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Effects of selective breeding for voluntary exercise, chronic exercise, and their interaction on muscle attachment site morphology in house mice

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Abstract

Skeletal muscles attach to bone at their origins and insertions, and the interface where tendon meets bone is termed the attachment site or enthesis. Mechanical stresses at the muscle/tendon-bone interface are proportional to the surface area of the bony attachment sites, such that a larger attachment site will distribute loads over a wider area. Muscles that are frequently active and/or are of larger size should cause attachment sites to hypertrophy (training effect); however, experimental studies of animals subjected to exercise have provided mixed results. To enhance our ability to detect training effects (a type of phenotypic plasticity), we studied a mouse model in which 4 replicate lines of High Runner (HR) mice have been selectively bred for 57 generations. Selection is based on the average number of wheel revolutions on days 5 & 6 of a 6-day period of wheel access as young adults (6-8 weeks old). Four additional lines are bred without regard to running and serve as non-selected controls (C). On average, mice from HR lines voluntarily run ~3 times more than C mice on a daily basis. For this study, we housed 50 females (half HR, half C) with wheels (Active group) and 50 (half HR, half C) without wheels (Sedentary group) for 12 weeks starting at weaning (~3 weeks old). We tested for evolved differences in muscle attachment site surface area between HR and C mice, plastic changes resulting from chronic exercise, and their interaction. We used a precise, highly repeatable method for quantifying the three-dimensional (3D) surface area of four muscle attachment sites: the humerus deltoid tuberosity (the insertion point for the spinodeltoideus, superficial pectoralis, and acromiodeltoideus), the femoral third trochanter (the insertion point for the quadratus femoris), the femoral lesser trochanter (the insertion point for the iliacus muscle), and the femoral greater trochanter (insertion point for the middle gluteal muscles). In univariate analyses, with body mass as a covariate, mice in the Active group had significantly larger humerus deltoid tuberosities than Sedentary mice, with no significant difference between HR and C mice and no interaction between exercise treatment and linetype. These differences between Active and Sedentary mice were also apparent in the multivariate analyses. Surface areas of the femoral third trochanter, femoral lesser trochanter, and femoral greater trochanter were unaffected

by either chronic wheel access or selective breeding. Our results, which used robust measurement protocols and relatively large sample sizes, demonstrate that muscle attachment site morphology can be (but is not always) affected by chronic exercise experienced during ontogeny. However, contrary to previous results for other aspects of long bone morphology, we did not find evidence for evolutionary coadaptation of muscle attachments with voluntary exercise behavior in the HR mice.

KEYWORDS

artificial selection, bone, enthesis, exercise, locomotion, muscle attachment

1 | INTRODUCTION

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Bone is a dynamic and metabolically active organ composed of calcium phosphate minerals and type I collagen. Bone modeling and remodeling, the actions of osteoclasts and osteoblasts during bone resorption and formation, is essential for the mineral and mechanical homeostasis of the skeleton (Doherty et al., 2015; Frost, 2003; Katsimbri, 2017). Mechanical forces acting on the skeleton cause strain and microdamage to bone tissue, which is responded by osteocytes (mechanosensory cells that sense fluid flow associated with strain) that translate mechanical strain to biochemical signals, and initiates bone remodeling (Bonewald, 2007; Yu et al., 2017). On one hand, increases in mechanical load cause changes in shape and material properties of bones that lead to increased stiffness and strength (Frost, 2003; Hart et al., 2017; Ruff et al., 2006). On the other, structural and mechanical changes occur on the skeleton during paralysis, unloading, and/or disuse (lower levels of strain and stress), that leads to declines in bone mass and mechanical integrity (Kodama et al., 1999; Maupin et al., 2019; Morey-Holton & Globus, 1998; Ruff et al., 2006).

Physical conditioning (e.g., exercise through running, weightlifting) is important for the maintenance of adequate bone mass and strength. In mammals, exercise induces bone formation and retards bone loss, enhancing bone structure and ultimately strength (Eliakim et al., 1997; Lieberman, 2003; Plochocki et al., 2008; Rubin & Lanyon, 1984). For example, in studies of rats and mice, both voluntary wheel running and tower climbing required to obtain food can increase the thickness and mass of the tibia-fibula and femur (Mori et al., 2003; Newhall et al., 1991; Notomi et al., 2001).

The extent of training effects depend on genetic factors (Middleton et al., 2008a; Peacock et al., 2018), as well as age, sex, and epigenetic factors (discussed in Wallace et al., 2012). For example, one study used two outbred strains of mice (ICR vs. CD1) to examine the effects of exercise (30 min of treadmill-running, 5 days a week) on skeletal structure and mechanics (Wallace et al., 2015). ICR mice that ran had significantly improved diaphyseal bone quantity, enhanced trabecular morphology, and increased femoral mechanical strength (as compared with sedentary controls). However, CD1 mice that ran (same regime) had reduced femoral structural strength (diaphyseal resistance to fracture). As another example, Peacock et al. (2018) studied the effects of genetics and exercise

on bone properties in three inbred mouse strains: high bone density (C3H/He), low bone density (C57BL/6), and a high-runner strain homozygous for the *Myh4*^{Minimsc} allele (see Methods). Although several interstrain differences were observed, femoral bone cross-sectional geometry and bending mechanics were not significantly different between exercised (wheel access for a 7-week period) and sedentary mice.

The origins and insertions of muscles adhere to the skeleton directly by aponeurosis or via tendons, both of which are termed muscle attachments (or entheses) (Benjamin et al., 1986, 2002). Mechanical stresses at the muscle/tendon-bone interface are proportional to surface area of the bony attachment sites, such that larger entheses will distribute loads over a wider surface area (Biewener, 1992). Recent studies have investigated the micromechanics and microstructure of muscle entheses, finding variation in material properties (hard-to-soft interface) and collagen/fiber orientation that leads to concentrated compliance zones at the micrometer level when the attachments are loaded (Deymier et al., 2017; Rossetti et al., 2017). Therefore, as with other aspects of long bone morphology, one would expect that intense and sustained physical activity might cause growth and/or remodeling of attachment sites (i.e., training effects due to increases in muscle activation and/ or muscle size). This expectation has served as the basis for studies attempting to reconstruct the physical activity levels of an organism from fossilized bones based on their muscle attachment site morphology (Becker, 2020; Foster et al., 2014; Hawkey & Merbs, 1995; Karakostis et al., 2021; Schlecht, 2012). However, experimental studies of the effects of physical activity on muscle attachment site morphology have generated mixed results.

For example, mature female sheep wearing weighted backpacks were given treadmill exercise for one hour, 5 days/week, over 90 days. Muscle attachment site morphology did not differ between exercised and sedentary sheep, and muscle mass did not correlate with muscle attachment size or complexity within the sedentary group (not analyzed in the active group) (Zumwalt, 2006). In another study, muscle enthesis size, periosteal growth rate, and muscle architecture of the upper forelimb were compared among sedentary mice and those housed with either wheels or a climbing tower (exercise) for 11 weeks, beginning either at 25 or 46 days of age (Rabey et al., 2015). Both types of exercise increased the periosteal growth rate of the deltoid tuberosity and significantly altered fiber lengths

and physiological cross-sectional areas of the shoulder muscles; however, muscle attachment surface area, length, and diameter of the deltoid crest were not significantly altered (Rabey et al., 2015). Finally, muscle entheseal topography, diaphyseal bone dimensions, and trabecular architecture of the femur were compared in growing female turkeys that were either trained on a declined treadmill (inclined running group not included) or remained sedentary over a 10-week period (Wallace et al., 2017). Trabecular thickness of the distal femoral metaphysis (knee) and second moments of inertia of the femoral mid-shaft were increased in exercised turkeys, but muscle enthesis topography was unaltered (but see below).

Although the foregoing studies suggest that muscle attachment site morphology may be generally less plastic than other aspects of bone morphology (e.g., length, thickness, density), more recent studies indicate that it can respond to physical activity, loading, or direct muscle stimulation. For example, with the same individual turkeys as in Wallace et al. (2017), and an additional group of incline runners, Karakostis et al. (2019b) found distinctive multivariate patterns involving three different entheses that distinguish controls from running groups (both inclined and declined running groups). Furthermore, a study of adult rats found that in vivo electrical muscle stimulation (over 28 days) caused changes (relative to non-stimulated controls) that differed among muscle entheses and reflected repetitive muscle recruitment (Karakostis et al., 2019a).

Here, we compare mice from four replicate lines that have been selectively bred for high levels of voluntary activity (wheelrunning behavior: High Runner or HR lines) with those from four non-selected Control (C) lines (Careau et al., 2013; Swallow et al., 1998). All four HR lines evolved rapidly and reached selection limits after ~17-27 generations, depending on replicate line and sex (Careau et al., 2013; Garland et al., 2011), at which point HR mice run approximately three-fold more wheel revolutions per day than C mice. These high levels of physical activity should enhance statistical power to detect training effects (phenotypic plasticity) in muscle attachment site morphology. In addition, we tested for differences between the HR and C lines to study possible coadaptation of the skeleton with physical activity behavior.

Although previous studies of HR mice have found size and shape differences between the long bones of HR and C mice (e.g., see Garland & Freeman, 2005; Wallace et al., 2010; Schwartz et al., 2018), which is part of their overall "mobility" phenotype (Wallace & Garland, 2016), no studies of muscle enthesis morphology are available for this animal model. Thus, our objectives were to quantify any evolved differences in muscle attachment site morphology between HR and C mice, plastic changes resulting from chronic exposure to exercise (active vs. sedentary experimental treatments), and potential genotype-by-environment interactions, as have been observed for some other skeletal traits (Middleton et al., 2008a). We used a precise, highly repeatable method to measure the three-dimensional (3D) surface area of muscle attachment sites that was first introduced in a study of human hand entheses (Karakostis & Lorenzo, 2016). Since then, this method, named "Validated Entheses-based Reconstruction of Activity" (V.E.R.A.) (Karakostis & Harvati, 2021),

has been applied successfully in other experimental studies using small mammals and birds (Karakostis et al., 2019a; b). Our large sample size, which is substantially greater than in other experimental studies of muscle entheses, allows us to reliably apply robust linear statistical models and probability testing.

ANAT

We had several hypotheses regarding the effects of exercise and genetics on muscle attachment morphology. 1: HR mice will have evolved larger (increased surface area) muscle attachment sites, which would reduce stress acting on the muscle insertion sites, a lower-lever trait that may be beneficial for endurance running. 2: HR and C Mice housed with wheels throughout ontogeny will have enlarged muscle attachments due to the dynamic loads experienced when running (cf., Roach et al., 2012). 3: HR mice may have altered phenotypic plasticity, which would be detected as a statistical interaction between the main effects of linetype and activity housing condition (i.e., a genotype-by-environment interaction). Hypothesis 3 implies comparing the alternatives of "more pain, more gain" versus the "principle of initial value." In the former, one expects a greater amount of training effort (e.g., greater daily wheel-running distance) to be associated with a stronger training response. Given that HR mice run more than C mice, one might generally expect to find larger training effects in the former, regardless of the trait in question (e.g., see Garland & Kelly, 2006). On the other hand, the principle of initial value expects an inverse relationship between the initial value of a trait and the magnitude of response to training (Koch et al., 2005; Middleton et al., 2008a). Thus, if mice from the HR lines were to have innately larger muscle entheses (i.e., without training), then we would expect any increase caused by training to be blunted as compared with the effect observed for mice from the non-selected Control lines.

2 | METHODS

2.1 | High Runner mouse model

Mice from the 4 High Runner (HR) lines are bred for high voluntary wheel running and are compared with 4 non-selected Control (C) lines (Swallow et al., 1998). The founding population was 224 laboratory house mice (Mus domesticus) of the outbred, genetically variable Hsd:ICR strain (Harlan-Sprague-Dawley). Mice were randomly bred for two generations and then separated into 8 closed lines, which consist of 10 breeding pairs per line per generation. During the routine selection protocol, mice are weaned at 21 days of age and housed in groups of 4 individuals of the same sex until 6-8 weeks of age. Mice are then housed individually in cages attached to computer-monitored wheels (1.12 m circumference, 35.7 cm diameter, and 10 cm wide wire-mesh running surface) with a recording sensor that counts wheel revolutions in 1-min intervals over 6 days of wheel access (Careau et al., 2013; Hiramatsu, 2017; Swallow et al., 1998). In the HR lines, the highest-running (average revolutions during days 5 and 6 of a 6-day trial) male and female from each family are chosen as breeders. Running on days 5 and 6 is

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used as the selection criterion to avoid potential effects of neophobia. In the C lines, a male and a female are randomly chosen from each family. Sibling mating is not allowed. Mice are kept at room temperatures of approximately 22°C, with ad lib access to food and water. Photoperiod is 12L:12D.

2.2 Mouse specimens, physical activity, and wheel access

We studied 100 female mice (evenly sampled from the 8 lines except for line 6: see Section 2.3) from generation 57 of the selection experiment (Copes et al., 2015, 2018). We chose female mice to remove the confounding effects of skeletal sexual dimorphism (Castro & Garland, 2018; Nieves et al., 2004) and because female mice generally run more revolutions per day and at higher average and maximum speeds in our study system. Briefly, mice were housed individually beginning at weaning (21 days of age), with food and water ad lib. At approximately 24 days of age, half of the mice were given access to wheels (attached to their cages as described above) for 12 weeks (see Figure 1). Furthermore, physical activity measures within the home cage were recorded daily using passive infrared motion detection sensors over a 23.5 hour period (Copes et al., 2015, 2018). Across the 12 weeks of either wheel access (Active group) or being housed without wheels (Sedentary), 3 individual mice died of natural causes and so were not included in our study. Therefore, our sample consisted of 25 Sedentary C mice, 23 Sedentary HR mice, 24 Active C mice, and 25 Active HR mice (Figure 1). None of the mice in this study were used as breeders for the selection experiment.

All experiments were approved by the University of California, Riverside Institutional Animal Care and Use Committee.

The wheel running and home-cage activity data sampled over 12 weeks are presented and analyzed in Copes et al. (2018). As expected, HR mice ran significantly further than C mice each week, due to both longer duration of running and higher average running speeds. Mice with wheel access had lower home-cage activity than Sedentary mice. Sedentary HR mice had significantly higher levels of home-cage activity than Sedentary C mice throughout the course of the experiment, due to moving during more intervals each day. Among Active mice, linetype did not have a significant effect on home-cage activity measures (Copes et al., 2015, 2018; Lewton et al., 2019).

2.3 Dissection and bone preparation

At 15 weeks of age, 97 mice were euthanized, weighed, and dissected for tissues (Figure 1). The mass of the triceps surae muscle was used to identify individual mice with the mini-muscle phenotype (Garland et al., 2002). In our selection experiment, the "mini-muscle" phenotype occurred in a subset of the mice, characterized by a 50% reduction in triceps surae and total hindlimb muscle mass, primarily caused by a significant reduction in type IIb muscle fibers (Guderley et al., 2006; Talmadge et al., 2014). The phenotype is caused by a novel intronic single nucleotide polymorphism in the Myosin heavy polypeptide 4 gene (Kelly et al., 2013) that behaves as a Mendelian recessive allele. This allele was present in the starting population of 224 mice at a frequency of ~7%, and population-genetic modeling

Timeline and experimental design



24 Active C mice 25 Active HR mice

and dissected at Week 15. Bones were prepped using Tergazyme



283

indicates that the mini-muscle phenotype was (unintentionally) under positive selection in the HR lines (Garland et al., 2002). The mini-muscle phenotype eventually became fixed in one HR line, but remains polymorphic in another. In our sample of 97 mice (not all of which had data for all traits), the number of mini-muscle individuals was all 11 in HR line #3 and 5 of 11 in HR line #6. After dissection of the triceps surae muscles, mice were disemboweled and the carcasses were soaked in a 1% solution of enzymatic detergent (Tergazyme) to dissolve flesh from bone (Copes et al., 2018; Selvey et al., 2018) (Figure 1).

2.4 | Selection of muscle entheses

For this study, we analyzed muscle entheses on the humerus and femur because they are the largest of the mouse long bones, with a considerable amount of attached muscle mass that is activated during exercise (Bab et al., 2007; Benjamin et al., 2002; Charles et al., 2016). Previous studies of mice and rats have routinely shown that long bones respond to mechanical loading, often achieved through exercise (Mori et al., 2003; Newhall et al., 1991; Plochocki et al., 2008; Yang et al., 2007), including in the HR and C mice (e.g., see Kelly et al., 2006; Middleton et al., 2008b; Wallace et al., 2012; Young et al., 2009). Furthermore, the few experimental studies that have investigated the effects of exercise on muscle attachment site morphology have included those found on long bones (Karakostis et al., 2019a; b; Rabey et al., 2015; Wallace et al., 2017; Zumwalt, 2006). However, muscles that attach on limb bones can have different roles during exercise (e.g., the quadratus femoris muscle functions to stabilize the hip and to counter the medial rotation generated by the gluteal muscles during extension) and may not be directly involved with load bearing per se.

When choosing which muscle attachments to analyze (especially considering the use of dry bone specimens), certain criteria were deemed necessary for measurements to be taken, including: 1) the enthesis must be clearly defined and homologous across specimens (e.g., see Bab et al., 2007; Karakostis et al., 2018); 2) the enthesis must not be damaged across multiple specimens (e.g., the femoral distal condyles frequently broke off); and 3) the muscles that attach on the enthesis must be clearly defined in terms of function and morphology in mice (see below). The muscle attachment sites that met these criteria for the femur include the femoral lesser trochanter, which serves as the insertion point for the iliacus muscle (origin is on the iliac crest of the pelvis) and functions to flex the hip joint (activated during the swing phase), the femoral third trochanter which serves the insertion point for the quadratus femoris muscle (origin is on the pubis bone of the pelvis) and functions to stabilize and rotate the hip joint laterally, and the femoral greater trochanter, which serves as the insertion point for the middle gluteal muscles (origin is on the lateral aspect of iliac crest) and functions to extend the hip joint (activated during the stance phase and providing propulsion) (Charles et al., 2016). On the humerus, the humeral deltoid tuberosity is a clearly defined, prominent ridge that serves as the insertion point for the spinodeltoideus, superficial pectoralis, and acromiodeltoideus muscles, with the spinedeltoideus inserting along most of the lateral surface and the superficial pectoralis and acromiodeltoideus inserting along the medial surface (Bab et al., 2007; Rabey et al., 2015). Although the deltoid muscles (spinedeltoideus and acromiodeltoideus) function primarily as shoulder extensors (activated during the swing phase), the pectoralis muscle (superficial pectoralis) functions during retraction, bringing the mouse forward during locomotion on the supporting limb. All the muscles considered are either involved in weight bearing, stabilization, rotation, retraction and/or protraction during mouse locomotor behavior (Clarke & Still, 1999).

2.5 | µCT scanning and image segmentation

As described by Copes et al. (2018), the right femur and humerus were µCT scanned at 12-µm resolution using a small animal preclinical microtomography scanner Viva-CT40, Scanco Medical AG. (Basserdorf, Switzerland) housed at the University of Calgary. Calgary, Alberta. For each specimen, the raw data were reconstructed as 16-bit TIFF image sequential stacks using ImageJ software (Schneider et al., 2012). Image stacks were imported into Thermo Scientific AMIRA 5.6 Software, Thermo Fisher Scientific (Waltham, Massachusetts, USA) for visualization and segmentation. The Isosurface module was used to create surface renderings of the humerus and femur to examine the external morphology of the muscle entheses. Next, an orthographic perspective (objects are displayed proportional to their true size) was used and each bone was virtually re-oriented along its long axis using Align Principal Axes. Afterwards, the OrthoSlice module was used to segment out the total bone area for each bone and 3D surface models were created and exported as.stl files using the Label Field module (cf., Schwartz et al., 2018).

2.6 | 3D reconstruction of muscle entheses

The 3D surface models of the right humerus and femur were separately imported and rendered into MeshLab (CNR-INC, Rome, Italy). Following the V.E.R.A. method (Karakostis & Harvati, 2021), four entheseal surfaces were delineated using image filtering techniques based on surface elevation, coloration, and surface complexity (Karakostis & Harvati, 2021; Karakostis et al., 2018, 2019a, b, 2021; Karakostis & Lorenzo, 2016). In this study, we provide a pipeline figure of the V.E.R.A method based on the humerus deltoid tuberosity (Figure 2). Furthermore, we illustrate the delineated models of all the muscle attachments described above (see 2.4: Selection of muscle entheses) (Figure 3). In this study, we could not include the *"equalize vertex colors"* filter because our scans lacked color information and primarily focused on the presence of distinctive surface elevation (i.e., projecting, or depressed bone surface) and irregularities (Karakostis & Lorenzo, 2016; Karakostis et al., 2021) (Figure 2a).



Caudo-lateral view

Caudo-lateral view

FIGURE 2 Illustration of the applied V.E.R.A. 3D measuring protocols (Karakostis & Harvati, 2021; Karakostis & Lorenzo, 2016). (a) 3D surface models of the right humerus bone (shown in left) and the right humerus deltoid tuberosity (shown in right) in the caudal-lateral view using MeshLab. Color information was not available for these scans. (b) Application of the "Discrete Curvatures" filter, a surface curvature filter that color-mapped the 3D surface of the right humerus deltoid tuberosity depending on its elevation and irregularity. 3D surface models of the right humerus deltoid tuberosity are examined over 360° and are depicted in the caudal-lateral (shown in right) and ventrallateral (shown in left) views. (c) Selection of the right humerus deltoid tuberosity's border and interior surface based on the application of the "Discrete Curvatures" filter (elevation and irregularities are shown in red and blue pigmentation for these specimens). 3D surface models of the right humerus deltoid tuberosity are examined over 360° and are depicted in the caudal-lateral (shown in right) and ventral-medial (shown in left) views. (d) Additional selection of a very thin flat region (red arrow) around the right humerus deltoid tuberosity (muscle attachment). The 3D surface model of the right humerus deltoid tuberosity was examined over 360° and is depicted in the caudal lateral view. Subsequently, the area selection was inverted to the rest of the bone surface (using the "Invert Selection" option), which was removed. (e) Color-mapping of the muscle attachment surface based on the application of the "Curvature Principal Directions" filter (followed by selecting "Principal Component Analysis"), a filter that highlights the surrounding flat region (shown in dark blue) of the right humerus deltoid tuberosity based on the principal direction of curvature. The 3D surface model of the right humerus deltoid tuberosity was examined over 360° and is depicted in the caudal lateral view. (f) Removal of the flatter area surrounding the right humerus deltoid tuberosity. The "Compute Geometric Measurements" filter was used to measure the external surface area of the muscle entheses in mm² (shown in the left). The 3D surface model of the right humerus deltoid tuberosity was the superimposed on the bone model (Figure 1a) for final verification (shown in the right)

Previous inter-method tests have confirmed the precise applicability of this methodology on 3D scans lacking color information (Karakostis et al., 2018). For each of the muscle attachments, we applied the "Discrete Curvatures" filter, a surface curvature filter in MeshLab that color-maps the 3D surface of the bone depending on its elevation and irregularity (Figure 2b). After this, the "Z-Painting" tool was used to model the general boundary of the muscle attachment (Figure 2c) (including a flatter region circling it) (Figure 2d) followed by using the "Invert Selection" filter to crop it from the

Caudo-lateral view

rest of the whole bone 3D surface model. The "Curvature Principal Directions" filter was applied on the individual muscle attachment models (Figure 2e) to separate out the flatter area (shown in dark blue) surrounding the entheseal surface (Figure 2f). Before quantifying the delineated muscle attachment 3D surface models, we transformed the scale on MeshLab based on linear measurements from the whole bone 3D surface models. Finally, the "Compute Geometric Measurements" filter was used to measure the external surface area of the muscle attachments in mm² (Karakostis & Harvati, 2021;



FIGURE 3 (a) 3D surface models of the humerus deltoid tuberosity on the humerus (shown in right) and the delineated muscle attachment (shown in left) using MeshLab. (b) 3D surface models of the femoral lesser trochanter on the femur (shown in right) and the delineated muscle attachment (shown in left) using MeshLab. (c) 3D surface models of the femoral third trochanter on the femur (shown in right) and the delineated muscle attachment (shown in left) using MeshLab. (d) 3D surface models of the femoral greater trochanter on the femur (shown in right) and the delineated muscle attachment (shown in left) using MeshLab

Trait	N	Linetype	Activity	Activity*Linetype	Mini-Muscle	Body Mass
Degrees of Freedom		1,6	1, 6	1, 6	1,~62-80	1, ~62-68
Body Mass	97	0.0938-	0.0922-	0.5230	0.4307-	
Humerus Deltoid Tuberosity (mm ²)	80	0.3705-	0.0346+	0.9894	0.3045-	0.0088
Femoral Third Trochanter (mm ²)	80	0.3478-	0.3818-	0.4567	0.0073-	0.0358
Femoral Lesser Trochanter (mm ²)	86	0.9791-	0.8989-	0.4195	0.5155+	0.2144
Femoral Greater Trochanter (mm ²)	91	0.7878+	0.4467+	0.6389	0.7576+	0.0302
PC 1	64	0.4275	0.3102	0.1213	0.0315-	
PC 2	65	0.3283	0.6678	0.9300	0.0927	
PC 3	65	0.2731	0.2406	0.3385	0.5004	
PC 4	65	0.5523	0.0212-	0.6398	0.8259	
PC 5	65	0.5475	0.2148	0.5231	0.1797	

TABLE 1 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

Signs following p values indicate direction of effect: + indicates HR lines > C or active > sedentary mice or mini > than non-mini.

Karakostis et al., 2018, 2019a, b, 2021; Karakostis & Lorenzo, 2016) (Figure 3).

Repeatability of measurements 2.7

All measurements were blind with respect to activity, linetype, and mini-muscle status. Prior to taking measurements from the segmented 3D models, we tested repeatability across a sub-sample of 10 specimens to account for intra- and inter-observer measurement error, akin to other studies using the V.E.R.A. approach, which found

that the maximum mean precision error was 0.62% (see Karakostis & Lorenzo, 2016). The repeatability test used to check inter-observer error involved only two observers (FAK and AAC). Furthermore, each specimen was measured twice, and both observers applied the V.E.R.A method on these specimens over two days. More specifically, we used a repeated-measures ANOVA for the inter-observer measurements and non-parametric paired tests (Wilcoxon) across all possible repetitions. In all scenarios, the p values were >0.05, indicating repeatability. In our study, AAC delineated and guantified all the muscle attachments using the V.E.R.A. approach. In addition, we did not measure any individual muscle enthesis that were damaged

(e.g., while being prepped using Tergazyme) and/or had a significant amount of osteoporosis (e.g., see Supplemental Material), which resulted in variable sample sizes for both univariate and multivariate analyses. Final samples sizes are indicated in Table 1.

2.8 | Statistical analysis

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We used the MIXED Procedure in SAS (SAS Institute, Cary, NC, USA) to apply nested analysis of covariance models with replicate line as a random effect nested within linetype, yielding 1 and 6 d.f. for testing the effect of linetype (Houle-Leroy et al., 2000, 2003; Swallow et al., 1999). Likewise, the main effects of activity (wheel access) and the interaction between activity and linetype were tested with 1 and 6 d.f. (see Copes et al., 2018; Lewton et al., 2019). The main effect of the mini-muscle phenotype (see Methods) was included and tested relative to the residual variance with 1 and ~62–80 d.f. (depending on the muscle enthesis). All analyses of muscle entheses included body mass (recorded at dissections) as a covariate.

In addition, we used measures of physical activity as covariates. We went back to the primary paper for this data set (Copes et al., 2018) and used the weekly average activity variables, as reported in their supplemental materials (e.g., wk8run6, wk8htot6). We then summed these variables across all 12 weeks to obtain a measure of the total "volume" of wheel-running distance and of spontaneous physical activity in the home cages. We used these as covariates (along with body mass) for each of the four attachment measures. We analyzed the sedentary and active mice separately, the former using only cage activity.

For multivariate analyses, the four entheseal surface area measurements were subjected to principal components analysis (PCA) that included body mass (5 principal components total). PCA is an exploratory technique used to reveal multivariate patterns of variation in a sample, without using *a priori* group classification (Field, 2013). Scores on the five PCs were subjected to the same analyses described above for the individual measures. Sample sizes were reduced because computation of PC scores requires all measurements for a given mouse (i.e., listwise deletion of missing data).

In all analyses (univariate or multivariate), outliers were removed when the standardized residual exceeded ~3.0 and we used an α of <0.05 for statistical significance. For univariate analyses, two low outliers were removed for the humerus deltoid tuberosity (MouseID = 60653 and 60625) and one low outlier for the femoral third trochanter (MouseID = 60714). For multivariate analyses, one low outlier was removed from PC1 (MouseID = 60235). All p values reported are two-tailed (Table 1).

3 | RESULTS

Significance levels from ANCOVAs of muscle entheses (using body mass as a covariate) and from ANOVAs using PC scores are presented in Table 1. Table 2 presents least square means and Table 3 shows the results of a PCA of body mass and the four attachment areas.

	Control Lin	les			High-Runr	ner Lines			Mini-Mus	cle		
	Sedentary		Active		Sedentary		Active		Normal		Mini	
Trait	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body Mass (g)	28.24	1.28	26.93	1.28	24.79	1.13	24.14	1.13	26.53	0.78	25.52	1.28
Humerus Deltoid Tuberosity (mm^2)	6.71	0.28	7.15	0.29	6.39	0.24	6.83	0.24	6.93	0.16	6.61	0.29
Femoral Third Trochanter (mm 2)	2.94	0.19	2.71	0.18	2.63	0.15	2.61	0.15	3.04	0.08	2.41	0.20
Femoral Lesser Trochanter (mm^2)	1.12	0.11	1.16	0.09	1.17	0.09	1.11	0.09	1.09	0.06	1.18	0.12
Femoral Greater Trochanter (mm^2)	3.53	0.20	3.58	0.19	3.52	0.16	3.71	0.16	3.54	0.09	3.62	0.20

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

TABLE 3 Principal components analysis of body mass and the four muscle attachment areas along with component correlations with principal components (factor loadings)

PC	Eigenvalue		% of Variance		Cumulative %
1	2.03		40.6		40.6
2	1.42		28.5		69.1
3	0.61		12.1		81.2
4	0.49		9.9		91.1
5	0.45		8.9		100.0
Trait	PC 1	PC 2	PC 3	PC 4	PC 5
Body Mass	0.775	-0.267	0.368	-0.104	-0.427
Humerus Deltoid Tuberosity (mm²)	0.832	0.002	-0.014	-0.378	0.406
Femoral Third Trochanter (mm ²)	0.514	-0.686	-0.106	0.476	0.167
Femoral Lesser Trochanter (mm ²)	0.311	0.789	0.391	0.328	0.144
Femoral Greater Trochanter (mm ²)	0.613	0.509	-0.555	0.080	-0.223

Scores on all five PCs were analyzed statistically (see Table 1).



FIGURE 4 Humerus deltoid tuberosity surface area in relation to body mass. Larger mice had larger deltoid tuberosities, and Active mice (housed with long-term wheel access) had significantly larger muscle attachments for a given body size when compared with Sedentary mice (see Table 1 for statistical analyses)

3.1 | Body size

Body mass was not significantly different when comparing HR vs C mice, active vs sedentary mice, or mini-muscle mice vs normalmuscled individuals (Table 1). However, HR mice tended to be lighter (p = 0.0938) than C mice and active mice tended to weigh less than sedentary mice (p = 0.0922).

3.2 | Muscle attachments

Figures 2 and 3a show a representative deltoid tuberosity image. With body mass as a covariate, mice from the active group had significantly larger humerus deltoid tuberosities (increased surface areas) than sedentary mice (p = 0.0346; Figure 4), with no significant linetype effect or interaction between activity and linetype (see Table 1 for full statistical results and Table 2 for least squares means for the experimental groups). Mice with the mini-muscle phenotype had significantly smaller femoral third trochanters (p = 0.0119) when compared with normal-muscled individuals (Figure 5a), with no significant linetype or activity effect. Morphology of the femoral lesser trochanter and femoral greater trochanter were not affected by chronic wheel access and did not differ between HR and C mice (Figure 5b,c).

For the mice with wheel access, neither measure of physical activity was ever a statistically significant predictor of attachment surface area. Similarly, for the mice without wheels, homecage activity was ever a significant predictor of attachment surface area. WILEY-ANATOMICAL

Journal of Anatomy

CASTRO ET AL.



FIGURE 5 (a) Femoral third trochanter surface area in relation to body mass. Large mice had larger entheses and mini-muscle mice had significantly reduced entheses when compared with normal-muscled mice, with no significant effect of either linetype or activity. (b) Femoral lesser trochanter in relation to body mass. There were no significant effects of linetype, activity or body mass. (c) Femoral greater trochanter surface area in relation to body mass. Large mice had larger entheses, with no significant effect of either linetype or activity

ANATOMICAL SOCIETY-WILEY

3.3 | Principal components analysis

Table 3 shows the results of a PCA of body mass and the four attachment areas. PC 1 accounted for 40.6% of the total variance and mainly reflected humerus deltoid tuberosity surface area and body mass (loading strongly in the same direction). PC 2 accounted for 28.5% of the variance that contrasted the femoral lesser trochanter and femoral greater trochanter surface areas with the femoral third trochanter surface area. PC 3 (12.1% of variance) was mostly related to femoral greater trochanter surface area, PC 4 (9.9%) reflected a contrast between the deltoid tuberosity area and the other entheses, and PC 5 (8.9%) was mostly body mass and humerus deltoid tuberosity (Table 3).

Figure 6 shows scores for PC 1 differed significantly between mini- and normal-muscled mice (p = 0.0315), whereas scores for PC 4 (9.90% of total variance) showed an effect of activity (p = 0.0211, Table 1). Figure 6 shows scores for PC 1 against PC 4, separately for C and HR mice, illustrating the separation by mini-muscle status and activity group.

4 | DISCUSSION

We studied the sizes of four muscle entheses from a unique model system that includes four replicate High Runner (HR) lines of house mice that have been selectively bred for wheel-running behavior and four non-selected Control lines. For the present study, half of the experimental subjects were housed with wheels (Active group) and half without wheels (Sedentary group) for 12 weeks starting at weaning. Thus, we studied phenotypic plasticity, evolved differences, and their interaction. With body mass as a covariate, mice in the Active group had significantly larger humerus deltoid tuberosities when compared with Sedentary mice (i.e., a training effect or phenotypic plasticity). We did not find any overall differences between the HR and C lines, but the subset HR individuals with the mini-muscle phenotype had significantly reduced femoral third trochanters. Consistent with most previous studies of skeletal traits in the HR and C mice, we did not find evidence for differential training responses (i.e., any linetype and activity group interactions) (e.g., see Kelly et al., 2006; Middleton et al., 2008b; Wallace et al., 2012). We discuss our results primarily in the context of previous studies of the causal relationship between physical activity and muscle attachment morphology.

4.1 | Effects of chronic exercise: phenotypic plasticity

As reviewed in the Introduction, some of the previous experimental studies using sheep, mice, and turkeys have not found statistically significant effects of chronic exercise on muscle attachment morphology (Rabey et al., 2015; Wallace et al., 2017; Zumwalt, 2006). Results of those studies would not support use of muscle enthesis

morphology as an indicator of physical activity levels, e.g., in fossils. However, more recent studies that used a combination of precise 3D quantification (i.e., the V.E.R.A. protocol) and multivariate principal components analysis (rather than univariate analyses of single attachments) have found effects of physical activity on muscle attachment surface areas (see Introduction and Karakostis et al., 2019a; b). In the present study, we used this highly repeatable 3D method (see Karakostis & Harvati, 2021; Karakostis & Lorenzo, 2016), and included both univariate and multivariate analyses to facilitate comparison of our results with previous studies.

The humerus deltoid tuberosity is a clearly defined, prominent ridge that serves as an insertion point for three muscles of the forelimb (spinodeltoideus, superficial pectoralis, and acromiodeltoideus muscles) that are involved in shoulder extension (spinodeltoideus and acromiodeltoideus) and retraction (superficial pectoralis) during locomotion (Rabey et al., 2015 and Methods). In univariate analyses, with body mass as a covariate, mice in the Active group had significantly larger humerus deltoid tuberosities (increased surface area) when compared with Sedentary mice (Figures 3a and 4). In multivariate analyses. PC 4 scores, which represent variation in the deltoid tuberosity relative to the other entheses (Table 3), were also significantly different between Active and Sedentary mice (Table 1). A larger surface area of the deltoid tuberosity suggests increased surface area available to dissipate stress (force per unit area) that results from elevated muscular activity and/or increased muscle size (muscle dimensions were not quantified in this study). Thus, we provide clear evidence that physical activity levels experienced across post-weaning ontogeny can cause muscle attachment hypertrophy, although not necessarily in all attachments. In our experimental model, daily access to large, rat-sized wheels resulted in an enlarged muscle attachment site on the forelimb, presumably induced by muscular forces required to turn the wheels during initial acceleration, when running, when decelerating, and/or when climbing/hanging in wheels.

Although we found experimental evidence of muscle attachment hypertrophy caused by voluntary exercise, only one of four (humerus deltoid tuberosity) showed such an effect. This finding is consistent with previous studies reporting that certain attachment sites were influenced much more by physical activity than others (e.g., Karakostis et al., 2017, 2019a, b). These differential responses might, in part, be explained by differences in muscle function and loading during voluntary wheel running. For example, three muscles (spinodeltoideus, acromiodeltoideus, superficial pectoralis) insert on the humerus deltoid tuberosity, whereas the femoral greater, lesser, and third trochanters only have one muscle insertion. Moreover, all these muscles differ in patterns of activation and force production during locomotion (see Methods). Further, the forelimb and hindlimb in general must experience somewhat different loading patterns. Finally, the humerus and femur may inherently differ in their responsiveness to a given pattern and/or intensity of loading.

In any case, our results emphasize the point that, in studies attempting to interpret the physical activity levels of fossil specimens from muscle attachments (e.g., Becker, 2020; Foster et al., 2014;



FIGURE 6 Pairwise plots of scores on principal components (see Table 3). Mice from the Active group (housed with long-term wheel access: squares) had significantly lower scores for PC 4, and mice with the mini-muscle phenotype (all in the HR lines: gray symbols) had lower scores for PC 1 (Table 1)

Hawkey & Merbs, 1995; Karakostis et al., 2021; Schlecht, 2012) special caution is necessary in the selection of entheses analyzed. For instance, previous applications of the V.E.R.A. protocols to fossil hominin hand skeletons (Karakostis & Harvati, 2021; Karakostis et al., 2018) focused exclusively on entheses that have shown consistent differences across documented humans with distinct lifelong occupational activities (Karakostis et al., 2017). Similar recommendations have been made for any skeletal feature used for reconstructing activity in the past (e.g., see Copes et al., 2018; Lieberman et al., 2004; Peacock et al., 2018; Wallace et al., 2012).

4.2 | Exercise throughout ontogeny vs. exercise as adults

In our study, mice were granted wheel access shortly after weaning (~24 days of age) and throughout ontogeny (12-week treatment), which includes the critical period before sexual maturity, during which bones grow and are more likely to adapt to mechanical loads caused by exercise as compared with mature mice (e.g., see Gardinier et al., 2018). Similar to our study, Rabey et al. (2015) gave mice exercise (housed in caged with activity wheels or with 1 m tall wire-mesh tower) when they were either 25 or 46 days old (two cohorts). Likewise, Wallace et al. (2017) and Karakostis et al. (2019b) used 1-year old female Eastern wild turkeys (maturity occurs at ~15 months of age). However, others focused on adults: Karakostis et al. (2019a) used eight week old rats (maturity occurs at ~6 weeks of age) and Zumwalt (2006) used adult female sheep that were all at least four years of age (or older). Studies in rats and mice have shown that senescence leads to reductions in bone mass and mineral density, declines in maximal muscular force production, and changes in muscle properties (Ferguson et al., 2003; Hill et al., 2020; Holt et al., 2016; Horner et al., 2011; Sobolev et al., 2017), all of which could affect muscle entheses. However, experiments on the effects of exercise on muscle attachment morphology that include comparisons of young, mature, and aged animals are lacking.

4.3 | Effects of selective breeding for high voluntary wheel running

Selection experiments and experimental evolutionary approaches (Garland & Rose, 2009) are well-suited for study of microevolution and coadaptation of the skeleton with locomotor behavior, locomotor performance, and body size (Marchini et al., 2014; Middleton et al., 2008a). Here, we found no evidence of evolutionary coadaptation of muscle attachment sites with voluntary exercise behavior (HR vs. C) in mice. However, previous studies have shown several examples of skeletal changes in the HR lines of mice (Castro et al., 2021; Kelly et al., 2006; Schutz et al., 2014). For example, by generation 11, male and female HR mice had evolved larger knee and hip surface areas (adjusting for body size), which, all else being equal, would reduce stress (i.e., force per unit area) acting on limb joints for mice running long distances on wheels (Castro & Garland, 2018; Garland & Freeman, 2005). Another study of the same specimens from generation 11 found that femurs from HR mice have larger total nutrient canal area (due to increased average cross-section size but not the number of canals) than those from C mice (Schwartz et al., 2018). The fact that selection limits were not reached until ~17-27 generations suggests that further skeletal evolution was probable. Indeed, by generation 21, HR males (females were not studied) had larger femoral heads (adjusting for body size and as reported for generation 11), but also had evolved thicker femurs and tibia-fibulas (measured but not significantly different at generation 11), along with heavier feet and longer metatarsals and metacarpals (not weighed at generation 11) (Castro & Garland, 2018; Kelly et al., 2006; Young et al., 2009). However, at generation 68 few differences were found between HR and C mice, and some of the differences in bone dimensions identified in earlier generations were no longer statistically significant (Castro et al., 2021). We are not aware of any other selection experiments targeting locomotion or physical activity that have tested for correlated responses in muscle attachment sites.

ANATO

As compared with other aspects of skeletal anatomy, relatively few interspecific comparisons of mammals have involved muscle attachment sites, and those that did not directly tested for relations with aspects of locomotor performance or ecology (e.g., skeletal correlates with daily movement distances or maximal running sprint speeds, Garland & Janis, 1993; Harris & Steudel, 1997; Kelly et al., 2006). For example, muscle attachment sites are enlarged in the forelimbs of species of rodents and carnivorans that regularly dig and swim (Samuels et al., 2013; Samuels & Van Valkenburgh, 2008) and in the forelimbs of carnivorans that specialize on larger prey (Meachen-Samuels & Van Valkenburgh, 2009). Another study compared the hindlimb muscle attachment morphology of the extinct the Santacrucian (Early Miocene) sloths with that of extant Xenarthran species and found that the former has thicker and more robust muscle attachment sites, suggesting increased muscle size and force generation capabilities for climbing behaviors, regardless of their comparatively larger body size (Toledo et al., 2015). The evolutionary relationships between muscle attachment sizes and locomotor behavior or performance require further study.

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 drastic reduction of type IIb muscle fibers (Garland et al., 2002; Guderley et al., 2006; Talmadge et al., 2014). We found that mini-muscle mice

had significantly reduced surface areas of the femoral third trochanter when compared with normal muscled mice (Table 1, also reflected in scores on PC 1). Likewise, at generations 11 and 68 for both sexes, mini-muscle mice had significantly thinner femoral third trochanters (Castro & Garland, 2018; Castro et al., 2021). These results suggest that the quadratus femoris muscle, which functions to stabilize and rotate the hip, has reduced surface area for attachment at the insertion site, likely due to the greatly reduced hindlimb musculature.

4.5 | Experimental models and future directions

Future investigations of correlations between physical activity and muscle attachment size would benefit from a deeper understanding of how muscle entheses respond to the magnitude of applied loads (e.g., see Rossetti et al., 2017), as well as their frequency (for studies in long bones see Berman et al., 2019; DeLong et al., 2020; Hart et al., 2017; Yang et al., 2017). Moreover, prolonged paralysis or disuse of muscles have detrimental effects on bone mass and strength (unloading) (DeLong et al., 2020; Kodama et al., 1999; Morey-Holton & Globus, 1998), that may also characterize the bony response of attachments. For example, one study used 40 mature male mice to investigate the effects of unloading (achieved via paralysis of shoulder muscles for 21 days) on the mechanical properties of the humerus-supraspinatus muscle attachment, finding an increased risk of fracture and significant bone loss at the millimeter scale, as well as changes at the micro and nanometer scales (Deymier et al., 2019). In addition, a preliminary study comparing muscle attachment size and ground reaction forces in CD1 wild-type and myostatin-deficient (muscular hypertrophy) mice found that while myostatin-deficient mice had expanded muscle attachments (humerus deltoid tuberosity and femoral third trochanter), both groups experienced similar vertical ground reaction forces (Schmitt et al., 2010). Thus, the relationship between muscle enthesis size and limb loading experienced during locomotion is unclear, highlighting the need for future kinematic and biomechanical studies (e.g., see Abraham et al., 2021; Claghorn et al., 2017; Sparrow et al., 2017).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

LEC and TG provided the specimens. LEC provided the CT scans. AAC, FAK, and NLS developed the measurement protocol. AAC, FAK, HEM, and APT collected the data. AAC, TG, and FAK analyzed the data and drafted the manuscript. All authors edited the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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SUPPORTING INFORMATION

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