0049	0.4110	0.0026	0.4238	0.0059
FMW/F	ratio	0.1017	0.0025	0.1185	0.0024	0.1054	0.0024	0.1203	0.0023	0.1173	0.0013	0.1056	0.0028
TFW/T	ratio	0.0506	0.0010	0.0532	0.0010	0.0518	0.0010	0.0545	0.0009	0.0558	0.0006	0.0493	0.0011
TFDW/T	ratio	0.1381	0.0015	0.1425	0.0015	0.1395	0.0014	0.1436	0.0014	0.1409	0.0009	0.1410	0.0016
FM/(FL * FLW ²)	ratio	0.001288	0.000037	0.001122	0.000037	0.001235	0.000036	0.001053	0.000034	0.001118	0.000021	0.001231	0.000043
TM/(TL * TFLW ²)	ratio	0.002744	0.000058	0.002759	0.000057	0.002690	0.000055	0.002647	0.000052	0.002569	0.000027	0.002850	0.000069

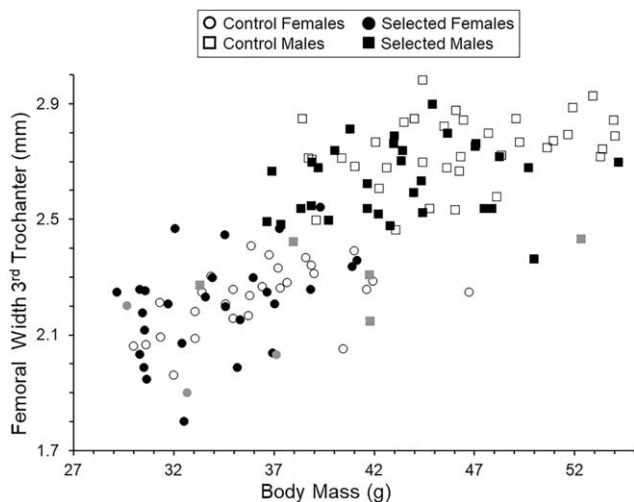


FIGURE 3 Mean femoral width at 3rd trochanter muscle scar in relation to body mass. Males had significantly thicker femoral width at 3rd trochanter muscle scar for a given body mass, suggesting increased robustness. In addition, mini-muscle mice had reduced femoral width measurements (see Table 2 for statistical analyses)

the body mass * linetype interaction was significant ($p = .0306$), whereas in males (Figure 6b) only the linetype effect was significant (after removing the body mass * linetype interaction, linetype $p = .0298$). Inspection of Figure 6 shows that female HR mice had larger femoral heads at larger body mass when compared with C female mice, whereas male HR mice had larger femoral heads than male C mice at all body masses.

In the interaction models for femoral proximal width measurements, the body mass * sex interaction was significant ($p = .0242$; supporting information Table S1). Analyses split by sex indicated that, for females, only the linetype effect was significant after removing the body mass * linetype interaction ($p = .0441$). Inspection of scatterplots

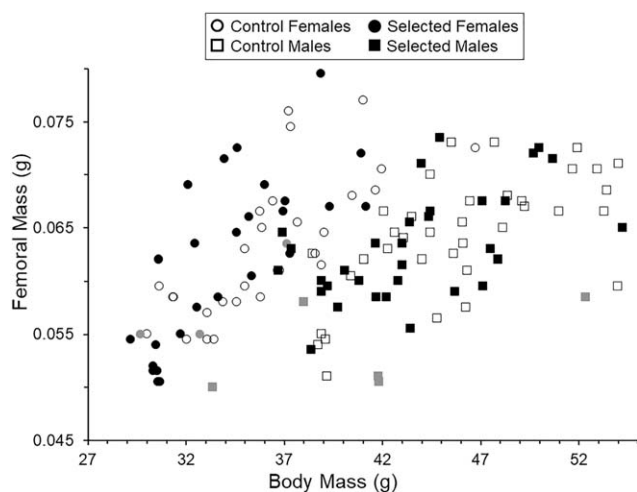


FIGURE 4 Mean femoral mass in relation to body mass. Males had significantly reduced femur mass for a given body mass, and mini-muscle mice had reduced femoral masses (see Table 2 for statistical analyses)

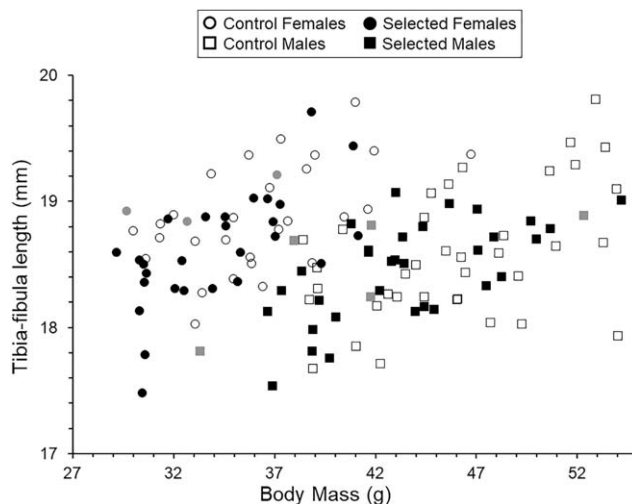


FIGURE 5 Mean tibia-fibula length in relation to body mass. Males had significantly shorter tibia-fibula lengths for a given body mass, and mini-muscle mice had increased tibia-fibula lengths (see Table 2 for statistical analyses)

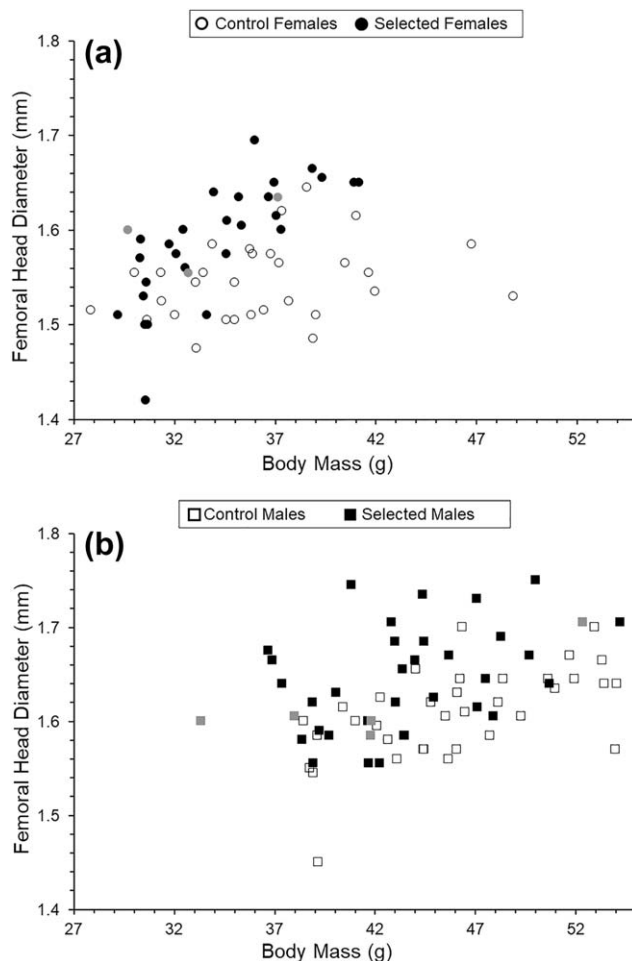


FIGURE 6 Mean anterior-posterior depth of femoral head in relation to body mass, separately by sex to illustrate the significant interaction between sex and body mass for both traits (statistical analyses are in Table S1). Note that these same data are shown in Figure 1

showed that female HR mice had wider proximal femurs, regardless of differences in body mass, when compared with C female mice, whereas male HR and C mice did not differ, regardless of body mass.

In the interaction models for tibia proximal depth with ranked values, the body mass * linetype * sex interaction was marginally significant ($p = .0663$; supporting information Table S1). Analyses split by sex indicated that, for females, the linetype effect was marginally significant after removing the body mass * linetype interaction ($p = .0673$). In males, the body mass * linetype interaction was significant ($p = .0324$) as was the linetype effect ($p = .0182$). Inspections of scatterplots (not shown) revealed that female HR mice tended to have deeper proximal tibias regardless of body mass, whereas for male the regression lines crossed at intermediate values of body mass, with a positive slope in C mice but a negative slope for HR mice.

In the interaction models for tibia fibula least depth, the body mass * sex interaction was strongly significant ($p = .0062$; supporting information Table S1). Analyses split by sex indicated that, for females, the body mass * linetype interaction ($p = .0483$), but this was not so for males. Inspections of scatterplots (not shown) revealed that female HR mice generally had deeper tibias at larger masses.

3.5 | Functional ratios and indicators of bone density

None of the ratios differed significantly between HR and C lines (Table 3). Mini-muscle mice had significantly increased T/F ratios, and the MT/F ratio ($p = .0674$) tended to be increased, suggesting increased distal limb elements relative to proximal ones. The distance from the femoral head to the 3rd trochanter muscle scar, divided by femur length (3rd/F), was significantly greater in mini-muscle individuals, indicating a change in the anatomical advantage of the quadratus femoris muscle (in-lever/out-lever; see above). Mini-muscle mice also had less robust femurs (FMW/F) and less robust tibia-fibulas (TFW/T). Mini-muscle mice tended to have reduced femoral distal widths. Finally, mini-muscle mice had increased $[FM/(FL * FLW^2)]$ and $[TM/(TL * TFLW^2)]$ ratios (Table 3), suggesting increased bone density. MT/F ratio and T/F ratio were significantly increased in males, suggesting increased distal limb elements relative to proximal ones. Males also had more robust femurs (FMW/F), more robust tibia-fibulas (TFW/T), and increased distal tibia-fibula robustness (TFDW/T; see Table 3). Finally, males had significantly decreased $[FM/(FL * FLW^2)]$, suggesting that femurs were less dense than for females (Table 3).

3.6 | Asymmetry

Directional asymmetry (supporting information Table S2) was significantly lower in HR mice for total leg length (2-tailed $p = .0217$), (see also Garland Jr. & Freeman, 2005) and for femur length ($p = .0311$), but not for tibia-fibula or metatarsal length (supporting information Table S2). The FA/DA ratio for the femur tended to be lower for HR mice ($p = .0510$). Fluctuating asymmetry was significantly lower in HR mice for tibia-fibula distal width ($p = .0108$). Tibia-fibula distal width also had increased levels of directional asymmetry in mini-muscle mice ($p = .0236$). Males had reduced directional asymmetry for 3rd

metatarsal length ($p = .0361$). Males also had reduced directional asymmetry for femoral least width ($p = .0454$), with a substantially greater reduction in HR lines than in Control lines (sex * linetype interaction $p = .0567$). However, this was not significant when analysis was split by sex (supporting information Table S1). HR males tended to have reduced directional asymmetry for tibia-fibula least width ($p = .0871$; supporting information Table S1), when compared with C lines (sex * linetype interaction $p = .0923$). This was also observed in FA/DA tibia-fibula least width (sex * linetype interaction $p = .0649$) and analyses split by sex for HR males ($p = .0396$; supporting information Table S1).

4 | DISCUSSION

We compared hindlimb bone dimensions and muscle masses of four replicate, selectively bred HR lines of mice with those from four non-selected Control lines at generation 11. We found several differences between the HR and C lines that can be interpreted as adaptive in the context of running long distances on a daily basis. We also found several differences between the subset of individuals that express the mini-muscle phenotype, caused by a Mendelian recessive allele (Kelly et al., 2013) and characterized by a 50% reduction in hindlimb muscle mass (Garland et al., 2002; Houle-Leroy et al., 2003), and wild-type (normal-muscle) individuals. Finally, we found differences between the sexes, including some unexpected interactions between bone dimensions and body size that differed between linetypes and/or between the sexes.

4.1 | Differences between high runner and control mice

In a preliminary analysis of a subset of the available bone measurements, Garland and Freeman (2005) reported increased anterior-posterior diameters of the femoral head, suggesting greater articular surface areas at the hip. In addition to confirming those results, our re-analysis also shows that HR mice have increased femoral distal widths and increased proximal tibia depths, suggesting larger knee surface areas. Functionally, larger articular surface areas may be related to increased joint mobility in mammals (Godfrey et al., 1995). We are not aware of previous studies of large or small-bodied mammals that have explored joint surface areas in relation to increased running ability (e.g., via increased stability), although studies of primates have associated joint surface areas with climbing (Godfrey, Sutherland, Boy, & Gombert, 1991). In the genus *Homo* (as compared with *Pan* and *Australopithecus*), greater articular surface areas of the femoral head, knees, sacroiliac joint, and lumbar centra (all judged relative to body mass, as in our analyses) are suggested to be adaptations for endurance running that increase shock absorption by expanding joint forces over larger surface areas, thus reducing joint stress from impact forces with the ground (Bramble & Lieberman, 2004). The same may be true for the hindlimbs of mice running at high speeds for many hours per day in large wheels (cf., Roach, Edke, & Griffin, 2012).

Previous studies of later generations of the selection experiment have reported increased femoral and tibiofibular mid-shaft diameters in

the HR mice (Kelly et al., 2006; Wallace, Tommasini, Judex, Garland & Demes, 2012), which may increase bone strength. We did not find this (Tables 1 and 3), conceivably because differences had not evolved to a statistically detectable degree by generation 11.

Finally, we need to qualify our conclusions regarding evolutionary changes in the bones of HR mice. As explained in the Methods, all of the mice studied here were given 6 days of wheel access when young adults, followed by housing without wheels until sacrifice at 232 days of age. Thus, bones may have been affected by wheel running during that brief period, even though the mice were sexually mature (Buie, Moore, & Boyd, 2008). Moreover, at least in later generations, HR mice are more active than C mice in home cages when housed without wheels (Copes et al., 2015; Malisch et al., 2009). Therefore, as noted previously (Kelly et al., 2006), some of the differences we measured between HR and C mice could be caused by the intermediate phenotype of elevated activity levels, rather than by genetic differences that directly affect bone properties. On the other hand, we have also shown that week-old mice (i.e., before they locomote) from generation 45 show differences in femoral characteristics (Wallace et al., 2010). Taken as a whole, we are confident that at least some of the observed differences in skeletal properties between HR and C mice represent evolved differences, not just the result of different activity levels acting across the lifespan (see also Garland & Freeman, 2005; Kelly et al., 2006; Middleton et al., 2010; Middleton, Shubin, et al., 2008; Middleton, Kelly, et al., 2008; Schutz, Jamniczky, Hallgrímsson, & Garland, 2014; Wallace et al., 2010, 2012; Young, Hallgrímsson, & Garland, 2009). Nevertheless, future studies should address the relationship between home-cage activity and bone properties by use of longitudinal sampling and also employ an immobilization model (Jämsä, Koivukangas, Ryhänen, Jalovaara, & Tuukkanen, 1999; Kodama et al., 1999).

4.2 | Sex differences

Sex hormones, growth hormones, mechanosensation, and insulin-like growth factors during puberty influence skeletal sexual dimorphism (Callewaert, Sinnesael, Gielen, Boonen, & Vanderschueren, 2010; Copes, Schutz, Dlugosz, & Garland, 2017). Further, given that female mice generally run more revolutions per day and at higher average and maximum speeds in our study system (see above), one might expect some degree of sex-specific response to selection. Indeed, several such examples have been reported, including the observation that female HR mice have evolved longer daily running distances almost entirely by increases in average running speed, whereas males also show increases in daily running duration (Garland, Kelly, et al., 2011). However, only one previous study of the HR mice has examined sex differences, with Garland and Freeman (2005) reporting that males had shorter leg lengths, femurs, tibia-fibulas, and metatarsal bones when accounting for body mass as a covariate, but higher MT/F ratios. In the present study, we confirm results and also report that males have higher T/F ratios, heavier hindlimb bones, and more robust femurs and tibia-fibulas (which may increase bone strength), the latter two findings consistent with studies on skeletal sexual dimorphism in Carnivora (Morris & Carrier, 2016), rats (Kim et al., 2003), and humans (Nieves et al., 2004).

Males also have shallower femurs indicating differences in the shapes of the hindlimb bones between the sexes. Males also had relatively wider distal femora and heavier hindlimb muscles when compared with females. Limb bone morphology differs between the sexes substantially, with males having seemingly more robust hindlimb bones and larger muscles than females.

4.3 | Interactions between linetype, sex, and body mass

Interaction models revealed interesting results regarding skeletal evolution, body size, and sexual dimorphism as it relates to selective breeding for high voluntary wheel running. For example, female HR mice have evolved larger femoral heads (Figure 6a), longer hindfeet, and deeper tibia-fibulas only at larger body masses, as compared with female control mice, whereas HR males have larger femoral heads than C males at all body masses (Figure 6b). In contrast, male HR mice have evolved altered tibia-fibula proximal depths that varied depending on body mass (supporting information Table S1). Thus, allometric relations have evolved in the HR mice, and in a sex-specific way. These results imply that the genetic correlations between bone dimensions and overall body size may be more labile than is commonly assumed (see also Marchini et al., 2014).

When interactions were observed and analysis was split by sex, additional main effects were in some cases discovered (supporting information Table S1). For example, female HR mice had wider proximal femurs and deeper proximal tibias (e.g., see above; near the hip and knee joint) than female control mice. Thus, sex-specific responses in the skeleton can occur even when the same selection is imposed on both sexes. In our case, we showed additional sex-specific skeletal adaptations for the selection of voluntary wheel-running, that was not previously investigated. More broadly, it seems prudent to include both sexes in skeletal evolutionary studies and comparative studies because there may be several sex-specific responses that may have important evolutionary implications. In fact, in lizard studies habitat use was a significant predictor of crus length in females but not in males (Olberding, Herrel, Higham, & Garland, 2015).

4.4 | Effects of the mini-muscle phenotype

As noted above, mini-muscle mice exhibit a 50% reduction in the triceps surae and total hindlimb muscle mass, caused by a significant reduction of type IIb muscle fibers (Guderley et al., 2006; Talmadge et al., 2014) and is evident in reduced gastrocnemius and quadriceps muscle mass. In a study of males from generation 21, mini-muscle mice were previously reported to share some traits with cursorial mammals, with thinner hindlimb bones, longer tibia-fibulas, and longer overall leg lengths (Kelly et al., 2006). In our analysis of mice from generation 11 (Table 2), mini-muscle individuals did not have significantly longer overall leg lengths, but did have longer distal limb bones relative to proximal bones (high MT/F [$p = .0674$] and T/F ratio [$p = .0069$]). A high T/F ratio and MT/F ratio may promote faster running on level ground by increasing arc of hindlimb movements (Chirchir, 2015 and references therein) and are often associated with increased locomotor speed and/

or efficiency (see Introduction). Mini-muscle mice have lighter hindlimb bones, as seen in many cursorial taxa, which, in principle, should reduce the muscular force required to overcome inertia through the swing phase of each stride (Carrano, 1999 and references therein), although mini-muscle individuals actually have increased costs of transport and reduced maximal sprint speeds (Dlugosz, Chappell, McGillivray, Syme, & Garland, 1993). Mini-muscle mice also have thinner hindlimb bones for many measurements which may reduce rotational inertia and be reflective of reduced bone mass (Young et al., 2014); see Table 2. Like cursorial lagomorphs (Young et al., 2014), mini-muscle mice have increased tibia–fibula distal widths (with body mass as a covariate), but not reduced distal limb bone robusticity (TFDW/T), suggesting the importance of maintaining bone strength at the distal limb.

Given that mini-muscle individuals have some skeletal traits similar to cursors, one might additionally expect their muscle insertion sites to be closer to the hip joints, which would serve to increase limb output velocity at the cost of force generation (Carrano, 1999; Young et al., 2014). In contrast to this expectation, mini-muscle mice have 3rd trochanter muscle insertion sites located more distally relative to the length of the femur. The 3rd trochanter muscle scar attaches the quadratus femoris (Charles et al., 2016) and a more distal muscle insertion site may allow for greater force generation (in-lever/out-lever) (Carrano, 1999) when the hip joint is rotated during running.

4.5 | Previous studies of the skeleton of HR mice & future directions

The present study clearly shows that the skeleton can evolve rapidly when selection acts on behavior. Several previous studies that examined later generations of HR and C mice bolster the current results. Middleton and colleagues gave female HR and C mice from generation 16 access to wheels for 20 months and found that the distal width of the femur was increased as a result of selective breeding, but the fracture characteristics of the femoral neck were not affected by selective breeding or wheel running, as compared with mice housed without wheels (Middleton, Shubin, et al., 2008). In addition, the cross-sectional area of the femoral mid-shaft was increased in the HR lines with wheel access, but decreased in the controls with wheel access (genotype-by-environment interaction).

Kelly et al. (2006), studied males from generation 21, half of which were housed with wheel access from weaning for 8 weeks. With body mass as a covariate, HR mice had larger femoral heads, heavier feet, and increased tibia–fibula and femoral thickness. Wheel access significantly increased hindlimb bone diameters, foot mass, and tibia–fibula mass in both HR and C lines, with no interaction between linetype and wheel access. Mice with the mini-muscle phenotype had significantly longer and thinner tibia–fibula and femoral bone measurements. However, none of the experimental factors affected the MT/F ratio. Another analysis of this sample of mice used μ CT of femoral morphology at two cortical sites and one trabecular site (Wallace, Tommasini, Judex, Garland, & Demes, 2012). HR mice had femurs with enlarged (wider) shafts, increased marrow areas, and altered mid-diaphysis shape which increased moments of inertia (resistance to bending/stress).

Mini-muscle mice had reduced cortical bone area, trabecular thickness, and altered shaft shape (Wallace et al., 2012). Wheel running led to moderate periosteal enlargement but increased endocortical expansion, leading to thinner cortices and reduced metaphysis bone area. However, trabecular morphology, moments of inertia, and mid-diaphysis bone area were unaffected by exercise (Wallace et al., 2012). An additional study using this sample found that HR mice have altered semicircular canal shape (Schutz, Jamniczky, Hallgrímsson, & Garland, 2014).

Finally, at generation 37, adult (79 days of age) female HR and C mice were housed with or without wheel access for 13–14 weeks. Both linetype and presence of the mini-muscle phenotype were significant predictors of femoral cortical cross-sectional anatomy. However, nano-indentation (micro-scale organization of materials) to measure compressive stiffness at the femoral mid-diaphysis indicated no significant effect of linetype and exercise on mean stiffness (Middleton et al., 2010).

Most studies of skeletal material from the HR selection experiment have examined aspects of the hindlimb bones (but see Copes, Schutz, Dlugosz, & Garland, 2018; Schutz et al., 2014). A more comprehensive view of skeletal evolution in these unique lines of mice will require consideration of the forelimbs, the pectoral and pelvic girdles (e.g., see Schutz et al., 2009), the axial skeleton, and their functional associations with ligaments, tendons, and muscles. Beyond this, we will need biomechanical studies to measure kinematics and forces during wheel running, as well as studies that attempt to relate morphology to gait and stride differences (e.g., see Claghorn et al., 2017; Sparrow et al., 2017).

ACKNOWLEDGMENTS

We thank Patricia A. Freeman for obtaining measurements and for comments on the manuscript (Dr. Freeman declined to be a coauthor on this manuscript). We also thank an anonymous reviewer, Campbell Rolian, Nicholas Schwartz, and Amanda Smolinsky for helpful comments on the manuscript. Special thanks go to Jessica Tingle for helpful discussions and revisions throughout the writing process. Supported by N.S.F. grants to T.G., most recently DEB-1655362.

ORCID

Theodore Garland  <http://orcid.org/0000-0002-7916-3552>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Castro AA, Garland T. Evolution of hindlimb bone dimensions and muscle masses in house mice selectively bred for high voluntary wheel-running behavior. *Journal of Morphology*. 2018;279:766–779. <https://doi.org/10.1002/jmor.20809>

Supplemental Table 2. Analyses of asymmetry. Significance levels (P values; bold indicates P < 0.05 or P < 0.1 for interaction terms) are from two-way nested analysis of variance models implemented in SAS PROC MIXED. Signs after P values indicate direction of effect: + indicates HR > C, Male > Female, or Mini-muscle > normal muscle.

Trait	N	Linetype	Sex	Sex*Linetype	Mini-Muscle
<i>Degrees of Freedom</i>		1, 6	1, 6	1, 6	1, ~19
DA Leg	134	0.0217-	0.4412+	0.3885	0.2340-
DA Femur L	137	0.0311-	0.9000-	0.1286	0.4762-
DA Tibia-fibula L	137	0.8981+	0.8958+	0.6055	0.4298-
DA 3rd Metatarsal	135	0.1398+	0.0361-	0.2592	0.7564+
DA Femoral Head	137	0.6190-	0.1875+	0.6215	0.8323-
DA Femoral Least Width	139	0.8551-	0.0454-	0.0567	0.8748-
DA Femoral Distal Width	135	0.1530-	0.7582+	0.5918	0.9447-
DA Tibia-fibula Least Width	136	0.4843-	0.6799-	0.0923	0.4814+
DA Tibia-fibula Distal Width	136	0.6963-	0.5590+	0.6426	0.0236+
FA Leg	134	0.0865-	0.2770-	0.7845	0.2586+
FA Femur L	138	0.0993-	0.2472-	0.9080	0.2131+
FA Tibia-fibula L	139	0.2538-	0.8835-	0.8816	0.8535+
FA 3rd Metatarsal	135	0.7827-	0.0806+	0.1785	0.4161-
FA Femoral Head	138	0.8409+	0.7462+	0.7363	0.1165+
FA Femoral Least Width	139	0.9712+	0.2062+	0.3773	0.4716+
FA Femoral Distal Width	135	0.3993-	0.6279+	0.9473	0.7901-
FA Tibia-fibula Least Width	136	0.3849+	0.2557+	0.1546	0.1934-
FA Tibia-fibula Distal Width	136	0.0108-	0.8846-	0.8235	0.0666+
FA/DA Leg	133	0.1230-	0.1783+	0.3295	0.2375-
FA/DA Femur L	135	0.0510-	0.4426+	0.2369	0.3549-
FA/DA Tibia-fibula L	133	0.5766-	0.2693-	0.5146	0.7018-
FA/DA 3rd Metatarsal	127	0.6255+	0.1990-	0.4861	0.4614+
FA/DA Femoral Head	128	0.6587-	0.1250+	0.5736	0.8764-
FA/DA Femoral Least Width	132	0.7714-	0.0743-	0.0997	0.9553+
FA/DA Femoral Distal Width	135	0.6163-	0.2418-	0.9592	0.2141-
FA/DA Tibia-fibula Least Width	133	0.2623-	0.9409-	0.0649	0.3467+
FA/DA Tibia-fibula Distal Width	126	0.9550+	0.5792+	0.7144	0.3473+