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Reduction of Type IIb Myosin and IIB Fibers in Tibialis Anterior Muscle of Mini-Muscle Mice From High-Activity Lines

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ABSTRACT Selective breeding of laboratory house mice (*Mus domesticus*) for high voluntary wheel running has generated four replicate lines that show an almost threefold increase in daily wheel-running distances as compared with four nonselected control lines. An unusual hindlimb ''mini-muscle'' phenotype (small muscles, increased mitochondrial enzyme levels, disorganized fiber distribution) has increased in frequency in two of the four replicate selected lines. The gene of major effect that accounts for this phenotype is an autosomal recessive that has been mapped to a 2.6335 Mb interval on MMU11, but not yet identified. This study examined the tibialis anterior muscle to determine whether changes in muscle fiber types could explain such modifications in muscle size and properties. Although selected and control lines did not exhibit systematic differences in the fiber types present in the tibialis anterior muscle, as assessed by electrophoresis of myosin heavy chains (MHC) and by histochemistry, mini-muscle mice lacked type IIB fibers a nd the corresponding MHCs. Mini-muscle tibialis show increased activities of hexokinase and citrate synthase compared with the normally sized muscles, likely the result of the modified fiber types in the muscle. The mini-muscle phenotype is the major means through which selective breeding for high wheel running has modified the functional capacities of the hindlimb muscles, as normally sized tibialis anterior muscles from control and selected lines did not show general differences in their enzymatic capacities, MHC profiles or fiber type composition, with the exception of an elevated hexokinase activity and a reduced GPa activity in the selected lines. *J. Exp. Zool. 311A:189–198, 2009.*

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Complex behavioral phenotypes involve components of both physical ''ability'' and mental ''motivation'' to perform the behavior. Selective breeding for a voluntary behavior in a controlled environment can reveal alterations in both motivation and ability (Rhodes and Kawecki, 2009; Swallow et al., 2009). As an example, selective breeding for wheel running in mice started in 1993 (Swallow et al., '98). Eight lines were created; half of them were bred without regard to wheel running, whereas breeders in the other four were the mice that ran the most on days 5 and 6 of a 6-day exposure to activity wheels at age 6–8 weeks. After ten generations, the mean number of revolutions run per day had increased by 75% in the selected compared with control lines, for both male and female mice (Swallow et al., '98).

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Selected mice covered a greater distance mainly because they ran faster and not because they spent more time running in the activity wheel (Girard et al., 2001). Wheel running by the selected lines increased until approximately generation 16, at which time when it reached almost three times more revolutions per day as compared with the control lines (Rhodes et al., 2001). Among other changes, selection for wheel running has reduced body size and the whole-animal cost of transport, and increased maximal oxygen consumption during forced treadmill exercise (VO_2 max) (Swallow et al., '98; Rezende et al., 2006). Alterations in neurobiology and motivation for wheel running have also been demonstrated (Rhodes et al., 2005; Belke and Garland, 2007; Keeney et al., 2008).

This study is part of an effort to determine the effect of this selective breeding for high voluntary wheel running on muscle structure and physiology. One entirely unexpected result of the artificial selection experiment has been the appearance of mice with smaller-than-normal triceps surae and thigh muscles in two of the selected lines (Garland et al., 2002). Animals with the mini-muscle phenotype have also larger hearts and double the mass-specific activity of mitochondrial enzymes in hindlimb muscles (Garland et al., 2002; Houle-Leroy et al., 2003), among various other pleiotropic effects (Kelly et al., 2006; Rezende et al., 2006). The mini-muscle phenotype is the result of a Mendelian recessive allele that has been mapped to a 2.6335 Mb interval on MMU11, but not yet identified (Hannon et al., 2008; Hartmann et al., 2008). Hierarchical models, including the effects of genetic drift and selection, approximated the frequency of the allele in the initial population at 7%, and indicated that it must have been favored by the artificial selection protocol (Garland et al., 2002). Both triceps surae mass and body-mass-corrected triceps surae mass are significantly reduced as compared with normal-sized muscles by the postnatal age of 2 weeks (Middleton et al., 2008).

Guderley et al. (2006) examined whether the mini-muscle phenotype leads to changes in fiber size, number, or ultrastructure. Electron microscopic examination of the plantaris muscle showed that mini-muscles had distinct attributes relative to normal muscles. Mini-muscle plantaris had a high frequency of small fibers (cross-sectional areas 1,000 μm^2) and a low frequency of intermediate fibers (2,000–3,000 μm^2), but tended to have higher frequencies of the largest fibers ($>$4,000 μm^2) than mice with normally sized muscles. Analysis of the myosin isoforms in the gastrocnemius showed that "mini-muscles" had a decreased proportion of isoforms (myosin heavy chain (MHC) and MLC) typical of fast glycolytic fibers (type IIB) and increased levels of the myosin isoforms typical of oxidative fibers (Guderley et al., 2008). These results suggest that the mini-muscle phenotype is caused by a reduction in the proportion of type IIB fibers. As these microscopy studies did not identify fiber types, the data could also reflect changes in the expression of myosin isoforms in the mini phenotype. The fiber typology and structure of red and white muscle samples from the gastrocnemius muscle was changed in the mini-muscle mice, with increases in the proportion of oxidative fibers (type I or IIA, depending on the line) in red muscle and a drastically altered cellular arrangement in white muscle samples (Guderley et al., 2008). Given the range of fiber types, large size, and heterogeneity of the gastrocnemius muscle, our previous studies could not definitely ascertain whether the mini-muscle phenotype was associated with a loss of type IIB fibers.

In this study, we determined the impact of selection for high voluntary wheel running and, in particular, the effects of the mini-muscle phenotype on the organization of the tibialis anterior muscle. We chose the tibialis anterior as it is mainly composed of type IIB fibers ($\sim$55%) and IIb MHC ($\sim$70%), the remaining consisting of type IID and IIA fibers and IId and IIa MHCs in mice (Hamalainen and Pette, '93; Allen et al., 2001). In addition, the small size of this muscle makes an overall characterization of its fiber type composition highly accurate. Our hypotheses were (1) that the tibialis anterior of mice from the selected lines would show characteristics reminiscent of trained muscle, and (2) that tibialis anterior from mice expressing the mini-muscle phenotype would show a markedly lower mass, lower levels of type IIb MHCs, a reduced proportion of type IIB fibers, and a higher oxidative capacity. Accordingly, we examined the electrophoretic profiles of MHCs, the fiber typology, and the enzymatic profiles of the tibialis anterior muscle in mice from all four selected and four control lines after 35–36 generations of the selection experiment. Fiber typing was accomplished using myosin ATPase and NADH tetrazolium reductase activity staining to compare with results from Allen et al. (2001). Our enzymatic profiling (HK, CS, GP, LDH, CCO) focused upon the enzymes that were most altered in mini-muscle phenotype in our previous studies (Houle-Leroy et al., 2003; Guderley et al., 2006). To isolate the effects of selection from those of

exercise training, all mice were housed without wheel access. Our results showed a dramatic reduction in the abundance of type IIB fibers and the corresponding expression of the IIb MHC isoform in the tibialis anterior from mini-muscle mice, but virtually no general differences in the fiber type composition between mice with normal-size muscles from control and selected lines.

MATERIALS AND METHODS

Selective breeding protocol

Female mice were sampled from the 35th ($n = 42$) and 36th ($n = 14$) generations of selective breeding for voluntary wheel running as described by Swallow et al. ('98). Founders for the selection experiment were from the outbred Hsd:ICR strain of house mice (*Mus domesticus*). Selective breeding was interrupted from generation 32 to 35 to transfer the colony from the University of Wisconsin–Madison to the University of Riverside, California. Mice were weaned at 21 days of age and housed 4/cage without wheel access, other than during routine wheel testing for 6 days at ~6–8 weeks of age (Swallow et al., '98). After initial establishment, lines had been randomly assigned to selection versus control treatment groups, and those designated 3, 6, 7, and 8 were selected for high running on days 5 and 6 of the 6-day wheel exposure, whereas lines 1, 2, 4, and 5 were bred without regard to wheel running, thus providing controls. Although such control lines should be similar to the initial base population, random genetic drift and/or adaptation to slightly different husbandry conditions might cause them to deviate. We sampled 6–7 mice from each line, except for line 6. Because mice in line 6 express both the mini-muscle and the normal phenotypes, we sampled six individuals of each phenotype. Line 3 had gone to fixation for the mini-muscle phenotype by approximately generation 36 (Syme et al., 2005).

Tissue sampling

At the time of the sacrifice (mean age = 200 days, range = 193–203 days), the hindlimb muscles were removed, the tibialis anterior was dissected, weighed, and then frozen in liquid nitrogen before storage at −80°C. Enzyme activities (HK, CS, GP, LDH, CCO) were measured using spectrophotometric assays (Beckman DU 640, Beckman Coulter, Brea, CA) following the conditions in Houle-Leroy et al. (2000).Temperature during assays was maintained at 37°C by a circulating water bath. Assays were run in duplicate and the linearity of reaction was maintained for at least 4 min.

Histochemical analysis

During the sacrifice, muscles reserved for histochemical staining were mounted on cork board, immersed in isopentane cooled on liquid nitrogen, and then stored at −80°C. In preparation for staining, individual muscle sections were encased in optimal cutting temperature compound (Tissue-Tek, Sakura, Japan) and cryocut at −20°C (Minotome, Damon/EC Division Minotome, Needham Heights, MA). Slices were collected on slides and left to air dry before staining.

Myosin ATPase staining

The myosin-ATPase stain is a colorimetric reaction resulting from the precipitation of cobalt (Co) and inorganic phosphate (Pi), the latter originating from the hydrolysis of ATP. Sections (10 μm in thickness) were stained (20 mM sodium barbital, 10 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM NaN_3, 1 mM ATP, 7.6% ethanol) at pH 9.4 for 55 min at 37°C. After washing in 1% $CaCl_2$ and incubating for 3 min in $CoCl_2$ (2%) slides were rinsed with distilled H_2O. Quick immersion in ammonium sulfide (0.0018% v/v) was the final step before washing in distilled H_2O and dehydrating in acetone, ethanol, and clearing in xylene.

NADH tetrazolium reductase staining

The NADH tetrazolium reductase stain was used to demonstrate the activity of the mitochondrial electron transport chain. The oxidative capacity of fibers was assessed by the relative intensity of the colorimetric assay. Muscle sections (10 μm in thickness) were incubated in 200 mM Tris-HCl, 1.2 mM Nitro blue tetrazolium chloride (NBT), 1.1 mM NADH, at pH 7.4 for 35 min at 37°C. Then the sections were dehydrated in acetone and left to air dry before being cleared in toluene and fixed.

Stained sections were viewed by light microscopy (Leitz Dialux 20, Wetzlar, Germany) and images were captured by a digital camera (Sony CCD-IRIS SSC-C350, Tokyo, Japan). Percent distribution of each fiber type was analyzed using NIH Image Analysis Software (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) after classifying the fibers according to their NADH tetrazolium reductase activity.

Myosin heavy chain SDS-gel electrophoresis

MHC isoforms were partially purified from frozen (−80°C) muscle by isolating myofibrils by centrifugation. Muscle (~3 mg; dilution 1/10 (mass/volume)) was homogenized with a hand-held glass–glass homogenizer in 250 mM sucrose, 100 mM KCl, 5 mM ethylene-diamine-tetraacetic acid disodium salt (EDTA-2Na+), 20 mM Tris-HCl at pH 6.8. Phenylmethylsulfonyl fluoride (PMSF; 0.2 mM) was added to the buffer at the time of homogenization. Samples were centrifuged at 10,000 g for 10 min at 5°C. After discarding the supernatant, the pellet was resuspended in 200 µl of 175 mM KCl, 2 mM EDTA-2Na+, 0.50% Triton X-100 and 20 mM Tris at pH 6.8. Centrifugation was repeated and the supernatant discarded. Sample pellets were resuspended in 100 µl of 150 mM KCl and 20 mM Tris at pH 7.0.

Sample protein concentrations were measured using the Bradford protein assay with bovine serum albumin as a standard. Samples were diluted 1:1 with sampling buffer (1.0% 2-mercaptoethanol, 4.0% SDS, 16.0% Tris (pH 6.8), 20% glycerol, and 0.2% bromophenol blue) and H_2O to attain a final protein concentration of 15 µg protein per 10 µl. The myofibrillar preparation was then heated at 100°C for 2 min and either stored at −80°C or directly applied to a polymerized gel.

Vertical mini-gel composition (0.75 mm thick; Bio-Rad Mini-PROTEAN II Cell) was modified from Bamman et al. ('99). The separation gel contained 30% glycerol, 7.2% acrylamide:bis-acrylamide (37.5:1), 300 mM Tris pH 8.8, 100 mM glycine, 0.4% SDS, 0.6% APS, 0.1% TEMED, and 11% H_20. The stacking gel contained 35% glycerol, 6% acrylamide:bis-acrylamide (37.5:1), 138 mM Tris pH 6.8, 5 mM EDTA disodium salt pH 7.0, 0.5% SDS, 0.07% ammonium persulfate (APS), and 0.11% TEMED. A higher concentration than that in the literature was used for acrylamide in the stacking gel and for glycerol in both gels because this improved resolution of IIb and IId MHC isoforms in our hands. The upper migration buffer consisted of 1% SDS, 0.2 M glycine, and 0.25 M Tris. Proteins were migrated for 35 hr at 90 V in an ice-filled cooler. Gels were stained in Biosafe Coomasie Blue for 1 hr before destaining and drying between cellophane membranes. Densitometry was carried out on scanned gels (UMAX Vista-S6E with transmission scanning module). An optical density plot was made from these images. The total optical density of each electrophoretic band was computed and used to calculate a percentage of MHC isoforms using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Biochemicals and chemicals

Reagents were purchased from Sigma Chemical Co. (St-Louis, MO), Boehringer Mannhein Co. (Montreal, CA), and BioRad Laboratories Ltd. (Mississauga, CA).

Statistical analysis

Two-way mixed-model nested analysis of covariance (ANCOVA) models were implemented with SAS PROC MIXED (SAS Institute, Cary, NC). Replicate lines ($n = 8$) were treated as a random effect and nested within line type (control or selected). Mini-muscle was an additional factor, and body mass was a covariate. Degrees of freedom for testing the line type effect were always 1 and 6. All analyses of tibialis anterior enzyme activities were performed using rank-transformed data as normality of residuals and the homogeneity of variance among groups were difficult to attain by other transformations. Statistical significance was judged at $P < 0.05$, and all P values presented are for two-tailed tests.

RESULTS

Body and tibialis anterior masses

As shown in Table 1, mice from selected lines had on average a body mass that was 6 g less (linetype $F(1, 6) = 14.78$, $P = 0.0085$) than that of control lines, and the difference was not attributable to the mini-muscle phenotype ($F(1, 47) = 0.43$, $P = 0.5139$).

In a two-way ANCOVA, tibialis anterior mass was positively affected by body mass ($F(1, 46) = 4.99$, $P = 0.0304$) and negatively affected by the mini-muscle phenotype ($F(1, 46) = 19.80$, $P < 0.0001$), but did not differ generally between selected and control lines ($F(1, 6) = 0.73$, $P = 0.4243$). However, as shown in Figure 1, the relationship between tibialis anterior mass and body mass has a shallower slope in mini-muscle individuals as confirmed by ANCOVA (line type $F(1, 6) = 0.01$, $P = 0.9316$; mini $F(1, 45) = 3.47$, $P = 0.0691$; body mass $F(1, 45) = 1.32$, $P = 0.2560$; "mini"*body mass interaction $F(1, 45) = 7.24$, $P = 0.0100$). A similar result has been reported for medial (but not lateral) gastrocnemius muscle

TABLE 1. Body and tibialis anterior muscle masses

	Control lines				Selected lines				
					Mini-muscles		Normal muscles		
Line	1	2	4	5	3	6	6	7	8
n	*6*	*6*	*7*	*6*	*7*	*6*	*6*	*6*	*6*
Body mass (g)	39.4±2.2	35.2±1.7	39.4±1.5	35.7±1.1	32.1±2.1	33.7±1.0	33.9±1.4	30.2±1.5	29.8±1.0
Tibialis mass (mg)	53.2±1.6	48.1±2.2	54.9±2.1	53.6±0.9	37.7±1.6	36.1±2.4	53.6±3.8	43.6±3.7	44.0±1.7

Values are simple means±SE; *n*, number of mice. Mean age was 200 days, range = 193–203 days. See text for results of two-way, nested analysis of variance (body mass) and covariance (tibialis mass). For the selected lines, the data are separated according to the mini-muscle phenotype, which has become fixed in line 3 and which is present at an intermediate frequency in line 6.

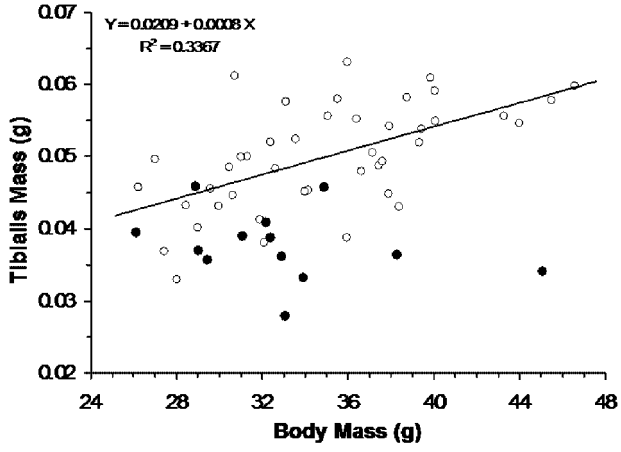

Fig. 1. Mass of the tibialis anterior muscle in relation to body mass for normal (open circles and solid line) and mini-muscle (closed circles) individuals. Analysis of covariance indicates that the relations differ significantly in slope (see text).

(Guderley et al., 2006; see also Fig. 1B in Guderley et al., 2008).

Enzyme activities

As suggested by results obtained for mixed hindlimb muscle samples of mice from the 14th generation of this experiment (Houle-Leroy et al., 2000), most enzyme activities (U/g) in tibialis anterior muscle were not significantly affected by selection as revealed by two-way ANCOVA (Table 2). However, the activity of rank-transformed HK was higher in selected than control lines (two-tailed $P = 0.0344$). In addition, when statistical analyses were performed without body mass as a covariate, mice from the selected lines had reduced GPa activity (ranked, line type $P = 0.038$).

Confirming earlier results for the impact of the mini-muscle phenotype on enzyme activities in hindlimb muscles (Houle-Leroy et al., 2003), and more specifically in the plantaris and gastrocnemius (Guderley et al., 2006), tibialis anterior from mice with the mini-muscle phenotype had significantly higher activities of HK and CS ($P = 0.030$ and 0.003, respectively). The other enzyme activities were not statistically affected by the mini-muscle phenotype (Table 2). Body mass only had a significant effect (positive) on the activity of GPtot ($P = 0.033$). When statistical analyses were performed without body mass as a covariate, the mini-muscle factor significantly increased GPtot ($P = 0.046$), HK ($P = 0.030$), and CS ($P = 0.004$) activities.

Electrophoretic profiles of myosin heavy chains

Identification of the isoforms of MHCs was based on molecular mass and on comparison with published results (Talmadge and Roy, '93). In agreement with Hamalainen and Pette ('93), our preparations of tibialis anterior muscle from normal size mice had detectable levels of only type IIb and IId MHCs (Fig. 2A,B).

In agreement with, but more dramatic than what we previously observed in the gastrocnemius (Guderley et al., 2006), the mini-muscle phenotype significantly modified the MHC composition of tibialis anterior muscle: mini-muscle samples were found to express only type IId MHC isoforms at detectable levels (Fig. 2B, Table 3). Beyond this effect, MHC composition was not statistically affected by body mass or line type.

Muscle fiber typology

Myosin-ATPase staining could not resolve type II fiber types, as the shades of gray were too close for reliable differentiation (Fig. 3). However, NADH tetrazolium reductase staining revealed major differences in the intensity of mitochondrial activity in tibialis anterior fibers, with two major classes of staining intensity. The more intensely

TABLE 2. Enzyme activities (U/g muscle) of tibialis anterior muscle

	Control lines				Selected lines				
					Mini-muscles		Normal muscles		
Line	1	2	4	5	3	6	6	7	8
n	*6*	*6*	*7*	*6*	*7*	*6*	*6*	*6*	*6*
GPa	23.7±2.3	23.3±4.0	33.5±6.5	33.0±7.6	23.6±2.8	35.0±5.1	16.6±5.3	19.7±4.1	20.7±4.2
GPtot	67.7±17.8	100.8±55.6	137.3±42.3	71.2±15.3	130.7±56.1	63.5±4.1	56.0±3.4	54.8±9.7	48.0±5.3
HK	5.05±0.80	3.88±0.49	4.73±0.23	4.68±0.25	7.42±0.76	9.96±0.96	7.16±0.53	6.45±0.73	4.37±0.71
CS	80.3±8.2	65.1±82.1	82.1±13.7	92.5±2.0	123.7±4.8	134.3±19.9	88.0±14.9	103.5±13.0	88.4±13.8
LDH	772±55	868±105	750±55	781±96	804±49	646±40	877±75	775±28	716±59
CCO	17.2±4.6	7.0±1.5	4.7±1.0	2.5±0.5	8.2±3.3	12.0±4.5	12.1±2.8	5.3±1.5	9.5±5.3

Values are means±SE; *n*, number of mice. Two-way ANCOVAs were performed after rank transformation for all enzyme activities, $n = 56$. F and P values are listed in the following order: Line type, mini-muscle factor, body mass. GPa: $F(1,6) = 2.66$, $P = 0.1539$, $F(1,6) = 2.85$, $P = 0.0984$, $F(1,44) = 1.05$, $P = 0.3109$. GPtot: $F(1,6) = 0.02$, $P = 0.8979$, $F(1,6) = 3.27$, $P = 0.0775$, $F(1,44) = 4.84$, $P = 0.0332^*$. HK: $F(1,6) = 7.43$, $P = 0.0344^*$, $F(1,6) = 5.05$, $P = 0.0297^*$, $F(1,44) = 3.53$, $P = 0.0669$. CS: $F(1,6) = 0.57$, $P = 0.4804$, $F(1,6) = 9.59$, $P = 0.0034^*$, $F(1,44) = 0.61$, $P = 0.4398$. LDH: $F(1,6) = 0.25$, $P = 0.6349$, $F(1,6) = 0.88$, $P = 0.3522$, $F(1,44) = 0.68$, $P = 0.4142$. CCO: $F(1,6) = 2.86$, $P = 0.1415$, $F(1,6) = 2.43$, $P = 0.1259$, $F(1,44) = 2.05$, $P = 0.1595$. For the selected lines, the data are separated according to the mini-muscle phenotype.

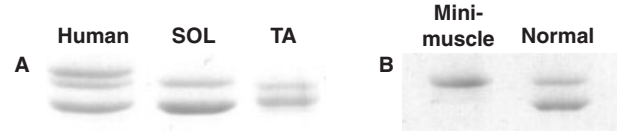

Fig. 2. (**A**) Sample gel showing myosin heavy chain (MHC) separation by SDS-PAGE electrophoresis. From left to right are MHC isoforms from human vastus lateralis (shown as a size standard), mouse soleus (SOL), and mouse tibialis anterior (TA) muscles. Four bands are distinguishable in mouse samples, as shown by the absence of overlap between the bands of the soleus and tibialis anterior samples. From top to bottom, bands in mouse samples are MHC isoforms IIa, IIx, IIb and I. (**B**) Representative gel showing major MHC isoforms from tibialis anterior muscle of mini-muscle and normal mice. In these samples, only isoforms IIx and IId were detectable by gel electrophoresis.

staining fibers were classified as NADH-TR positive fibers, corresponding to type IIA (the smallest and most intensely stained) and IID fibers, whereas the large and weakly staining fibers were classified as type IIB. Given the objectives of the study and the difficulties in clearly distinguishing between IIA and IID fibers, we chose to report on IIA+IID composition.

In normally sized muscles from control and selected lines, type IIA+IID fibers were generally smaller than the less intensely staining type IIB fibers. In normally sized muscles from both control and selected lines, approximately 60% of the fibers were NADH-TR positive (IIA+IID; Fig. 2, Table 3), whereas in mini-muscle tibialis from lines 3 and 6, all fibers were of this type. Statistical analysis showed no impact of line type ($F(1,6) = 0.67$, $P = 0.4443$) or body mass ($F(1,44) = 0.53$, $P = 0.4685$) upon the rank-transformed % NADH-TR positive fibers, but a strong impact of the mini-muscle factor ($F(1,44) = 29.19$, $P < 0.0001$). Results (not shown) were similar when body mass was not used as a covariate.

DISCUSSION

This study demonstrates that selective breeding for high voluntary wheel running has changed fiber MHC expression, typology, and metabolic potential of the tibialis anterior muscle primarily through the dramatic modifications that accompany the mini-muscle phenotype. Our results suggest that in mice with the mini-muscle phenotype, type IIb MHC expression and IIB fibers are lost in the tibialis anterior muscle (Fig. 2, Table 3). Two lines of evidence support this conclusion: (1) only type IId MHCs were detectable by electrophoretic separation of MHCs in mini-muscle tibialis anterior and (2) all fibers in mini-muscle tibialis anterior showed relatively intense staining for NADH tetrazolium reductase, whereas in mice with the normal phenotype, tibialis was found to possess significant numbers of fibers that stained much less intensely (i.e. type IIB). The higher activity of the mitochondrial enzyme CS in the mini-muscle tibialis together with higher HK activity are also consistent with the enhanced oxidative capacity that would be expected with the loss of type IIB fibers, which we suggest is a primary mechanism underlying the mini-muscle phenotype.

MHCs, as the most common proteins in skeletal muscle and the major component of the sarcomeric thick filaments, are the primary characteristic used to identify fiber types. The functional properties of the MHCs establish the contractile

TABLE 3. Tibialis anterior myosin heavy chain (MHC) isoform proportion assessed by electrophoresis and fiber type composition measured by histochemistry of mice from control and selected lines

	Control lines				Selected lines				
					Mini-muscles		Normal muscles		
Line	1	2	4	5	3	6	6	7	8
n	6	6	7	6	7	6	6	6	6
Myosin heavy chain (MHC) composition (%)									
IIb	68.7±5.1	73.0±4.4	80.0±2.6	66.8±5.9	ND	ND	68.3±2.7	69.5±3.5	59.7±7.6
IId	31.3±5.1	27.0±4.4	20.0±2.6	33.2±5.9	100.0±zero	100.0±zero	31.7±2.7	30.5±3.5	40.3±7.6
Fiber type distribution (%)									
IIB	45.1±3.9	43.6±3.4	45.5±9.1	37.4±5.1	ND	ND	33.4±9.6	44.4±1.1	41.6±4.5
IIA+IID	54.9±3.9	56.4±3.4	54.5±9.1	62.6±5.1	100.0±zero	100.0±zero	66.6±9.6	55.6±1.1	58.4±4.5

Values are means±SE; *n*, number of mice (total $n = 56$); ND, not detected. See text regarding statistical analyses. For the selected lines, data are separated according to mini-muscle phenotype status.

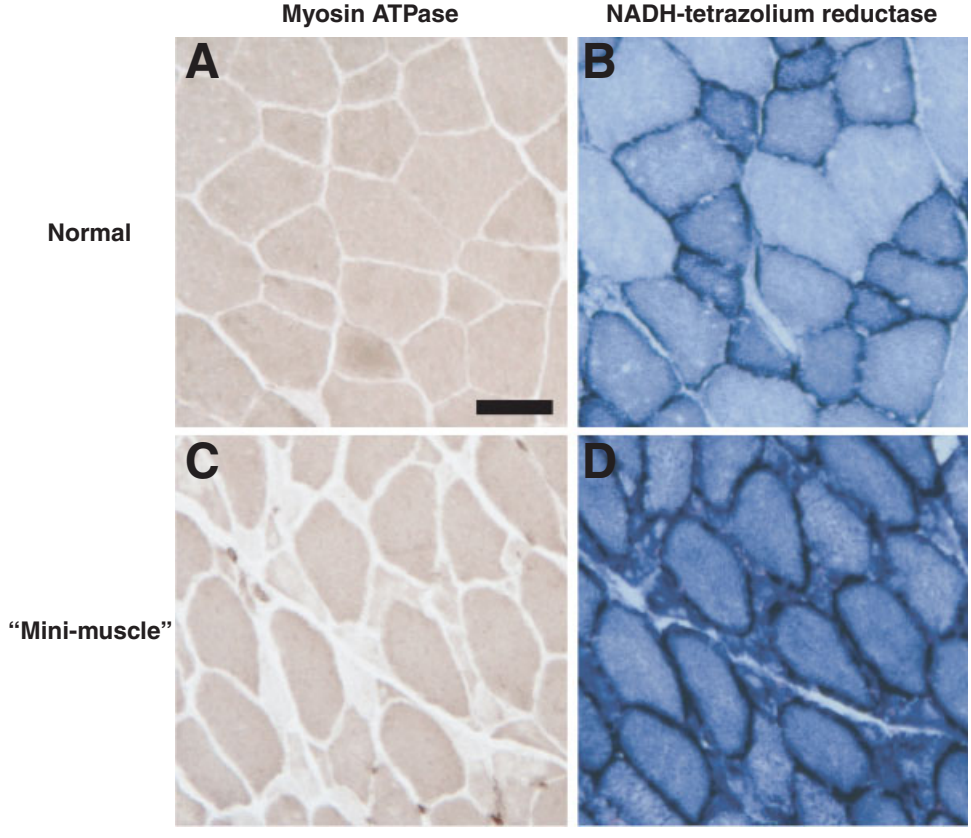

Fig. 3. Representative photomicrographs of histochemical staining for myosin ATPase and NADH tetrazolium reductase in tibialis anterior from normal (**A** and **B**) and mini-muscle (**C** and **D**) mice. Darker staining for NADH-tetrazolium reductase indicates a greater oxidative potential (i.e., more mitochondria). The bar is 50 μm in length.

properties of the fibers: these, in turn, are closely linked with their metabolic capacities. Typically, hindlimb muscles from adult mice contain four MHC isoforms identified as slow oxidative (type I), fast oxidative (IIa), fast glycolytic (IIb), and the form that is intermediate between IIa and IIb (IId). In the mouse tibialis anterior the proportion of IIa plus IId MHC isoforms typically lies between 23–29% (Allen et al., 2001), and our data from nonmini-muscle mice are consistent with this range (Table 3). The proportion of MHC isoforms in muscle correlates with the maximum contraction

velocity of the cell or muscle, reflecting the myosin ATPase activity of each isoform (Barany, '67). Rivero et al. ('98) confirmed that, in rat muscle, fiber size, SDH, and GPD activity are associated with the type of MHC expressed in the fibers. Small fibers typically have type I or IIa MHCs and high oxidative capacities, whereas the large fibers have a low oxidative capacity and express type IIb MHCs.

If the hypothesis by which we explain the mini-muscle phenotype is correct, then muscles rich in type IIB fibers should display a greater loss of mass than muscles with lower levels of type IIB fibers when compared with control mice. Consistent with this prediction, the soleus muscle, with its content of primarily type I and IIA fibers, is actually greater in mass, whereas the gastrocnemius and plantaris muscle are markedly smaller in the mini-muscle phenotype (Syme et al., 2005; Guderley et al., 2006). The fact that soleus is larger in mini-muscle individuals, rather than just not being smaller, may reflect a compensatory hypertrophy that occurs during ontogeny. The IIb MHC content and the proportion of type IIB fibers in the mouse tibialis anterior muscle ranges from 35–60% (Wernig et al., '89; Hamalainen and Pette, '93; Burkholder et al., '94; Allen et al., 2001); thus, we would predict a marked loss in tibialis anterior mass in any model with type IIB fiber type loss or atrophy. Comparison of normal and mini-muscle individuals within selected line 6 most clearly demonstrated the impact of the mini-muscle phenotype: mini-muscle tibialis were 67% of the size of normal-size muscles (Table 1). In both control and selected lines, we found that normally sized muscles contained approximately 40% type IIB fibers in the tibialis anterior; the observed loss in tibialis anterior mass in mini-muscle mice thus corresponds very closely with the proportion of type IIB fibers we observed.

Our electrophoretic separation and quantification of MHC isoforms provided an estimate of the overall proportion of fiber types within the tibialis anterior, and allowed comparison with results from histochemical classification of fiber types using NADH tetrazolium reductase. As indicated by previous studies (Talmadge and Roy, '93; Rivero et al., '98; Allen et al., 2001), we found that the myosin ATPase stain did not provide a clear separation between types IIB and IID fibers. On the other hand, NADH tetrazolium reductase staining did allow classification according to the oxidative potential of the fibers. Confirmation of the validity of our classification with this stain comes from the relationship between staining intensity and fiber size. Generally, and as observed here, the smaller type IID fibers have a higher staining intensity for NADH-tetrazolium reductase activity relative to the larger type IIB fibers (Hamalainen and Pette, '93). The absence of MHC IIb and the abundance of oxidative fibers in mini-muscles mice lend support to the hypothesis that IIB fibers are markedly reduced in these individuals (Guderley et al., 2006).

The higher oxidative potential of ''mini-muscle'' fibers as revealed by the NADH tetrazolium reductase is correlated with an increase in the activities of HK and CS. CS occurs in the mitochondrial matrix, whereas HK is both cytosolic and associated with the intermembrane space in mitochondria. The role of hexokinase in phosphorylating glucose suggests an enhanced capacity for use of blood-borne glucose through aerobic glycolysis. Previous characterization of the enzymatic activities of mini-muscle mice showed a more generalized increase in activities of mitochondrial enzymes than we found in this study. The small size of the tibialis anterior muscle led to greater experimental variability during measurements than we experienced with the pooled hindlimb muscles, the gastrocnemius or plantaris muscles (Houle-Leroy et al., 2003; Guderley et al., 2006). Further, the levels of mitochondrial enzymes were generally lower in this more glycolytic muscle compared with the plantaris and gastrocnemius (Guderley et al., 2006).

The various lines of evidence we present in this study, from histochemical characterization of fiber types, to electrophoretic evaluation of MHC isoforms, to the characterization of enzymatic activities support the hypothesis that the mini-muscle gene changes muscle structure by interfering with the production and/or maintenance of type IIB fibers. The manifestations of the mini-muscle phenotype vary somewhat among muscles and between the two selected lines in which it occurs. In plantaris muscle, light and electron microscopic examination discovered atypical, myofibril containing ''mini-cells,'' primarily in the outer cell layers (Guderley et al., 2006), whereas samples of white gastrocnemius are full of highly atypical disorganized regions preventing quantification of fiber types (Guderley et al., 2008). In earlier generations, when it was possible to compare individuals with normal size and mini-muscles within both of the selected lines 3 and 6, line-specific differences were apparent in the ultrastructure and MHC isoform distribution of

mini-muscles (Guderley et al., 2006). Thus, the specific manifestations of the mini-muscle allele, when present in the homozygous state, depend upon the genetic environment in which it is expressed, both at the level of the selected line and of the specific muscle. The mini-muscle phenotype seems to be the major means through which selection for high wheel running has modified the functional capacities of the hindlimb muscles, as normal-sized muscles from control and selected lines have so far only been shown to differ in HK activity (and possibly GPa activity: see section ''Results'') in terms of their enzymatic capacities, and did not differ statistically in their MHC isoform profiles or their fiber type composition. The modifications of muscle capacities that occur in the mini-muscle phenotype do not require expression of the selected behavior (i.e. wheel running). Other differences in muscle metabolic capacities between selected and control mice with normal-sized muscles appear after days (Gomes et al., in revision) or weeks (see Houle-Leroy et al., 2000) of wheel access, but not when animals are housed without wheel access.

In closing, we attempt to place the present results in a broader biological context. The nature of genetic variation that underlies within-population phenotypic variation in quantitative traits (e.g. voluntary activity levels, body size, muscle size, or fiber type composition) continues to be an important and very active area of research (e.g. see Hoekstra and Coyne, 2007; Roff, 2007; Lynch and Wagner, 2008; Stern and Orgogozo, 2008), even for such well-studied animals as mice (Eisen, 2005). Beyond this, comes the question of what sorts of alleles are important in determining the response to selection (see foregoing references), and whether the answer might differ for natural versus laboratory-maintained populations. Another question of ongoing debate is whether the outcome of any particular type of selection may be predictable or reproducible in terms of either the subordinate phenotypes involved or their underlying genes. Experimental evolution offers a powerful approach for addressing such questions (Garland and Rose, 2009). Results from selective breeding for high voluntary activity levels in mice demonstrate that at least one gene of major effect has been involved, i.e. the Mendelian recessive allele that underlies mini-muscles (Garland et al., 2002; Hannon et al., 2008; Hartmann et al., 2008). However, the ''use'' of this allele by individual replicate lines has been unpredictable: two lines exhibited an increase in frequency (one to fixation) whereas two did not. Ongoing mapping efforts should eventually reveal whether it lies in a protein-coding region or in a *cis*-regulatory region (Hoekstra and Coyne, 2007; Lynch and Wagner, 2008; Stern and Orgogozo, 2008).

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LITERATURE CITED

Allen DL, Harrison BC, Maass A, Bell ML, Byrnes WC, Leinwand LA. 2001. Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. J Appl Physiol 90:1900–1908.

Bamman MM, Clarke MS, Talmadge RJ, Feeback DL. 1999. Enhanced protein electrophoresis technique for separating human skeletal muscle myosin heavy chain isoforms. Electrophoresis 20:466–468.

Barany M. 1967. ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50:197–218.

Belke TW, Garland Jr T. 2007. A brief opportunity to run does not function as a reinforcer for mice selected for high daily wheel-running rates. J Exp Anal Behav 88:199–213.

Burkholder TJ, Fingado B, Baron S, Lieber RL. 1994. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. J Morphol 221:177–190.

Eisen EJ. 2005. The mouse in animal genetics and breeding research. London: Imperial College Press. p ix–364.

Garland Jr T, Rose MR. 2009. Experimental evolution: concepts, methods, and applications of selection experiments. Berkeley, CA: University of California Press.

Garland Jr T, Morgan MT, Swallow JG, Rhodes JS, Girard I, Belter JG, Carter PA. 2002. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267–1275.

Girard I, McAleer MW, Rhodes JS, Garland Jr T. 2001. Selection for high voluntary wheel-running increases speed and intermittency in house mice (*Mus domesticus*). J Exp Biol 204:4311–4320.

Gomes FR, Rezende EL, Malisch JL, Lee SK, Rivas DA, Kelly SA, Lytle C, Yaspelkis III BB, Garland Jr T. In revision. Glycogen storage and muscle glucose transporters (GLUT-4) of mice selectively bred for high voluntary wheel running. J Exp Biol.

Guderley H, Houle-Leroy P, Diffee GM, Camp DM, Garland Jr T. 2006. Morphometry, ultrastructure, myosin isoforms, and metabolic capacities of the ''mini muscles'' favoured by selection for high activity in house mice. Comp Biochem Physiol 144:271–282.

Guderley H, Joanisse DR, Mokas S, Bilodeau GM, Garland Jr T. 2008. Altered fibre types in gastrocnemius muscle of high wheel-running selected mice with mini-muscle phenotypes. Comp Biochem Physiol 149:490–500.

Hamalainen N, Pette D. 1993. The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit. J Histochem Cytochem 41:733–743.

Hannon RM, Kelly SA, Middleton KM, Kolb EM, Pomp D, Garland Jr T. 2008. Phenotypic effects of the ''mini-muscle'' allele in a Large HR x C57BL/6J mouse backcross. J Hered 99:349–354.

Hartmann JT, Garland Jr T, Hannon RM, Kelley AE, Munoz G, Pomp D. 2008. Fine mapping of ''mini-muscle,'' a recessive mutation causing reduced hindlimb muscle mass in mice. J Hered 99:679–687.

Hoekstra HE, Coyne JA. 2007. The locus of evolution: evo devo and the genetics of adaptation. Evolution 61:995–1016.

Houle-Leroy P, Garland Jr T, Swallow JG, Guderley H. 2000. Effects of voluntary activity and genetic selection on muscle metabolic capacities in house mice *Mus domesticus*. J Appl Physiol 89:1608–1616.

Houle-Leroy P, Guderley H, Swallow JG, Garland Jr T. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. Am J Physiol 284:433–443.

Keeney BK, Raichlen DA, Meek TH, Wijeratne RS, Middleton KM, Gerdeman GL, Garland Jr T. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716:rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behavior. Behav Pharmacol 2008.

Kelly SA, Czech PP, Wight JT, Blank KM, Garland Jr T. 2006. Experimental evolution and phenotypic plasticity of hindlimb bones in high-activity house mice. J Morphol 267:360–374.

Lynch VJ, Wagner GP. 2008. Resurrecting the role of transcription factor change in developmental evolution. Evolution 62:2131–2154.

Middleton KM, Kelly SA, Garland Jr T. 2008. Selective breeding as a tool to probe skeletal response to high voluntary locomotor activity in mice. Integr Comp Biol 48:394–410.

Rezende EL, Gomes FR, Malisch JL, Chappell MA, Garland Jr T. 2006. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. J Appl Physiol 101:477–485.

Rhodes JS, Kawecki TJ. 2009. Behavior and neurobiology. In Garland Jr T, Rose MR, editors. Experimental evolution: concepts, methods, and applications of selection experiments. Berkeley: University of California Press.

Rhodes JS, Hosack GR, Girard I, Kelley AE, Mitchell GS, Garland Jr T. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 158:120–131.

Rhodes JS, Gammie SC, Garland Jr T. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integr Comp Biol 45:438–455.

Rivero JL, Talmadge RJ, Edgerton VR. 1998. Fibre size and metabolic properties of myosin heavy chain-based fibre types in rat skeletal muscle. J Muscle Res Cell Motil 19:733–742.

Roff DA. 2007. A centennial celebration for quantitative genetics. Evolution 61:1017–1032.

Stern DL, Orgogozo V. 2008. The loci of evolution: how predictable is genetic evolution? Evolution 62:2155–2177.

Swallow JG, Carter PA, Garland Jr T. 1998. Artificial selection for increased wheel-running behavior in house mice. Behav Genet 28:227–237.

Swallow JG, Hayes JP, Koteja P, Garland Jr T. 2009. Selection experiments and experimental evolution of performance and physiology. In Garland Jr T, Rose MR, editors. Experimental evolution: concepts, methods, and applications of selection experiments. Berkeley: University of California Press.

Syme DA, Evashuk K, Grintuch B, Rezende EL, Garland Jr T. 2005. Contractile abilities of normal and ''mini'' triceps surae muscles from mice (*Mus domesticus*) selectively bred for high voluntary wheel running. J Appl Physiol 99:1308–1316.

Talmadge RJ, Roy RR. 1993. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. J Appl Physiol 75:2337–2340.

Wernig A, Irintchev A, Wasserschaff M. 1989. Prolonged running does not improve muscle coordination after cross-union of tibial and peroneal nerves in mice. Brain Res 489:352–354.