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# Morphometry, ultrastructure, myosin isoforms, and metabolic capacities of the “mini muscles” favoured by selection for high activity in house mice

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## Abstract

Prolonged selective breeding of mice (*Mus musculus*) for high levels of voluntary wheel running has favoured an unusual phenotype (“mini muscles”), apparently caused by a single Mendelian recessive allele, in which most hind-limb muscles are markedly reduced in mass, but have increased mass-specific activities of mitochondrial enzymes. We examined whether these changes reflect changes in fibre size, number or ultrastructure in normal and “mini-muscle” mice within the two (of four) selectively bred lines (lab designations L3 and L6) that exhibit the phenotype at generations 26 and 27. In both lines, the gastrocnemius and plantaris muscles are smaller in mass (by >50% and 20%, respectively) in affected individuals. The mass-specific activities of mitochondrial enzymes in the gastrocnemius and plantaris muscles were increased in the mini phenotype in both lines, with stronger effects in the gastrocnemius muscle. In the gastrocnemius, the % myosin heavy chain (MHC) IIb was reduced by 50% in L3 and by 30% in L6, whereas the % MHC IIa and I were higher, particularly in L3. Fibre number in the plantaris muscle did not significantly differ between mini and normal muscles, although muscle mass was a significant positive correlate of fibre number. Small fibres were more abundant in mini than normal muscles in L3. Mitochondrial volume density was significantly higher in mini than normal muscle fibres in L3, but not in L6. Microscopy revealed a surprising attribute of the mini muscles: an abundance of small, minimally differentiated, myofibril-containing cells positioned in a disorderly fashion, particularly in the surface layer. We hypothesise that these unusual cells may be satellite cells or type IIb fibres that did not complete their differentiation. Together, these observations suggest that mice with the mini phenotype have reduced numbers of type IIb fibres in many of their hind-limb muscles, leading to a decrease in mass and an increase in mass-specific aerobic capacity in muscles that typically have a high proportion of type IIb fibres. Moreover, the several statistically significant interactions between muscle phenotype and line indicate that the effect of the underlying allele is altered by genetic background.

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## 1. Introduction

Adaptive phenotypic evolution in response to natural selection is a central and undisputed paradigm underlying contemporary biology, but its direct study is difficult. Experimental evolution protocols provide a means of evaluating adaptive

mechanisms under more-or-less defined and controlled selective regimes (Bennett, 2003; Garland, 2003; Folk and Bradley, 2005; Swallow and Garland, 2005). For example, selective breeding of house mice for high voluntary wheel running was used to create four replicate lines (Swallow et al., 1998a) that run approximately 170% more revolutions/day as compared with four randomly bred control lines (e.g., see Garland et al., 2002; Garland, 2003; Belter et al., 2004; Rezende et al., 2006b). As a group, the four selected lines exhibit a number of characteristics that appear to represent adaptations for high levels of

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sustained, aerobically supported wheel running. These include higher whole-animal maximal oxygen consumption ( $VO_2\text{max}$ , especially in males: Swallow et al., 1998b; Rezende et al., 2006a,b), increased insulin-stimulated glucose uptake in extensor digitorum longus muscle (Dumke et al., 2001), and more symmetrical hind-limb bones and larger femoral condyles (Garland and Freeman, 2005; Kelly et al., in press).

In addition to traits that differ consistently between the four selected and four control lines, some distinct responses have occurred among the replicate selected lines. Such differences are expected because random genetic processes (founder effects, genetic drift, mutation) will necessarily alter the gene pool within each line (isolated population) at the same time that any adaptive responses to selection are occurring. The most dramatic example of among-line variation in the wheel-running selection experiment is that two selected lines have evolved a high frequency of a “mighty mini-muscle” phenotype, whose primary characteristic is an approximate halving of hind-limb muscle mass with a coincident doubling of mass-specific activities of mitochondrial enzymes (Houle-Leroy et al., 2003). The phenotype appears attributable to an autosomal recessive allele that was present in the base (starting) population at a frequency of about 7% (Garland et al., 2002). The fact that two of the selected lines have never shown the phenotype is presumably attributable to random genetic drift, i.e., as a rare allele, it was lost by chance before selection could increase its frequency (see Garland et al., 2002). Interestingly, although the mini phenotype has been favoured by selection, the absolute running performance (revolutions/day) of these mice usually does not differ statistically from that of selected mice with normally sized muscles (Garland et al., 2002; Houle-Leroy et al., 2003; Swallow et al., 2005). However, individuals with the mini phenotype often run significantly faster on wheels as compared with selected individuals with normally sized muscles (Kelly et al., in press; see also Syme et al., 2005), and they have higher  $VO_2\text{max}$  during forced exercise tests in hypoxia (Rezende et al., 2006a). Therefore, whatever the characteristics that led it to be favoured by selection for high wheel running, the mini-muscle phenotype represents what may be considered an alternate solution to the physiological challenge imposed by the selection regime, not necessarily a better one as compared with normal-sized hind-limb muscles (Garland, 2003).

Recently, we reported contractile characteristics of soleus and medial gastrocnemius muscles from selected lines L3 (all mini individuals) and L6 (both normal and mini) (Syme et al., 2005). We found that soleus of mini individuals was actually larger than for normal mice. In spite of the increased mass, contractile characteristics of the soleus did not significantly differ between mini and normal mice. However, medial gastrocnemius muscles of mini mice exhibited slower twitches, a more curved force–velocity relationship, produced about half the mass-specific isotonic power, 20–50% of the mass-specific cyclic work and power, and fatigued at about half the rate of normal muscles (i.e., had greater endurance). Gastrocnemius muscles of mini individuals also exhibit increased glycogen concentration as compared with the normal phenotype (Gomes et al., 2004).

The primary goal of the present study was to evaluate mechanisms that may underlie the altered size, aerobic capacity, and contractile properties of mini muscles. For example, the reduced mass could reflect a reduction in fibre number and/or fibre size. The increased aerobic capacity of mini muscles could reflect a greater mitochondrial volume density in the fibres. We evaluated these hypotheses using a combination of biochemical, morphometric, and ultrastructural techniques. Previous biochemical studies of the mini muscles examined the “triceps surae” complex (gastrocnemius, soleus, plantaris: Belter et al., 2004) or mixed hind-limb muscle as a pool (Houle-Leroy et al., 2000, 2003; but see Gomes et al., 2004). Thus, to identify the structural and physiological changes associated with the expression of the mini-muscle allele, we compared specific individual muscles in mice with mini muscles with those of their line-mates that had normally sized muscles. Our first step was to compare the mass of the gastrocnemius, plantaris, and soleus muscles. Next we compared the enzymatic profiles of the muscles that were smaller in the mini phenotype. Given the small size, cylindrical shape, and homogeneity of fibre distribution in the plantaris muscle, we chose it rather than the larger gastrocnemius to compare fibre morphometry and ultrastructure of mini- and normal-sized muscles (see also Discussion). We quantified the number of muscle cells in cross-sections, compared the frequency distributions of fibre sizes, and evaluated whether such ultrastructural characteristics as mitochondrial and myofibrillar volume density and mitochondrial cristae density differ between mini- and normally sized muscles. As a first step towards evaluating whether differences in fibre type proportions might underlie the mass reduction, we characterised the myosin heavy and light chain (MHC and MLC) composition in the gastrocnemius. By carrying out these measurements in both selected lines that expressed the mini phenotype, we also evaluated whether mini muscles show the same characteristics in both lines or whether the effect of the allele depends on genetic background.

## 2. Material and methods

### 2.1. Animals and the selection experiment

Swallow et al. (1998a) provide full details of the selection experiment, which involves four lines of mice (*Mus musculus*) selectively bred for high voluntary wheel running and four additional lines maintained as controls. Mice used for our metabolic, morphometric, and ultrastructural determinations were 55 males aged between 152–169 days and sampled from generation 26 of the artificial selection experiment for increased voluntary activity on running wheels. For the characterisation of myosin heavy and light chain profiles, we used females aged between 126–208 days (mean of 154 days) from generation 27. These mice were collected from the two selected lines (L3 and L6) that exhibit the mini-muscle phenotype (Garland et al., 2002; Houle-Leroy et al., 2003). Mice were weaned at 21 days of age and housed with three same-sex siblings in a standard clear plastic cage, other than

during routine wheel testing for six days (see Swallow et al., 1998a).

## 2.2. Sampling strategy

As a prelude to the morphometric and ultrastructural analysis, we determined body mass, mass of the gastrocnemius, plantaris, and soleus muscles, and enzyme activities in the gastrocnemius and plantaris muscles in 33 mice from L3 and 22 from L6 (generation 26). The large size and heterogeneous fibre distribution in the gastrocnemius muscle led us to choose the more cylindrical plantaris for quantitative microscopic comparison of mini and normal muscles. Morphometric analysis using light microscopy examined the plantaris muscles of 24 of the mice from L3 (equal numbers for the two phenotypes) and 12 from L6 (equal numbers for the two phenotypes). Of these 36 mice, 24 (12 per line with equal numbers for each phenotype) were used for ultrastructural analysis of plantaris fibres with electron microscopy.

For the analysis of myosin isoform expression profiles, we used 19 mice from L3 and 25 from L6 from generation 27. The body mass and mass of the gastrocnemius, plantaris, and soleus muscles from both hind limbs were determined in 32 mice. Of these, 4 in L3 and 5 in L6 had the mini phenotype.

## 2.3. Muscle dissection

Mice were killed by cervical dislocation after CO<sub>2</sub> anaesthesia. Within ~ 5 min of death, the muscles of the left “triceps surae” (which includes lateral and medial heads of the gastrocnemius, plantaris, and soleus) were weighed separately and fixed in glutaraldehyde/formaldehyde solution for electron microscopy. Within ~25 min of death, the muscles of the right triceps surae were weighed separately, frozen on dry ice, and placed at –80 °C for enzymatic determinations. The enzyme activities we measured were equivalent to those obtained in a previous study in which samples were rapidly frozen for measurements of enzyme activities (Houle-Leroy et al., 2000). The muscle samples for electron microscopy were transported in cacodylate buffer to Université Laval, whereas frozen samples were transported in liquid nitrogen.

For our myosin profiling (generation 27 males), left and right gastrocnemius, plantaris, and soleus muscles were dissected and weighed, then quick-frozen in liquid nitrogen and stored at –80 °C. Only gastrocnemius muscles were used for myosin analyses.

## 2.4. Enzyme activity measurements

Muscle extracts were prepared following Houle-Leroy et al. (2000). The enzymes, citrate synthase, cytochrome C oxidase, hexokinase, pyruvate dehydrogenase, phosphofructokinase-1 and lactate dehydrogenase (CS, CCO, HK, PDH, PFK, LDH), were assayed using the following conditions.

CS: 100 mM Tris–HCl, 0.2 mM acetyl CoA, 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1 mM oxaloacetate (omitted for control), pH 8.0.

CCO: 0.1 M KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>PO<sub>4</sub>, 0.1 mM cytochrome C reduced with sodium hydrosulfite (NA<sub>2</sub>S<sub>2</sub>O<sub>4</sub>), pH 7.0. After reduction of cytochrome C with sodium hydrosulfite, the excess was removed by bubbling with air. Reactions were run against a control of 0.1 mM cytochrome C oxidised with 50 μM K<sub>3</sub>Fe(CN)<sub>6</sub>.

HK: 50 mM Triethanolamine–HCl, 8 mM MgCl<sub>2</sub>, 0.5 mM NADP, 8 mM ATP, excess levels of glucose-6-phosphate dehydrogenase (4 U), 4 mM glucose (omitted for control), pH 7.6.

PDH: 50 mM Tris–HCl, 0.5 mM EDTA, 0.2% Triton X-100, 1 mM MgCl<sub>2</sub>, 1 mg mL<sup>-1</sup> bovine serum albumin, 2.5 mM NAD, 0.1 mM CoA, 10 mM oxalate, 0.6 mM *p*-iodonitrotriazolium violet (INT), 0.2 mM thiamine pyrophosphate, 6 U lipoamide dehydrogenase, 6 mM pyruvate (omitted for control), pH 7.8.

PFK: 50 mM Triethanolamine–HCl, 5 MgCl<sub>2</sub>, 50 mM KCl, 4 mM ATP, 0.28 mM NADH, 3.5 mM fructose-6-phosphate (omitted for control), excess levels of aldolase (1 U), triosephosphate isomerase (50 U), and α-glycerophosphate dehydrogenase (8 U), pH 7.6.

LDH: 50 mM Triethanolamine–HCl, 0.28 mM NADH, 2.4 mM pyruvate (omitted for control), pH 7.6.

## 2.5. Myosin isoform expression: homogenisation and protein determination

Gastrocnemius muscles were homogenised (50 g per 100 mL) on ice in a buffer consisting of 50 mM Tris–HCl, 0.25 mM sucrose, 5 mM EDTA, and 1% (w/v) SDS using a Tekmar Tissuemizer. The resulting homogenate was then centrifuged for 10 min at 1200 g at 4 °C in a Beckman bench top centrifuge (GS15R). Total muscle protein concentration was determined using the Bio Rad Protein Assay Kit (based on the Bradford method). Samples were read at 595 nm using a Shimadzu UV-2101PC spectrophotometer at room temperature following the kit's instructions.

## 2.6. Analysis of MHC and MLC isoform expression

Myosin heavy chain (MHC) isoform content of muscle homogenates was determined using a modification of the SDS-polyacrylamide gel electrophoresis technique of Talmadge and Roy (1993; see Diffie et al., 2002). Muscle homogenates were combined with sample buffer (8 M urea, 2 M thiourea, 0.05 M Tris pH 6.8, 75 mM dithiothreitol, 3% sodium dodecyl sulfate [SDS], and 0.05% bromophenol blue), heated (3 min at 100 °C), and loaded onto polyacrylamide gels (total protein load 4–10 μg per sample). Stacking gels were composed of 5% acrylamide/bis (50:1), 25% glycerol, 87.5 mM Tris (pH 6.7), 5 mM EDTA, and 0.5% SDS. Resolving gels were composed of 8% acrylamide/bis (50:1), 30% glycerol, 0.2 M Tris (pH 8.8), 0.1 M glycine, and 0.4% SDS. Gels were run using SE 200 Tall (10×12 cm) Mighty Small Mini-Vertical Units (Hoefer) with 0.75 cm thick spacers, and an EPS 301 power supply (Amersham Pharmacia Biotech). The upper running buffer consisted of 100 mM Tris (base), 150 mM glycine, and 0.1%

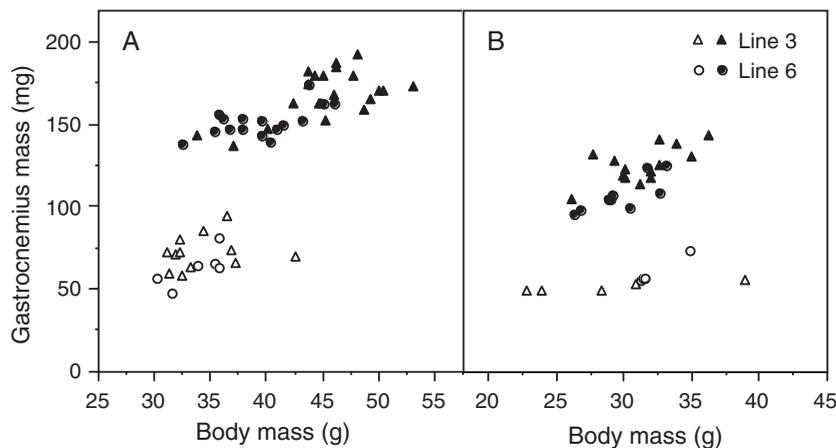


Fig. 1. Relationship between body mass and gastrocnemius mass for male mice from generation 26 (A) and female mice from generation 27 (B). Black symbols (normal muscle phenotype); white symbols (mini-muscle phenotype).

SDS. The lower running buffer consisted of 50 mM Tris (base), 75 mM glycine, and 0.05% SDS. The gels were run at 135 V (constant voltage) for 43–48 h at 4 °C. Gels were silver stained using Bio Rad Silver Stain Plus kit according to kit instructions. Stained gels were dried, then scanned into bitmap file format (.bmp) using an Epson Perfection 1200 Photo-scanner with its transparency adapter (back-lit). Density of bands was quantified using Un-Scan-It gel quantification software (Silk Scientific, Orem, Utah). Myosin light chain composition was analyzed using 18% SDS-polyacrylamide gels. Muscle homogenates (10 µg/lane) were loaded onto gels and run at 70 V (constant voltage) for 20 h at 4 °C. Gels were stained, dried, and scanned as described above. MHC and MLC isoforms were identified according to their electrophoretic mobilities relative to molecular weight standards (Talmadge and Roy, 1993).

### 2.7. Muscle fixation and embedding for microscopy

Muscles were fixed for ~24 h in 2.5% glutaraldehyde, 1.5% formaldehyde, 0.25 mM CaCl<sub>2</sub>, and 0.1 M cacodylate buffer, pH 7.3. Each plantaris was subsequently cut into 2 blocks by making a transverse cut at approximately half the length of the muscle, and refixed for ~12 h. Blocks were then stored at 4 °C in cacodylate buffer until the next step (~1–2 week). After rinsing in cacodylate buffer, blocks were postfixated in 1% osmium tetroxide (OsO<sub>4</sub>) and 0.5% uranyl acetate and dehydrated in a graded ethanol series (30–100%). Then they were placed in a solution of (1 : 1) propylene oxide : epon and finally embedded in 100% epon (poly-bed), which polymerised after 3 days at 60 °C. Propylene oxide was used as infiltrating chemical.

### 2.8. Analysis of muscle morphometry

For light microscopy, semi-thin sections (500 nm thickness) of the plantaris blocks were cut transversally at a slightly oblique angle and coloured with 1% methyl blue and 1% azure II. Completely transverse sections of the plantaris muscle, made at

approximately the middle of the muscle, provided an overview of its structure. We counted the total number of muscle fibres in these sections at low magnification (100×). The cross-sectional area of plantaris fibres,  $a(f)$ , was assessed at a magnification of 200×. To obtain a representative sample of the fibres throughout the muscle, we drew 5 transect lines, equally spaced from the surface to the centre of the muscle on a photograph, which represented about 20% of the cross-section of the plantaris. We calculated  $a(f)$  of all cells intersected ( $57 \pm 2$ ) by the transects, using NIH software.

For ultrastructural evaluation, muscle sections were examined at higher magnification by electron transmission microscopy on a JEOL 1200 EX microscope. Organelle volume densities were obtained using stereological methods following a cascade sampling strategy with different magnifications (Weibel, 1979). Ultra-thin sections (60–70 nm thickness), taken in the middle of the plantaris cross-section, were placed on 200 mesh copper grids and treated with lead citrate and uranyl acetate. On one of these ultra-thin sections, 8 micrographs (each covering ~270 µm<sup>2</sup> of fibre) were taken randomly (starting at the same corner of the hexagon made by the grid mesh) at a magnification of 5000×. Micrographs were recorded on film and printed on paper at a final magnification of 12,000×. By point counting (Weibel, 1979), we estimated volume densities (Vv) per fibre volume of intracellular components [mitochondria, Vv (mt, f); myofibril, Vv (mf, f); nuclei, (n, f); and sarcoplasm, Vv (s, f), which included sarcoplasmic reticulum and T tubules, on each micrograph.

A second level of magnification (20,000×) was used to evaluate densities of mitochondrial cristae on  $15 \pm 1$  randomly selected subsarcolemmal mitochondria per muscle. Mitochondria were printed at a final magnification of 50,000× and we traced the outline of the cristae before undertaking counting. The surface density of the cristae [Sv (cr, mt); the ratio of the surface area of the mitochondrial inner membrane to the volume of the mitochondrion] was determined using line–intercept measurements (Weibel, 1979). No corrections were made for the effects of thickness or compression.

Table 1

Body mass and muscle masses of mice with mini vs. normal muscles from selected lines 3 and 6 (males from generation 26)

	Line 3		Line 6		<i>P</i> values		
	Mini	Normal	Mini	Normal	Mini	Line	Line*Mini
Body mass, g	34.3±1.0	45.2±1.0	33.7±1.0	33.7±1.0	0.0001	0.0126	0.0399
log <sub>10</sub> Body mass	1.53±0.012	1.65±0.009	1.53±0.017	1.59±0.011			
Mean gastrocnemius, mg	72.8±3.0	168.2±3.0	62.7±2.3	152.3±2.3	0.0001	0.0008	0.0716
log <sub>10</sub> Mean gastroc	1.89±0.012	2.20±0.010	1.83±0.016	2.18±0.008			
Mean plantaris, mg	15.2±0.4	20.2±0.5	15.4±0.8	18.4±0.3	0.0149	0.9185	0.9645
log <sub>10</sub> Mean plantaris mass	1.22±0.014	1.27±0.012	1.22±0.019	1.27±0.010			
Left soleus, mg	12.0±0.4	11.3±0.2	12.5±0.8	10.4±0.5	0.0111	0.7682	0.1441
log <sub>10</sub> Left soleus mass	1.08±0.022	1.04±0.018	1.11±0.028	1.01±0.015			

N=55.

Raw means±S.E.M. are shown in addition to log<sub>10</sub> adjusted means±S.E.M. from two-way ANCOVA models that included the interaction between muscle phenotype and line, as well as log body mass (except for body mass itself) and age as covariates.

Age was never a significant (*P*<0.05) covariate for any trait. As would be expected, log body mass was a positive predictor of all log muscle masses, although the significance level varied (*P*=0.0002 for gastrocnemius, *P*=0.0001 for plantaris, *P*=0.3794 for soleus).

## 2.9. Statistical analyses

The General Linear Models (GLM) procedure in JMP and the Mixed procedure in SAS (both SAS Institute) were used to apply analysis of covariance (ANCOVA) models to our data. We performed two-way ANCOVAs to test effects of both phenotype [mini-muscle phenotype (MMP) vs normal muscle phenotype (NMP)] and line (L3 vs L6). Both muscle pheno-

type and line factors were considered fixed effects, and we also tested for phenotype\*line interactions, with all terms tested over the residual error term. Log transformations were used for body and muscle mass data to improve linearity and homoscedasticity of bivariate relations and normality of residuals. Body mass and age were included as covariates. Because body mass differed between lines 3 and 6 for generation 26, all the ANCOVA models were tested with and

Table 2

Body mass, muscle masses, and myosin heavy and light chain composition (%) in gastrocnemius muscle of mice with mini vs. normal muscles from selected lines 3 and 6 (females from generation 27)

	Line 3		Line 6		<i>P</i> values		
	Mini	Normal	Mini	Normal	Mini	Line	Line*Mini
Body mass, g	29.0±2.9	31.4±0.7	32.2±0.9	29.8±0.8	0.8146	0.4168	0.0669
log <sub>10</sub> Body mass	1.45±0.022	1.50±0.013	1.51±0.025	1.47±0.017			
Mean gastrocnemius, mg	50.3±2.2	121.4±3.0	57.4±3.9	106.9±3.4	0.0001	0.2103	0.0029
log <sub>10</sub> Mean gastroc. mass	1.72±0.013	2.08±0.008	1.74±0.015	2.03±0.010			
Mean plantaris, mg	7.9±0.6	11.8±0.6	9.6±1.3	10.3±0.5	0.0040	0.8451	0.0503
log <sub>10</sub> Mean plantaris mass	0.91±0.033	1.07±0.019	0.97±0.037	1.00±0.024			
Mean soleus, mg	7.2±0.15	7.4±0.4	10.2±1.8	6.6±0.4	0.0098	0.2987	0.0130
log <sub>10</sub> Mean soleus mass	0.87±0.033	0.87±0.019	0.98±0.036	0.82±0.024			
Myosin heavy chains <sup>a</sup>							
% MHC I	19.2±1.68	13.6±1.69	20.0±1.94	16.5±1.81	0.0382	0.2874	0.5682
% MHC IIA	26.7±0.69	20.5±0.69	22.7±0.79	20.9±0.74	0.0003	0.0233	0.0098
% MHC IIB	24.8±1.87	45.6±1.89	28.9±2.16	41.6±2.02	0.0001	0.9729	0.0628
% MHC IIX	29.3±1.36	20.4±1.37	28.4±1.57	21.0±1.47	0.0002	0.9127	0.5896
Essential light chains <sup>a</sup>							
% MLC 1F	54.8±0.95	70.8±0.86	59.5±0.97	70.4±1.07	0.0001	0.0455	0.0195
% MLC 1S	35.7±1.08	21.3±0.98	30.4±1.11	21.8±1.22	0.0001	0.0470	0.0198
% MLC 3F	9.5±0.80	7.9±0.72	10.1±0.82	7.8±0.90	0.0598	0.7199	0.6654
Regulatory light chains <sup>b</sup>							
% MLC 2F	67.6±1.05	75.4±0.93	63.5±1.05	73.2±1.16	0.0001	0.0105	0.3732
% MLC 2S	32.4±1.03	24.6±0.93	36.5±1.05	26.8±1.16			

For body and muscle masses, raw means±S.E.M. are shown in addition to log<sub>10</sub> adjusted means±S.E.M. from two-way ANCOVA models that included the interaction between muscle phenotype and line, as well as log body mass (except for body mass itself) and age as covariates.

Age was never a significant (*P*<0.05) covariate for any trait. As would be expected, log body mass was a positive predictor of all log muscle masses, although the significance level varied (*P*=0.0001 for gastrocnemius, *P*=0.1414 for plantaris, *P*=0.1123 for soleus). For myosin heavy and light chains, log body mass was significant only for MHC IIA (*P*=0.0196, positive partial regression coefficient).

<sup>a</sup> For the MHC and essential MLCs, raw values sum to 100%, so statistical tests are not independent among the individual types. Tests for all types are presented for completeness.

<sup>b</sup> Raw values for the two regulatory MLCs sum to 100%, so statistical tests are not independent between the two types. *P* values are shown only for one type because they are identical for the other.

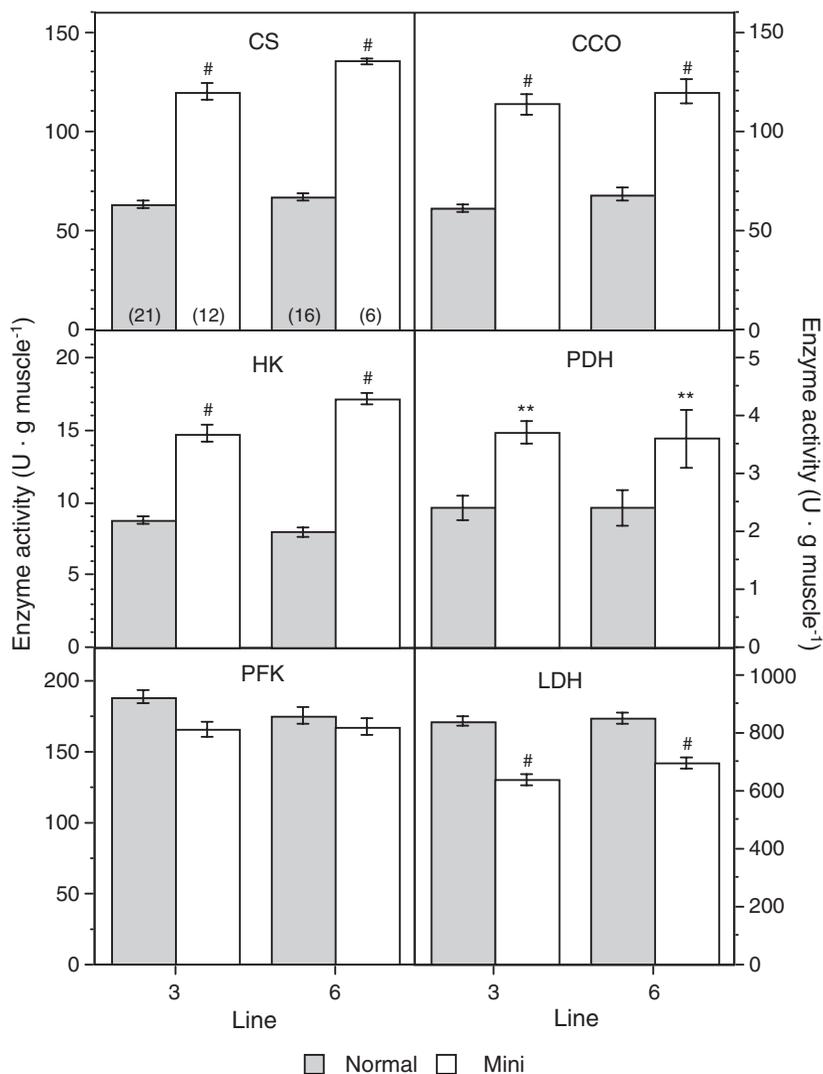


Fig. 2. Enzyme activities of gastrocnemius of male mice from generation 26. Values are means  $\pm$  SE. The number ( $n$ ) for each sub-group is indicated in parenthesis and was similar for the enzymes assayed. Activities were expressed per g of fresh muscle. Probabilities associated with the effect of the mini-muscle phenotype were \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; # $P \leq 0.0001$ . Covariates in these models were body mass and age. When the body mass was not included in the model, the probability associated with the mini-muscle effect was stronger (see Results).

without body mass as a covariate for the biochemical and histomorphometric data.

### 3. Results

#### 3.1. Body and muscle masses

In generation 26, of the 55 experimental male mice (33 from L3, 22 from L6), 18 individuals (12 from L3, 6 from L6) showed the mini-muscle phenotype (Fig. 1; open symbols). The body mass of these individuals was significantly lower ( $P < 0.0001$ ) than that of mice with normally sized muscles from these selected lines, and the difference was greater in L3 (Table 1, interaction  $P = 0.039$ ). Results were different for the females of generation 27, as body mass did not differ between mice with the mini phenotype (9 of 32) and those with normal muscles.

In both generations 26 and 27, the reduction of muscle mass in the mini phenotype was particularly pronounced for gastro-

cnemius (approximately 50% reduction), with the plantaris showing a reduction between 8% and 30% (Table 1). On the other hand, the mass of the soleus muscle was greater in mice with the mini phenotype than in normal mice. In the generation 27 females, the statistical interaction between mini and line was significant for gastrocnemius and soleus, and almost significant for plantaris mass (Table 2). The reduction of the gastrocnemius was stronger in L3 whereas the increase in the soleus was stronger in L6.

#### 3.2. Specific enzyme activities

In the gastrocnemius (Fig. 2), mice with the mini phenotype showed significantly higher mass-specific activities ( $P < 0.0001$ , except for PDH,  $P < 0.01$ ) for mitochondrial enzymes (CS: 90% and 103%; CCO: 86% and 76% and PDH: 54% and 50%, L3 and L6, respectively) and HK (68% and 115%, L3 and L6, respectively) than mice with normally

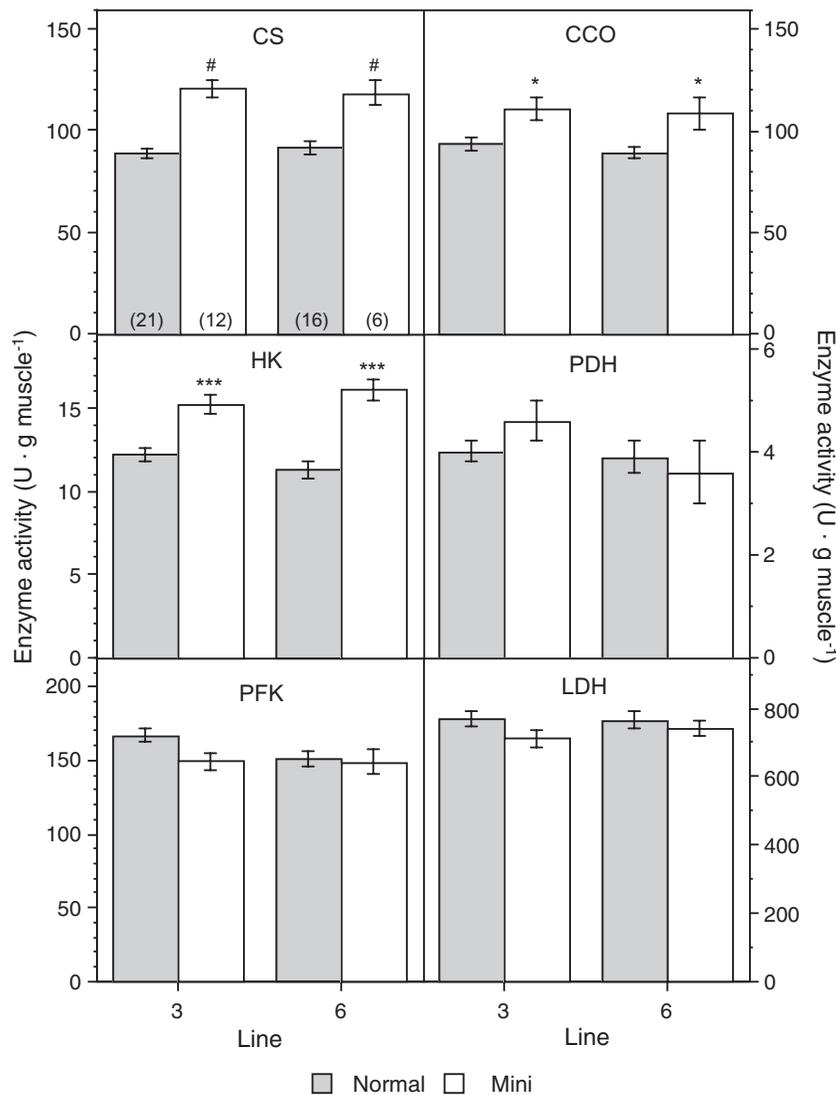


Fig. 3. Enzyme activities of plantaris of mice from generation 26. Values are means  $\pm$  SE. The number ( $n$ ) for each sub-group is indicated in parenthesis and was similar for the enzymes assayed. Activities were expressed per g of fresh muscle. Probabilities associated with the effect of phenotype were \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; # $P \leq 0.0001$ . Covariates used in these models were body mass and age. When the body mass was not included, the probability associated with the mini-muscle effect was stronger (see Results).

sized muscles. LDH showed the opposite effect, with significantly lower activity ( $P < 0.0001$ ) in mini mice. PFK activity tended to be lower in mice with the mini phenotype (12% and 5%, L3 and L6, respectively) ( $P = 0.05$ ). These patterns are consistent with the enzymatic differences between mini and normal muscles observed at generation 14 (Houle-Leroy et al., 2003). No significant interactions between line and mini phenotype were observed for enzyme activities in the gastrocnemius muscle.

In the plantaris (Fig. 3), CS, CCO, and HK showed a significant effect of phenotype ( $P < 0.0001$ ,  $P < 0.05$ , and  $P < 0.001$ ; CS, CCO, and HK, respectively). Specific activities of these enzymes were higher in mini than normal muscles (CS: 36% and 30%; CCO: 18% and 21%; HK: 25% and 42%, for L3 and L6, respectively), but the differences were less pronounced than in the gastrocnemius muscle. In L3, PDH and PFK activities in plantaris showed similar patterns (but not significant) to those

observed in the gastrocnemius. LDH activity did not differ between normal and mini muscles in either L3 or L6. None of the enzyme activities in the plantaris muscle showed a significant interaction between line and phenotype. Thus, the mini phenotype increases the specific activity of mitochondrial enzymes in the gastrocnemius and plantaris muscles in both selected lines in which it is expressed.

### 3.3. Myosin heavy and light chain isoforms

The myosin heavy chain compositions in gastrocnemius muscle differed markedly between mice with the mini and normal phenotypes in both L3 and L6 (Table 2). Mice with mini muscles showed a lower % MHC IIB than mice with normally sized muscles, but contained more MHC I, MHC IIA and MHC IIX. The most dramatic difference was observed in the % MHC IIB, with an average reduction of 38% in mini muscles. When a significant

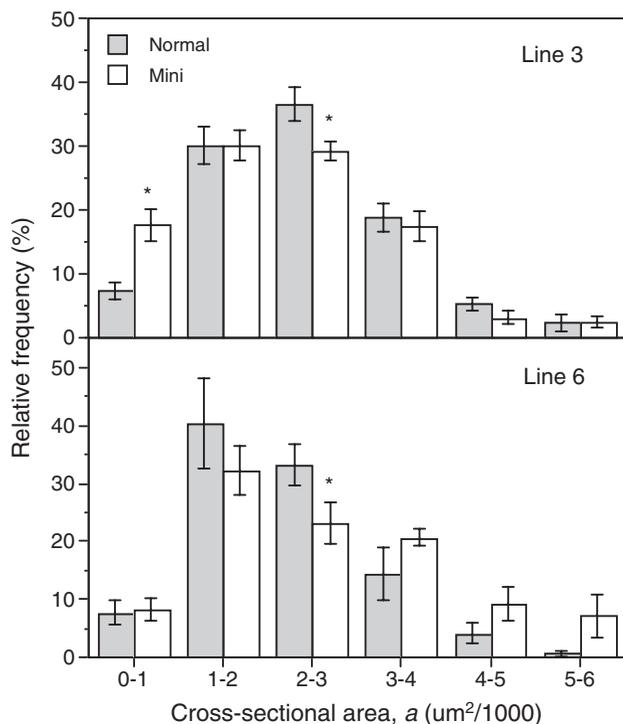


Fig. 4. Relative frequencies of fibres with different cross-sectional areas in cross-section of plantaris of mice from generation 26. Values are means  $\pm$  SE.  $n = 12$  for each phenotype in L3;  $n = 6$  for each phenotype in L6. Significant effects of the mini-muscle phenotype ( $*P < 0.05$ ) were only obtained without body mass as a factor in the statistical model.

interaction occurred between mini and line (MHC IIA,  $P = 0.0098$ ), the mini phenotype had a stronger influence in L3 than in L6.

The myosin light chain composition was also strongly influenced by the mini phenotype (Table 2). For the essential light chains, the % MLC 1F was reduced in mini muscles while the % MLC 1S was increased. These changes were significantly stronger in L3 than in L6 (interaction  $P = 0.0195$  and  $0.0198$ , respectively). The proportion of the slow form of the regulatory light chain was significantly increased in mini muscles (Table 2). The changes in MHC and MLC composition suggest a reduction in the proportion of fast fibres in the gastrocnemius of mice with the mini-muscle phenotype. These changes seem more pronounced in mice in L3 than in L6.

### 3.4. Morphometry: light microscopy of the plantaris muscle

The total number of muscle fibres in cross-sections of plantaris did not differ ( $P > 0.05$ ) between the mini and normal phenotypes or between lines. In L3, the number of cells was  $769 \pm 24$  and  $794 \pm 26$  (mini and normal, respectively). However, in L6, mini muscles tended to have a lower number of cells than normal muscles ( $690 \pm 14$  vs  $799 \pm 35$ , mini and normal, respectively). Plantaris mass was a significant covariate ( $P = 0.026$ ) of fibre number in the 2-way ANCOVA and was positively correlated with the number of muscle fibres ( $F_{1, 34} = 7.65$ ;  $P < 0.01$ ;  $r^2 = 0.18$ ).

To assess whether reduction in fibre size could explain the decreased size of the plantaris in the mini phenotype, we

evaluated the cross-sectional area of fibres along transects within the muscle cross-section (see Materials and methods). In both mini and normal muscles,  $\sim 75\%$  of fibres ranged between  $1000$ – $4000 \mu\text{m}^2$  (Fig. 4). In L3, mice with the mini phenotype had higher frequencies of the smallest fibre size ( $0$ – $1000 \mu\text{m}^2$ ) and lower frequencies of intermediate fibres ( $2000$ – $3000 \mu\text{m}^2$ ) compared with normal mice. In L6, mice with the mini phenotype showed lower frequencies of intermediate size fibres ( $2000$ – $3000 \mu\text{m}^2$ ) but tended to have higher frequencies of the largest fibres ( $>4000 \mu\text{m}^2$ ). The differences in frequencies of these fibre sizes were significant ( $P < 0.05$ ) in ANCOVA models without body mass, but not significant when body mass was included as a covariate.

Inspection of plantaris cross-sections revealed a particularity of the mini-muscle phenotype present in all mice with this phenotype. In contrast to the ordered, relatively continuous and concentric organisation of fibres in mice with the normal phenotype, fibre organisation in the plantaris of mice with the mini phenotype was disordered, especially in the surface layer (Fig. 5A). In this zone, all mini muscles exhibited very small cells, which were rare in normal muscles. The abundance of these cells may have been underestimated at the magnification used to count the fibres in cross-sections, but these “mini” cells were easy to measure at the magnification used to assess fibre cross-sectional area. A spherical nucleus in the middle of these cells was easily distinguished (Fig. 5B). Higher magnification (Fig. 5C) revealed mitochondria and many myofibrils in these “mini” cells.

### 3.5. Stereology: electron microscopy

In plantaris fibres, volume densities ( $V_v$ ) of intracellular components [mitochondria (mt), myofibrils (mf) and sarcoplasm (s)] were estimated and expressed per fibre volume (Fig. 6). In L3, fibres from mini muscles had  $37\%$  higher  $V_v$  (mt, f) than those from normal muscles. In contrast, in L6 the two phenotypes did not differ in mitochondrial volume density. In the ANCOVA, both phenotype ( $P < 0.02$ ) and line ( $P < 0.03$ ) had a significant impact on the mitochondrial volume density. The significant interaction phenotype\*line ( $P < 0.01$ ) emphasised the different effects in the two lines. Positive relationships ( $P < 0.01$ ) were observed between  $V_v$  (mt, f) and the activities of CS, CCO, and HK with  $r^2 = 0.38$ ,  $0.26$ , and  $0.30$ , respectively.

Mini mice in L3 exhibited a  $6\%$  reduction of myofibrillar volume density,  $V_v$  (mf, f) (Fig. 6), which may reflect the additional space occupied by mitochondria. Again, phenotype ( $P < 0.02$ ) and the phenotype\*line interaction ( $P < 0.005$ ) were significant factors in the model, indicating that the mini phenotype did not have the same impact in the two lines. The significance of the mini factor for  $V_v$  (mf, f), was due to the marked decrease in mini-muscle mice from L3. On the other hand, no statistical differences were observed for  $V_v$  (s, f). Sarcoplasm is the intracellular component most susceptible to dilation or compression following muscle fixation and embedding, leading its values to vary considerably among individuals.

Mitochondrial cristae density  $S_v$  (cr, mt) did not differ consistently between mini- and normally sized muscles in the two lines. In L3, mitochondria in the normal phenotype had a

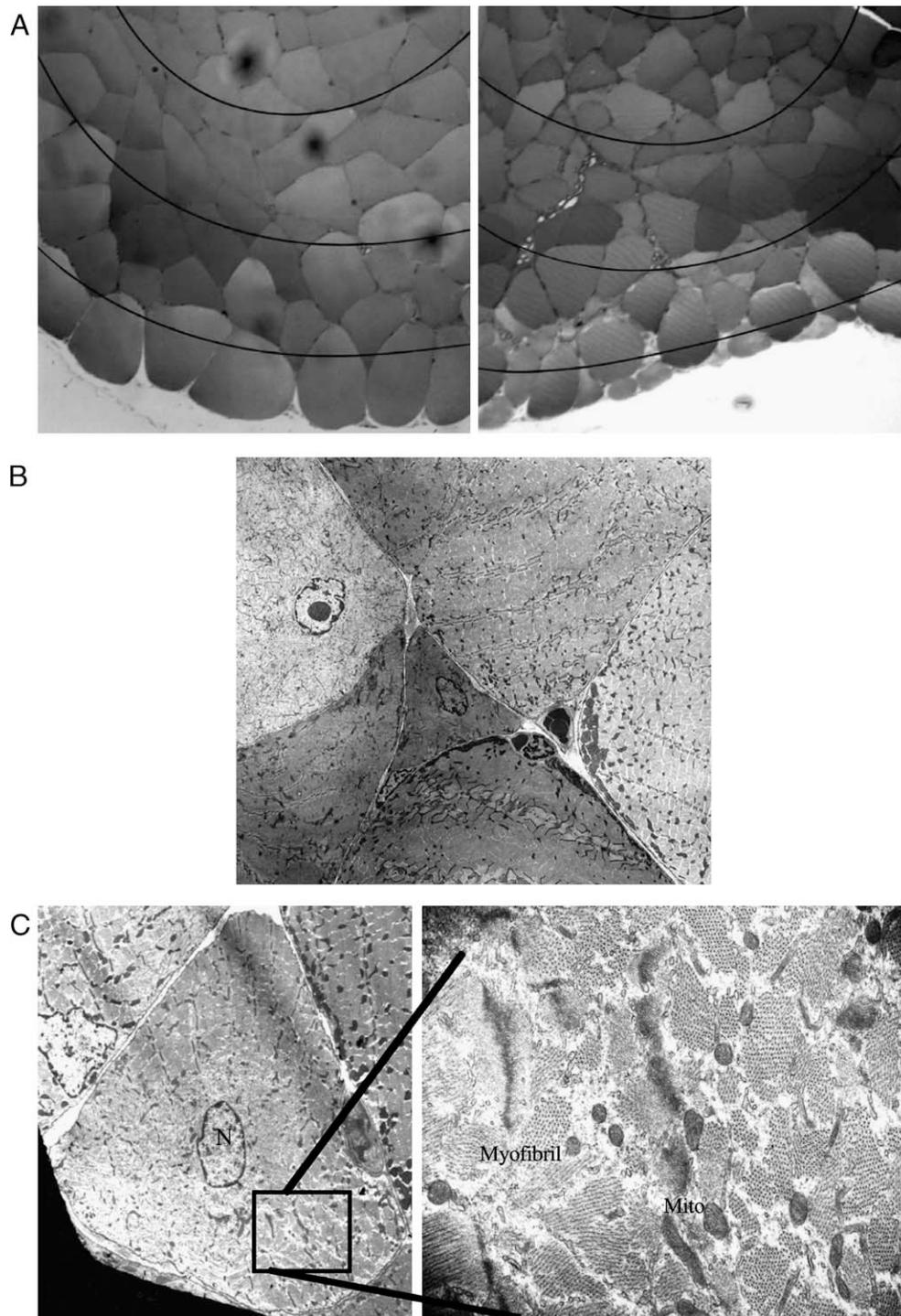


Fig. 5. Photographs of plantaris muscle (generation 26) showing (A) normal muscle phenotype (left panel) and mini-muscle phenotype (right panel). Transect lines used to quantify the frequency distribution of fibre sizes are shown. These photographs were taken at 200 $\times$  magnification. (B) Electron micrograph of mini-muscle phenotype showing a mini cell inserted among muscle fibres. Photograph taken at 2000 $\times$  magnification. (C) Electron micrograph showing an entire mini cell (right panel, taken at 2000 $\times$  magnification) and detail of myofibrils (Myo) and mitochondria (Mito) in the cytoplasm (right panel, photograph taken at 15000 $\times$  magnification).

cristae density of  $33.5 \pm 0.7 \mu\text{m}^{-1}$  ( $\pm$ S.E.M), whereas those from mini muscles had a value of  $31.2 \pm 0.8$ . In L6 the opposite tendency was observed with values of  $31.4 \pm 0.7$  and  $33.3 \pm 0.8$ , respectively. These contrasting tendencies led to a significant interaction between phenotype \* line ( $P < 0.02$ ), whereas neither

the effects of line nor phenotype were significant ( $P > 0.05$ ). We also examined whether the relationship between cristae density and mitochondrial volume density was similar between mice with the mini and normal phenotypes. Mini muscles from L3 typically had high  $V_v$  (mt, f) and low  $S_v$  (cr, mt) values,

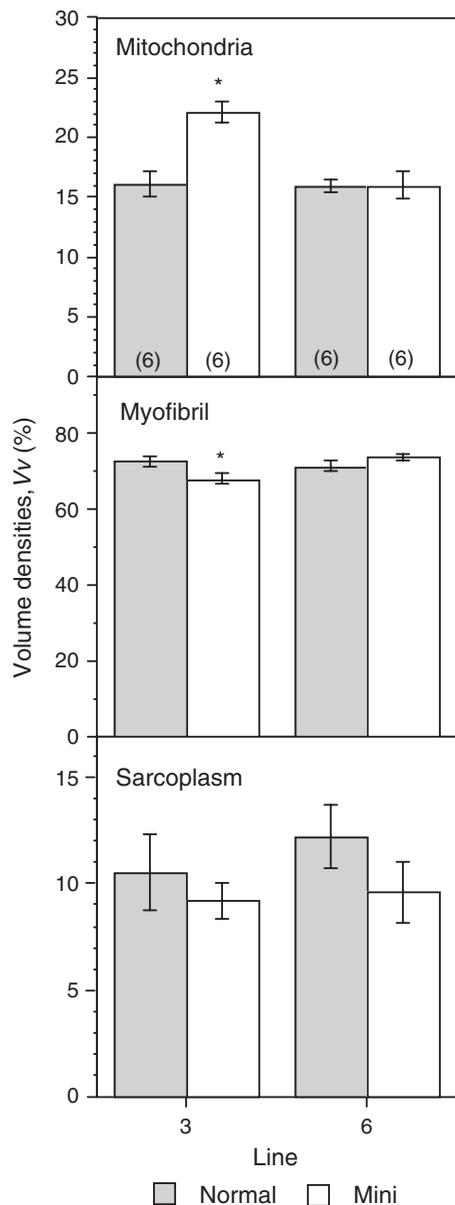


Fig. 6. Volume densities of organelles expressed per plantaris fibre volume in 24 mice (generation 26). Values are means  $\pm$  SE.  $n=6$  for each of the four sub-groups. A significant effect of the mini-muscle phenotype is indicated by \* ( $P < 0.05$ ). Covariates used in this model were body mass and age.

whereas mini muscles from L6 had high Sv (cr, mt) and low Vv (mt, f) values. The mice with the normal phenotype showed a positive relationship between Vv (mt, f) and Sv (cr, mt). However, these patterns are based on a fairly limited sample size (6 mice of each phenotype for each line).

#### 4. Discussion

The central finding of this study is that multi-generation selective breeding for high levels of voluntary running has favoured a phenotype in which hind-limb muscles that are usually rich in type IIb fibres decrease in mass and show an abundance of unusual “mini” cells. These cells contained myofibrils and

mitochondria, but were vastly smaller than typical muscle fibres. In the plantaris muscle, these “mini” cells primarily occurred in the outer fibre layers. Various lines of evidence suggest a decrease of type IIb fibres in the “mini” muscles. Accordingly, the gastrocnemius, which normally has a greater % type IIb fibres than the plantaris (Armstrong and Phelps, 1984), had a greater mass reduction than the plantaris. Furthermore, the myosin chains typical of type IIb fibres (MHC IIB, MLC 1F and MLC 2F) were markedly reduced in gastrocnemius muscles from mice with the mini-muscle phenotype. Concomitant with the decrease in mass, the specific activities ( $U\ g^{-1}$  muscle) of mitochondrial enzymes in the plantaris and gastrocnemius muscles increased, as previously found for pooled hind-limb muscles (without the triceps surae) (Houle-Leroy et al., 2003). The expression of the mini-muscle phenotype led to similar changes in mass-specific enzyme activities in L3 and L6. However, the proportions of MHC and MLC, and the histological and ultrastructural characteristics, differed more between mini muscle and normal muscles in L3 than in L6. Therefore, the specific manifestations of the mini-muscle allele are affected by the genetic background in which it is expressed.

Partly for logistical reasons, our compositional and microscopic analyses of the mini-muscle phenotype were based on different muscles. The gastrocnemius muscle normally contains regions that are rich in type IIb fibres. If the mini phenotype causes a loss in type IIb fibres, as we hypothesise, then the impact should be particularly pronounced in the gastrocnemius, making it the best muscle for the study of myosin isoform profiles. On the other hand, even in the mouse, the gastrocnemius is rather large for evaluation of fibre number. Further, the regional disparities in its fibre composition within the gastrocnemius render sampling for microscopic and ultrastructural quantification difficult. Instead, we chose the plantaris for our microscopic studies, given its small size, more homogeneous distribution of fibres, and mass decrease in the mini phenotype. Clearly, an important next step will be to examine whether the sections of the gastrocnemius muscle that are typically rich in type IIb fibres are preferentially affected by the mini phenotype and manifest the unusual small cells we found.

A decrease in mass of the mini muscles could occur by decreasing the number and/or size of the fibres. Although the number of muscle fibres in a cross-section of the plantaris muscle did not significantly differ between the mini and normal phenotypes, muscle mass was a significant positive correlate of fibre number. A systematic decrease in fibre cross-sectional area could also decrease overall muscle mass. Whereas the size distribution of the fibres differed between mini and normal muscles, the differences were line-specific and did not systematically indicate a decrease in fibre cross-sectional area. In L3, in which the mini phenotype became fixed in subsequent generations, mini muscles had more small fibres and fewer intermediate fibres, whereas in L6 mini muscles had fewer intermediate fibres (Fig. 4). Thus, fibre size was more strongly affected in L3 than in L6.

The specific activities of mitochondrial enzymes in the mini muscles could increase through increases in the aerobic capacity of individual fibres or through decreases in the proportion

of glycolytic type IIB fibres. Our data provide evidence for both mechanisms. A systematic reduction in the proportion of MHC IIB suggests a decrease in the proportion of type IIB fibres in the gastrocnemius muscles of mice with the mini phenotype, although this change could also reflect altered myosin isoform expression in existing fibres rather than a change in fibre “type.” MLC profiles also suggest a decrease of type IIB fibres in mini muscles, with stronger modifications in L3 than L6. In plantaris muscles of mice in L3 but not in L6, mini muscles have a greater mitochondrial volume density than normal muscles, which could be due to a loss of type IIB fibres or a generalised increase in the aerobic capacity of the fibres. Thus, although the overall expression of the mini phenotype followed similar patterns in L3 and L6, the lines differed in its cellular manifestations.

The most unusual attribute of the mini muscles was the presence of the many small cells. These cells look much like satellite cells (Hawke and Garry, 2001) or like myoblasts in an early state of differentiation. The presence of myofibrils suggests that they are muscle cells. The decrease in MHC IIB and the increase in the specific activities of mitochondrial enzymes in the mini phenotype suggests that these mini cells may represent muscle cells that did not complete their differentiation into type IIB fibres. A loss of type IIB fibres in the mini phenotype should reduce the concentration of myofibrils in mini muscles. In accordance with this expectation, Houle-Leroy et al. (2003) observed a lower concentration of myofibrillar protein in mini muscles.

In our study of generation 14, only our sample of L6 had sufficient individuals to characterise the impact of the mini phenotype (Houle-Leroy et al., 2003), although the few mini mice in L3 were similar to those in L6. The mini phenotype has gradually increased in frequency, with approximately half the mice in L3 and L6 showing the phenotype at generations 22, 26, and 27 (Garland et al., 2002; this study). Since then, L3 has become fixed for the mini phenotype (Syme et al., 2005; unpublished results). For the gastrocnemius, the impact of the mini phenotype on the enzymatic profiles was virtually identical in L3 and L6, whereas the myosin heavy and light chains showed line-specific responses. Effectively, when significant phenotype\*line interactions were found for the myosin isoforms (MHC IIa; MLC 1f; MLC 1s), the impact of the mini phenotype was always stronger in L3 than L6. For the plantaris, the impact of the mini phenotype on the enzymatic profile was similar for L3 and L6, except that the activity of the mitochondrial regulatory enzyme, PDH, was only higher in mini than normal muscles in L3. Line-specific responses were also apparent in several histological and ultrastructural characteristics. The mini phenotype only increased mitochondrial volume density in plantaris muscle fibres in L3. Small muscle fibres, including the mini cells, were more abundant in mini than normal muscles in L3 but not in L6. Although the mass-specific aerobic capacity of hind-limb muscles was consistently increased by the mini phenotype, the genetic background of L3 was associated with more extensive cellular modifications during the expression of the mini phenotype. The different dynamics of the mini phenotype in the two selected lines show that its expression, and possibly its adaptive

value, varies with genetic background. Demonstration of the mechanisms underlying multiple solutions to the physiological challenge imposed by the selection regime, such as presented by the mini phenotype in these two selected lines, is of central importance for evolutionary physiology (e.g., see Garland and Carter, 1994; Garland, 2003; Alfaro et al., 2004).

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