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Altered fibre types in gastrocnemius muscle of high wheel-running selected mice with mini-muscle phenotypes

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Abstract

Selective breeding of mice for high voluntary wheel running has favoured characteristics that facilitate sustained, aerobically supported activity, including a "mini-muscle" phenotype with markedly reduced hind limb muscle mass, increased mass-specific activities of oxidative enzymes, decreased % myosin heavy chain IIb, and, in the medial gastrocnemius, reduced twitch speed, reduced mass-specific isotonic power, and increased fatigue resistance. To evaluate whether selection has altered fibre type expression in mice with either "mini" or normal muscle phenotypes, we examined fibre types of red and white gastrocnemius. In both the medial and lateral gastrocnemius, the mini-phenotype increased activities of oxidative enzymes and decreased activities of glycolytic enzymes. In red muscle samples, the mini-phenotype markedly changed fibre types, with the % type I and type IIA fibres and the surface area of type IIA fibres increasing; in addition, mice from selected lines in general had an increased % type IIA fibres and larger type I fibres as compared with mice from control lines. White muscle samples from mini-mice showed dramatic structural alterations, with an atypical distribution of extremely small, unidentifiable fibres surrounded by larger, more oxidative fibres than normally present in white muscle. The increased proportion of oxidative fibres and these atypical small fibres together may explain the reduced mass and increased mitochondrial enzyme activities in mini-muscles. These and previous results demonstrate that extension of selective breeding beyond the time when the response of the selected trait (i.e. distance run) has levelled off can still modify the mechanistic underpinnings of this behaviour.

Keywords: Aerobic capacity; Artificial selection; Exercise physiology; Experimental evolution; Muscle metabolic capacities

1. Introduction

Selective breeding involves deliberately choosing individuals with a given trait (or combinations of traits) to produce the next generation. This approach allows the identification of aspects of behaviour, performance, morphology, and physiology that are genetically correlated with the trait under selection (Garland, 2003; Garland and Kelly, 2006). If selection is imposed at the level of behaviour or whole-organism performance, then the mechanistic underpinnings of evolutionary changes in that trait can be elucidated by examination of subordinate traits hypothesised to affect (or permit variation in) the higher-level phenotype (Swallow et al., 2005). As an example, beginning from a base population of outbred laboratory house mice, replicated selective breeding for high voluntary locomotor activity in wheels has resulted in greater running performance and maximal oxygen consumption (VO_{2max}) (Rezende et al., 2006a,b; Swallow et al., 1998a,b), smaller body mass (Swallow et al., 1999), increased insulin-stimulated glucose uptake in extensor digitorum longus muscle (Dumke et al., 2001), more symmetrical hind limb bones and

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larger femoral condyles (Garland and Freeman, 2005; Kelly et al., 2006), and greater muscle aerobic capacity in mice that are allowed access to wheels for several weeks (Houle-Leroy et al., 2000). Thus, the four replicate selected lines show several characteristics that appear to favour high levels of sustained, aerobically supported wheel running.

Selection for increased wheel running has also increased the frequency of a peculiar phenotype primarily affecting hind limb muscles in two of the four selected lines (Garland et al., 2002). This phenotype is characterised by a reduction of muscle mass (~50%), accompanied by increased mass-specific oxidative enzyme activities in mixed hind limb muscles (Houle-Leroy et al., 2003). In one of the selected lines, this phenotype has gone to fixation (all individuals now exhibit the phenotype), whereas in another it is still polymorphic (Rezende et al., 2006a,b; Syme et al., 2005). The phenotype is caused by an autosomal recessive allele that was present in the starting population at a frequency of approximately 7% (Garland et al., 2002). That this mini-phenotype only occurs in two of the four selected lines probably reflects the loss of this rare allele by random genetic drift during the early generations of selective breeding (Garland et al., 2002). Differences in the response of replicate lines to selection occur because random genetic processes will alter the gene pool within each line while the adaptive responses to selection are occurring. Although the distance run per day by mice with the mini-phenotype 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), mice with the mini-phenotype often run faster (Kelly et al., 2006; Syme et al., 2005) and have a higher VO_{2max} during forced exercise tests in hypoxia (14% O_2) (Rezende et al., 2006a) as compared with selected-line mice with muscles of normal size. Thus, the mini-phenotype seems to be an alternate, but not necessarily superior, phenotype with respect to the artificial selection protocol.

Hind limb muscles from mice with the mini-phenotype differ in their contractile, metabolic, and structural properties from those in mice with muscles of normal size. The reduction in mass with the mini-phenotype is greatest in muscles that normally contain a high proportion of glycolytic fast-twitch type IIB fibres in the normal phenotype (Guderley et al., 2006), with a greater mass reduction in the gastrocnemius than in the plantaris and an increased mass in the soleus. At the same time, the increase in mass-specific activities of mitochondrial enzymes with the mini-phenotype (Houle-Leroy et al., 2003) is greater in the gastrocnemius than the plantaris (Guderley et al., 2006). Gastrocnemius muscles of individuals with the mini-phenotype also exhibit increased glycogen concentrations as compared with those found in the normal phenotype (Gomes et al., 2004). For the plantaris muscle, fibre number and size do not systematically differ between individuals with the two phenotypes in a given line, but plantaris muscles from individuals with the mini-phenotype show many small, minimally differentiated myofibril-containing cells in their surface layers (Guderley et al., 2006). Despite its increased mass, the contractile properties of the soleus muscle did not differ between the normal and mini-phenotypes (Syme et al., 2005). However, the medial gastrocnemius muscle of mice with the mini-phenotype has slower twitches, a more curved force–velocity relationship, produces about half the mass-specific isotonic power, 20–50% of the mass-specific cyclic work and power, and fatigues at about half the rate of normal muscles (i.e. has greater endurance) (Syme et al., 2005). In the gastrocnemius, the % myosin heavy chain (MHC) IIb isoform was markedly reduced in mice with the mini-phenotype, whereas the proportions of MHC IIa and I isoforms were enhanced (Guderley et al., 2006). Together these results suggest that mice with the mini-phenotype have decreased the proportion of type IIB fibres in their hind limb muscles, leading muscles that typically have a high proportion of these fibres to reduce their mass, increase their mass-specific aerobic capacity, reduce their power production, and increase their endurance. The current study examines this hypothesis, by comparing the fibre types of the gastrocnemius muscles from mice that express the mini- and the normal phenotypes.

Specific genetic backgrounds can modulate the expression of particular genes, such as the allele that causes the mini-muscle phenotype. For example, the expression of the mini-phenotype differs somewhat between lines, with the decrease in % MHC IIb, the increase in % MHC IIa, and the increase in mitochondrial volume density being greater in one than the other (Guderley et al., 2006). In analogy, the "normal" phenotype in selected lines may demonstrate specific fibre type combinations that favour high levels of wheel running. In mammals, muscle fibres range from small oxidative, slow-twitch, type I red fibres to oxidative IIA fibres and large glycolytic, fast-twitch, type IIB white fibres (Torgan and Daniels, 2001). In rodents, type IIA fibres are the most oxidative and also the smallest, followed by type I (Azpiazu et al., 2000). The type IID (also known as IIX) fibres, assumed to exist only in small mammals, are intermediate between types IIA and IIB (Hamalainen and Pette, 1993). With this range of fibre types, multiple specific combinations could, in principle, enhance sustained wheel-running ability. Although the fibre types of medial gastrocnemius muscle were not statistically changed by 10 generations of selection for high voluntary wheel running (Zhan et al., 1999), the subsequent 25 generations of selective breeding could have differentiated the lines.

To evaluate whether mice with the mini-muscle phenotype have decreased the proportion of type IIB fibres and to assess whether the selection protocol has led to line-specific responses in fibre composition, we examined the medial (MG) and lateral (LG) sections of the gastrocnemius in mice after 35–36 generations of selection for voluntary wheel running. In rodents, both the MG and LG have mixed fibre compositions and possess "red" and "white" sections. The LG is somewhat richer in type IIB fibres than the MG (Burkholder et al., 1994), suggesting that metabolic and fibre type changes with the mini-phenotype should be more pronounced in the LG. We first examined the metabolic capacities of these portions to confirm the increased oxidative capacity that accompanied the mini-phenotype in previous generations (Guderley et al., 2006; Houle-Leroy et al., 2003). Further, we examined the fibre types in samples of red and white muscles from the gastrocnemius to assess whether the two types of muscles showed similar changes with selection and the mini-phenotype. We used the

MG for samples of red muscle and the LG for samples of white muscle, given that this allowed us to obtain relatively pure samples of these muscle types. Two histochemical techniques were used to determine fibre types (proportion and size). Identification of fibre types I and II was based on the pH lability of myosin ATPase (mATPase) and the subclasses of type II fibres were distinguished with NADH-tetrazolium reductase (NADH-Tr). The latter method reveals fibre oxidative capacity (IIA > I > IIB + D), the darkest fibres being the most oxidative. We evaluated muscle metabolic capacities using the glycolytic and mitochondrial enzymes that revealed the influence of the mini-phenotype in previous generations (Guderley et al., 2006; Houle-Leroy et al., 2003). The muscle phenotype can be altered by neural activity, hormonal profiles, and training levels (Pette, 2001). To limit such multiple influences, we focused on adult female mice that were housed without wheel access.

2. Materials and methods

2.1. The selection experiment and animals sampled

Swallow et al. (1998a,b) provide full details of the experiment, which involves four lines of mice (*Mus domesticus*) selectively bred for high voluntary wheel running and four additional lines maintained as non-selected controls. Mice used for our metabolic, morphometric, and ultrastructural determinations were females aged between 193 and 203 days (mean = 200 days) and sampled from generations 35 and 36. Mice were weaned at 21 days of age and housed (without wheel access) with three same-sex siblings per standard clear plastic cage, other than during routine wheel testing for 6 days at 5–7 weeks of age (see Swallow et al., 1998a). All the lines established in the selective breeding experiment were studied: lines designated 3, 6, 7, and 8 were selected for high levels of wheel running, whereas lines 1, 2, 4, and 5 were bred randomly with respect to wheel running and thus serve as controls for the effects of selective breeding. In general, the control lines should be similar to the initial base population, although random genetic drift or possibly adaptation to slightly different husbandry conditions may cause them to deviate. We examined 6 or 7 mice from each line, except for line 6. As mice in line 6 express both the mini- and the normal phenotypes, we sampled 6 individuals of each phenotype.

2.2. Muscle dissection

Mice were killed by cervical dislocation to avoid effects of pharmaceuticals. Within about 10 min of death, muscles in the right hind limb were prepared for histochemical study. A portion of red muscle in the medial gastrocnemius and one of white muscle in the lateral gastrocnemius were dissected, cut into longitudinal strips, small pieces mounted on cork board, frozen in isopentane (−160 °C) cooled on liquid nitrogen, and stored at −80 °C. All muscles of the left hind limb were weighed, the two sections of the gastrocnemius were separated according to their anatomical position and colour, frozen in liquid nitrogen, and placed at −80 °C for enzymatic and protein content determinations. The muscle samples were transported in dry ice to Université Laval for measurements.

2.3. Enzyme extraction and assay

We measured the maximal activities of the glycolytic enzymes: hexokinase (HK), glycogen phosphorylase (GP), phosphofructokinse (PFK) and lactate dehydrogenase (LDH) as well as those of the mitochondrial enzymes: cytochrome *C* oxidase (CCO) and citrate synthase (CS) following Houle-Leroy et al. (2000). Enzyme activities were measured using a UV/Vis spectrophotometer (Beckman DU 640) equipped with a temperature-controlled cell holder and circulating refrigerated water bath (Haake G8). All enzymatic assays were carried out at 37 °C using saturating concentrations of substrates and cofactors. CS and CPT activities were measured at 412 nm to detect the transfer of sulfhydryl groups to 5,5′-dithiobis 2-nitrobenzoic acid (DTNB). CCO activity was measured at 550 nm to follow the oxidation of reduced cytochrome *C*. PFK, LDH, HK, and GP activities were measured at 340 nm by following use or production of NADH or NADPH. The extinction coefficients for NAD(P)H, DTNB and cytochrome *C* were 6.22, 13.6 and 19.1 mL cm^{-1} μmol^{-1}, respectively. For all enzymes, reaction rates were linear for ≥4 min. Specific activities were expressed in international units (μmol substrate transformed to product/min) per gram of tissue wet mass.

The frozen gastrocnemius samples were ground into powder in liquid nitrogen and an aliquot was homogenised in a polypropylene vial (Wheaton Cryule cryogenic vial) with a glass grinder. We used a 1:10 (w/v) dilution in 100 mM K_2HPO_4/KH_2PO_4, 5 mM EDTA, 0.1 mM fructose-2,6-bisphosphate, 0.1 % Triton X-100, and 1 mM dithiothreitol, pH 7.2.

2.4. Histochemical analysis

Serial cross sections (8 μm) of isopentane-frozen muscle were cut with a microtome at −20 °C and mounted on coverslips for successive staining of myosin ATPase (mATPase) and NADH-tetrazolium reductase (NADH-Tr). Stained serial sections were observed under a light microscope (Leitz Dialux 20), which was connected to a CCD camera (Sony C-350). Photographed images were analysed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

For mATPase staining, sections were incubated for 55 min at 37 °C in 0.04 M sodium Barbital, 0.2 M $CaCl_2$, 0.1 M $MgCl_2$, 0.1 M NaN_3, 12.1 mM ATP, 20.6% ethanol, adjusted with 0.1 N NaOH to pH 9.4. Three successive 3 min incubations in 1 % $CaCl_2$, were followed by 3 min in 2% $CoCl_2$, then by washing with H_2O. Sections were dipped in $(NH_4)S$ and washed in tap water for 10 min. Sections were then dehydrated in ethanol, cleared in xylene, and embedded in Cytoseal mounting medium.

For NADH-Tr staining, sections were incubated for 35 min at 37 °C in 0.2 M Tris–HCl (pH 7.4), 1.5 mM NADH, and 1.5 mM nitrotetrazolium blue and then dehydrated through

serial dilutions of acetone and mounted in Cytoseal mounting medium. Based on the mATPase staining profile, fibres were classified as type I or II. These fibre types correspond to the categories slow and fast, or types 1 and 2 (Guderley et al., 2006; Zhan et al., 1999). NADH-Tr staining was used to distinguish type IIA from IIB+D and to determine average surface area of each fibre type (~25 fibres or more, if possible). Surface area was measured using NIH image. Types IIB and IID (also known as IIX) fibres were pooled in one category (IIB+D) since they could not be separated using these methods, showing similar size and colouration. For nuclear and general cell staining, Harris modified hematoxylin (Fisher SH30) and alcoholic eosin yellowish solution (Fisher SE22) were used as per the manufacturer's instructions.

2.5. Statistical analysis

This study had two aims. First, we sought to examine whether muscle metabolic capacities (enzyme activities) differed between mini- (line 3 and mini-individuals from line 6) and normal-sized muscles. Second, we sought to ascertain whether white and red gastrocnemius muscle samples differed in their fibre types between the selected and control lines. Our statistical analysis addressed these questions by simultaneously testing the effects of line type (selected vs. control) and the "mini" factor upon the various characteristics (enzyme activities and fibre type composition). Analyses were made using SAS PROC MIXED (SAS Institute, Cary, NC, USA) and a two-way mixed-model nested ANCOVA. Lines ($N=8$) were a random effect nested within line type (control or selected); an additional factor was the mini-phenotype (without or with); and body mass was used as a covariate. No other covariate was used as the mice were all sedentary females with approximately the same age at sacrifice. As in our previous studies of these lines of mice, degrees of freedom for testing the line type effect were always 1 and 6. Statistical significance was judged at $p<0.05$. As residuals of some of the traits in the ANCOVA models were not normally distributed, even with such transforms as square root or log, a rank transformation was applied to the data before the analysis (e.g. see Klomberg et al., 2002). In tables and figures, data are shown as raw (untransformed) means±standard errors, but the statistical results were obtained with the rank-transformed data.

3. Results

3.1. Body and muscle masses

As in previous generations in the selection experiment, the body mass of mice from the selected lines was reduced compared to that of mice from control lines (Dumke et al., 2001; Koteja et al., 1999; Malisch et al., 2007; Rezende et al., 2006a, b; Swallow et al., 1998a; 1999). This reduction was not attributable to the mini-phenotype (Table 1). As expected, mice with the mini-phenotype had medial (MG) and lateral gastrocnemius (LG) muscles that were roughly half the mass of those in mice with normally-sized muscles (Table 1). During dissection of the mini-mice, we observed that the white portions of the gastrocnemius muscles were proportionally more reduced than the red portions.

In addition to the effect of the mini-phenotype, the mass of the MG was lower ($p=0.0403$) in mice from the selected lines than in mice from control lines (Table 1). This effect of line type was not apparent for the lateral gastrocnemius. Mice from lines 3 and 6 with the "mini-muscle" phenotype did not show the positive correlation between body mass and gastrocnemius mass shown by the other mice, particularly for the MG (Fig. 1). For MG, the interaction between body mass and line type was highly significant ($p=0.0055$), but this was not the case for LG ($p=0.3223$) (Table 1). Within line 6, the MG of mice with the mini-phenotype weighed 41% of that of line-mates with normal-sized muscles, whereas for the LG this value was 50%.

3.2. Enzyme activities

Enzyme activities (U/g) in the MG were affected by the mini-factor, but not by line type (i.e. control vs. selected lines) (Table 2). The same statistical results were obtained with and without body mass as a cofactor. As expected, differences among lines were especially strong for mitochondrial enzymes (CPT, CS, and CCO) that were all higher in muscles from individuals with the mini-phenotype. LDH activities were also

Table 1
Impact of selective breeding for high voluntary wheel running on body mass (g) and mass of the medial (MG) and lateral gastrocnemius (LG) muscles

Control lines					Selected lines				
					Mini-phenotype		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	35.3±1.7	39.4±1.5	35.8±1.1	32.1±2.4	33.7±1.0	33.9±1.4	30.2±1.5	29.8±1.0
MG, mg	83.9±4.0	75.4±4.0	79.5±2.7	73.8±4.1	27.8±1.3	29.1±2.3	70.2±3.7	62.9±2.3	62.9±3.8
LG, mg	53.2±2.9	52.7±1.6	54.0±2.0	45.4±3.2	20.8±2.4	25.6±2.0	51.2±1.8	40.9±3.1	46.1±1.8

Values are simple means±SE, n = number of mice (56 total). For body mass, line type had a significant impact ($p=0.0085$) whereas the mini-factor did not ($p=0.5139$). For MG mass, body mass $p=0.002$, line type $p=0.0403$, and mini $p<0.0001$. If the body mass∗mini interaction was added to the model, it was highly significant ($p=0.0055$), with body mass $p=0.0099$, line type $p=0.1449$, and mini $p=0.6445$. For LG, body mass $p=0.0034$, line type $p=0.5462$, and mini $p<0.0001$. If the body mass*mini interaction was added to the model, it was highly not significant ($p=0.3223$), with body mass $p=0.0159$, line type $p=0.7152$, and mini $p=0.4132$.

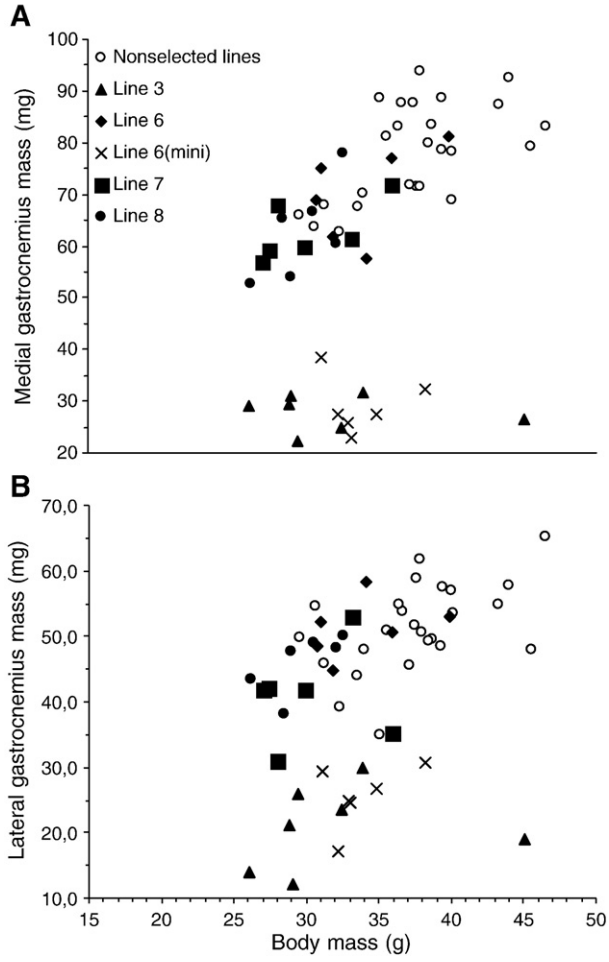

Fig. 1. Relationship between body mass and muscle mass for the medial (A) and lateral (B) portions of the gastrocnemius muscle for 56 mice. See Table 1 and text for statistical analyses.

modified by the mini-factor, being reduced in muscles from individuals with the mini-phenotype. In line 6, CPT, CS, and CCO activities in the MG were, on average, 88, 71 and 165% higher in the mini- than in the normal phenotype, whereas LDH activities were only 66% of those in normal muscles.

Enzyme activities in the LG were also strongly affected by the mini-phenotype, with significant effects for CPT, CS, CCO, and PFK both for models with and without body mass as a covariable (Table 3). When body mass was a covariable, body mass affected GPtot, and the mini-factor decreased LDH activity ($p=0.045$). When body mass was removed from the model, line type affected only CS activity and the mini-factor no longer affected LDH activity ($p=0.0503$) (Table 3). In line 6, the mini-phenotype led CPT, CS, and CCO in LG to increase, on average, by 141, 90 and 250% compared to the normal phenotype, whereas PFK and LDH activities in the mini-phenotype were only 88% and 76% of those in the normal phenotype. Thus, in line 6, the mini-factor increased mitochondrial enzyme activities more in the LG than the MG, as predicted from the typical fibre type composition of these sections of the gastrocnemius (Burkholder et al., 1994) (Tables 2 and 3).

3.3. Histochemical analysis of red gastrocnemius

We used the myosin ATPase stain to discriminate between type I and type II fibres (Fig. 2A, B), NADH-Tr reductase stain to assess fibre oxidative capacity (not shown), and hematoxylin–eosin staining for nuclei and for general resolution of cell structure (Fig. 2E, F). This allowed us to classify the fibres into type I, type IIA, and a combined category composed of types IIB+D. Both the mini-phenotype and selective breeding modified the fibre types of red muscle samples. The mini-factor increased the proportion of type I fibres in red muscle samples, both in ANCOVAs with and without body mass as a covariable (Table 4, $p=0.0406$). The mice from line 6 with the mini-phenotype showed particularly high % type I (25.7%), almost twice that (13.6%) of their line-mates with normally-sized muscles. The mini-factor also affected the proportion of type IIA fibres, again in both models with and without body

Table 2
Impact of selection for high voluntary wheel running and the mini-phenotype on enzyme activities (U/g muscle) of the medial gastrocnemius muscle

Control lines					Selected lines				
					Mini-phenotype		Normal muscles		
Line	1	2	4	5	3	6	6	7	8
n	6	6	7	6	7	6	6	6	5
CPT	0.46±0.03	0.31±0.04	0.36±0.07	0.32±0.11	0.94±0.07	0.98±0.09	0.52±0.07	0.34±0.05	0.38±0.05
CS	74±9	`63±8	60±5	72±18	140±10	151±7	88±6	71±6	51±6
CCO	20±6	11±3	10±2	9±2	35±5	45±5	17±3	10±3	12±4
PFK	149±25	119±15	84±16	142±18	79±18	134±11	143±10	107±17	113±18
HK	2.4±0.2	1.9±0.4	2.0±0.5	3.5±0.8	2.7±0.1	2.6±0.3	2.1±0.5	3.9±0.7	3.25±0.5
GPa	17±4	23±11	36±12	26±7	50±15	55±15	43±10	18±5	19±3
GPtot	172±63	104±7	129±11	125±11	139±18	113±8	148±9	109±10	106±12
LDH	1650±368	1378±220	787±123	840±167	440±44	625±40	940±44	1198±217	950±229

Values are simple means±SE, *n* = number of mice analysed. Statistical analyses used rank-transformed values. The enzymes measured were carnitine palmitoyl transferase (CPT), citrate synthase (CS), cytochrome *C* oxidase (CCO), phosphofructokinase (PFK), hexokinase (HK), glycogen phosphorylase a (GPa), total glycogen phosphorylase activity (GPtot), and lactate dehydrogenase (LDH).
ANCOVA models with and without body mass showed the same patterns: line type did not affect enzyme activities, with $F(1,6)<2.39$ and p values >0.17 for line type effects. The mini-factor was significant for CS, LDH, CCO, and CPT in both models with $F(1,45)$ values >12.65 and p values <0.0009. In none of these models did body mass significantly affect enzyme activity.

Table 3
The impact of selection for high voluntary wheel running and the mini-phenotype on enzyme activities (U/g muscle) of the lateral gastrocnemius muscle

Control lines					Selected lines				
					Mini-phenotype		Normal muscles		
Line	1	2	4	5	3	6	6	7	8
n	6	6	7	6	7	6	6	6	6
CPT	0.45±0.05	0.32±0.04	0.28±0.05	0.40±0.08	0.88±0.08	1.04±0.07	0.43±0.04	0.41±0.09	0.49±0.12
CS	73±4	57±4	67±5	68±6	118±13	139±5	73±5	75±5	74±10
CCO	13±1.5	13.3±4	14±3	18±5	38±6	49±6	14±2	19±2.6	34±7.2
PFK	96±19	98±16	131±11	109±18	49±6	85±10	97±20	121±13	114±9
HK	4.3±0.6	4.4±0.5	3.5±1.1	3.6±0.8	2.8±0.2	2.8±0.2	2.5±0.8	3.3±1.1	4.7±2.8
GPa	31±7	34±5	26±6	37±3	31±7	46±16	56±10	19±4	28±4
GPtot	168±33	126±16	127±9	115±9	131±11	142±7	146±8	107±5	94±12
LDH	950±130	1088±94	1065±118	898±85	903±165	694±40	917±77	949±86	1042±40

Values are simple means±SE, *n* = number of mice analysed. Statistical analyses used rank-transformed data. The enzymes measured were carnitine palmitoyl transferase (CPT), citrate synthase (CS), cytochrome *C* oxidase (CCO), phosphofructokinase (PFK), hexokinase (HK), glycogen phosphorylase a (GPa), total glycogen phosphorylase activity (GPtot), and lactate dehydrogenase (LDH).
ANCOVA models with and without body mass showed similar patterns: line type affected CS when body mass was removed from the model $F(1,6)=6.53$ and $p=0.0432$, increasing enzyme activity in selected lines. The mini-factor was significant for CS, CCO, PFK, and CPT in both models with and without body mass $F(1,46)$ values >9.94 and p values <0.0028. Body mass positively affected the activity of GP tot $F(1,6)=5.48$ and $p=0.0237$.

mass as a covariable (Table 4). However, the two lines with the mini-phenotype differed in the % type IIA fibres. Within line 6, individuals with the mini-phenotype had lower % type IIA than those with the normal phenotype (Table 4). Within line 3, all the mice demonstrated the mini-phenotype and resembled other selected mice, with type IIA fibres composing over half of their fibres. Mice in selected lines had a greater proportion of type IIA fibres than mice in control lines ($p=0.0405$) when body mass was absent as a covariate. With body mass in the model, this tendency was not significant ($p=0.0979$).

The size of the more oxidative fibre types was affected by both line type and the mini-factor. The mini-factor strongly and systematically increased the average surface area of type IIA fibres, both within line 6 and in comparison of mini-muscles with normally-sized muscles from the other lines (Table 4). Samples from line 3 had the largest type IIA fibres, with mean surface areas of 2454 μm^2 compared to mean surface areas around 1670 μm^2 for normally-sized muscles. In line 6, muscles from individuals with the mini-phenotype had surface areas of type IIA fibres that were, on average, 350 μm^2 larger than those of their line-mates with normal muscles (Table 4). In addition to the mini-effect, mice from selected lines had larger type I fibres than mice from control lines ($p=0.0394$ when body mass was included as a covariate). The difficulties in distinguishing types IIB and IID fibres led us to combine these categories. For this combined category, no differences due to selective breeding or to the mini-phenotype were apparent in the red gastrocnemius.

3.4. Histochemical analysis of the white gastrocnemius

Histochemical staining of white muscle samples from mice with the mini-phenotype (Fig. 2D, H) revealed a perplexing, atypical distribution of extremely small fibres of variable size and morphology. These cells were difficult to distinguish, and within some of these atypical cells variable staining intensities for myosin ATPase were observed, rendering classification impossible. For most of these small, unusual cells, hematoxylin staining showed peripheral nuclei, suggesting that they are indeed muscle cells. These fibres were impossible to count with optical microscopy and therefore were not quantified. These "mini-fibres" were surrounded by larger and more oxidative fibres than those normally found in white muscle. These findings were unprecedented, certainly unexpected, and may partly explain the impact of the mini-phenotype on the mass of the gastrocnemius.

The fibre types of the white muscle samples from normal-sized muscles (Fig. 2C, G) were not changed by selection, but some parameters were affected by the body size of the mice (Table 5). As expected in these white muscle samples, most fibres fell in the IIB+D category, and these covered over 90% of the area sampled. The surface area of the three types of fibres was affected by body mass as was the surface area occupied by type I fibres. Larger-bodied animals have larger type I and type IIA fibres whereas they have smaller type IIB+D fibres. The surface area occupied by type I fibres increased as animal size rose.

4. Discussion

Although phenotypic evolution in nature can be rapid (Hendry and Kinnison, 2001), adaptive evolution is typically accelerated during the initial generations of artificial selection protocols compared to rates in natural environments (Gingerich, 2001). Selection for voluntary running in house mice has been highly effective, leading to a threefold increase in distance run per day (Garland, 2003; Swallow et al., 1998a), while also changing such traits as body mass (Swallow et al., 1999), insulin-stimulated glucose uptake by locomotor muscles (Dumke et al., 2001), maximal aerobic capacity (Rezende et al., 2006a,b; Swallow et al., 1998b), circulating corticosterone levels (Malisch et al., 2007), and bone dimensions (Garland and Freeman, 2005; Kelly et al., 2006). In addition, two major muscle phenotypes occur in the selected lines: mice with muscles of normal size and mice with hind limb muscles approximately half this mass. In this study, we demonstrated

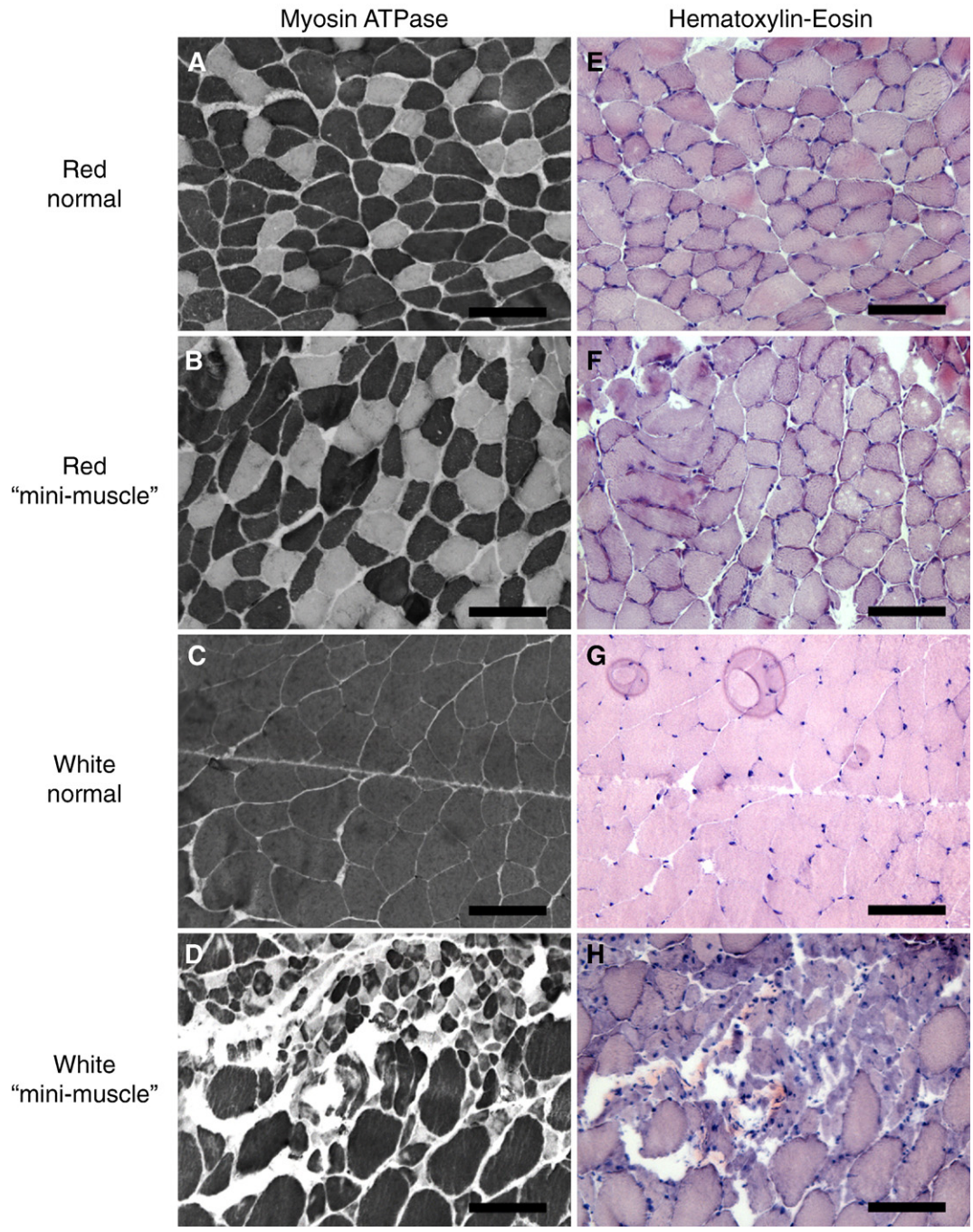

Fig. 2. Representative histochemical staining of red (MG) (A, B, E and F) and white (LG) (C, D, G and H) gastrocnemius muscle sections from mice with normal and mini-phenotypes. The proportions of oxidative type I (light coloured in A to D) and type II fibres (darker fibres in A to D) and the area of type II fibres increased significantly in the samples of red muscle from mice with the mini-phenotype, with distinct responses in lines 3 and 6 (see Table 4). A totally atypical distribution of small unidentifiable fibres was observed in white muscle samples from mice with the mini-phenotype (D, H). Bar = 100 μm.

that selection has favoured changes in the fibre type composition of red muscle samples, both through the effects of the mini-phenotype (which is caused by a Mendelian recessive allele when present in the homozygous condition) (Garland et al., 2002) and by other genetic mechanisms in selected lines without the mini-phenotype. In white muscle of the gastrocnemius, the selection protocol led to drastic structural modifications in individuals with the mini-phenotype but did not change fibre types in individuals with normal-sized muscle.

4.1. Mini-phenotype and changes in red and white muscles in gastrocnemius

Changes in fibre types seem to underlie the mini-phenotype. Red muscle samples from mice with the mini-phenotype had considerably larger type IIA fibres than those from muscles of normal size (Table 4). Further, the mini-phenotype was accompanied by an increase in the proportion of type I and type IIA fibres in red muscle (Table 4). In contrast to these

Table 4
Impact of the mini-phenotype and of selection for high voluntary wheel running on fibre type composition of portions of red muscle sampled from the medial gastrocnemius muscle

Control					Selected				
					Mini-phenotype		Normal muscles		
Line	1	2	4	5	3	6	6	7	8
n	6	5	4	5	6	4	6	5	3
Type I, %	8.1±1.4	10.8±2.6	18.0±5.3	13.0±1.7	17.9±3.6	25.7±5.4	13.6±3.5	8.0±2.2	19.1±2.9
Type IIA, %	44.7±3.7	49.9±3.4	48.8±4.8	52.2±1.3	56.7±1.4	41.4±1.7	55.4±3.9	56.4±3.3	62.3±3.0
Type IIB+D, %	47.2±3.9	39.2±5.2	33.2±5.1	34.8±1.7	25.3±2.7	32.9±4.5	31.0±7.0	35.5±4.0	18.5±5.9
Type I, mean surface area, μm^2	1902±125	1922±135	2114±206	1921±76	2624±220	2310±134	2222±98	1892±212	2421±22
Type IIA, mean surface area, μm^2	1672±109	1595±73	1763±20	1673±119	2454±105	2197±145	1857±98	1744±155	1972±95
Type IIB+D, mean surface area, μm^2	4265±422	3823±343	4194±401	4023±408	3997±172	3740±225	3774±192	4821±330	4471±297
Type I, % surface	5.6±1.1	9.0±2.4	16.0±6.2	10.4±2.0	16.9±4.7	22.7±5.9	12.8±3.8	5.6±1.8	18.8±4.0
Type IIA, % surface	26.5±3.6	32.6±4.4	32.4±2.7	34.7±1.5	48.1±1.8	32.9±1.8	42.0±6.1	35.0±3.8	48.9±5.0
Type IIB+D, % surface	67.8±4.3	58.4±6.5	51.6±6.7	54.9±2.9	35.0±4.1	44.4±5.9	45.2±9.6	59.3±4.9	32.2±8.9

Data are presented as simple means±SE, *n* = the number of mice analysed for each parameter. Statistical analyses used rank-transformed data.
ANCOVA models with and without body mass showed that the mini-factor significantly affected fibre types, increasing the surface area of type IIA fibres ($F=14.85$; $p<0.0005$) as well as the % type I and IIA fibres ($F>4.53$; $p<0.0406$). Mice from selected lines had significantly increased mean surface area of type I fibres, when body mass was included in the model ($F=6.89$, $p=0.0394$), and increased % type IIA fibres, when body mass was excluded from the model ($F=6.77$, $p=0.0405$).

moderate qualitative and quantitative modifications in the red muscle samples, dramatic changes of fibre structure occurred in white muscle samples. In mice with normally-sized muscles, white muscle samples were filled primarily with type IIB+D fibres. In mice with the mini-phenotype, a multitude of small, atypical cells was found instead of type IIB+D fibres (Fig. 2).

Reductions in type IIB+D fibres have many consequences. First, red muscle from "mini" muscles has an increased proportion of oxidative fibres that, in turn, are bigger than normal. Further, white muscle samples from "mini-muscles" are bereft of normal type IIB+D fibres and are filled by small, atypical cells. At generation 27, the two selected lines in which the mini-phenotype is expressed also had mice with muscles of normal size, allowing within-line comparisons. The "mini"-gastrocnemius had markedly lower levels of type IIB myosin heavy chains than that in normal muscles (Guderley et al., 2006), suggesting that the fibre type most affected by the mini-phenotype was type IIB. Electron microscopic comparison of the plantaris muscle in these lines revealed numerous, extremely small, myofibril-containing cells in the surface layers of the plantaris muscle from "mini" individuals (Guderley et al., 2006). While the arrangement of the unusual small cells in the plantaris differs considerably from what we demonstrated here for white gastrocnemius, we believe that these are two manifestations of a loss of type IIB fibres.

Both the medial and lateral gastrocnemius decreased in mass in the mini-phenotype, with the medial gastrocnemius losing the dependence of its size upon body mass (Fig. 1, Table 1). The increased activities of mitochondrial enzymes with the mini-factor in line 6 were more pronounced in the lateral than the

Table 5
Impact of selection for high voluntary wheel running on the fibre type composition of white muscle sampled from lateral gastrocnemius muscles

Control					Selected				
					Mini-phenotype		Normal		
Line	1	2	4	5	3	6	6	7	8
n	6	4	3	5	6	4	6	4	3
Type I, %	0.34±0.26	0	0	0.14±0.14	n/a	n/a	0	0	0
Type IIA, %	17.0±3.6	8.0±2.8	5.0±2.9	5.8±2.9	n/a	n/a	7.67±2.5	19.7±1.2	15.9±4.8
Type IIB+D, %	82.7±3.6	92±2.77	93.7±3.0	94.1±3.0	n/a	n/a	92.3±2.4	80.8±1.4	83.8±4.9
Type I, mean surface area, μm^2	1685±313			1750[a]	n/a	n/a			
Type IIA, mean surface area, μm^2	1973±46.6	1789±224.9	2147±172	1625±193	n/a	n/a	1475±152	1787±7.9	1640±311
Type IIB+D, mean surface area, μm^2	4386±292	3683±332	3695±256	3869±180	n/a	n/a	3121±178	3915±341	4061±491
Type I, % surface	0.18±0.16	0	0	0.3	n/a	n/a	0	0	0
Type IIA, % surface	8.3±1.5	4.1±1.4	3.5±1.3	2.8±1.5	n/a	n/a	3.8±1.2	10.1±0.8	7.6±2.7
Type IIB+D, % surface	91.5±3.8	95.9±1.6	96.5±1.3	97.2±1.5	n/a	n/a	96.2±1.2	90.0±1.0	92.3±2.7

Data are presented as simple means±SE, *n* = the number of mice analysed for each parameter. Statistical analyses used rank-transformed data. n/a indicates that measurements could not be made in the samples from mice with the mini-phenotype.
ANCOVA models with and without body mass showed that line type did not affect fibre types, but body mass did. Surface area of the three types of fibres was affected by body mass (positively for types I and IIA and negatively for types IIB/D) ($F>6.04$, $p<0.0223$) as was as the % type I (positively, $F=11.66$, $p=0.0025$) and the surface occupied by type I fibres (positively, $F=11.66$, $p=0.0025$).
[a] Only one individual in this line showed evidence of type I fibres.

medial gastrocnemius (compare Tables 2 and 3). As the lateral gastrocnemius contains more type IIB fibres than the medial in rodents (Hamalainen and Pette, 1993), the changes in enzyme activities are consistent with the hypothesis that the mini-phenotype is due to a loss of type IIB fibres. Our visual observation of a marked decrease in the size of the white muscle portions of the lateral and medial gastrocnemius in individuals with the mini-phenotype is telling, in light of our histochemical demonstration of a multitude of atypical small cells in white muscle samples. As both the lateral and medial gastrocnemius contain regions with red and white muscles, we assumed that the histochemical results apply to both muscles.

As for any technique, histochemical analyses have shortcomings that limit the conclusions that can be drawn. Complete histochemical characterisation of the gastrocnemius muscle, even when separated into its two sections, would require intensive sampling throughout its structure. This approach, particularly given the heterogeneity of this muscle, is incompatible with our study of a large number of individuals. Thus, we chose to characterise red and white muscle portions of the lateral and medial gastrocnemius, knowing that the information gleaned from these analyses is not equivalent to that obtained from a homogenate of the entire muscle (e.g. myosin heavy chain electrophoresis, enzyme activity measurements). It proved difficult to separate type IID from IIB fibres even with sequential sections stained by different methods. Type IID is intermediate (in size and oxidative capacity) between types IIB and IIA (Hamalainen and Pette, 1993). Therefore, we established whether types I and IIA increased with selection and the mini-phenotype, and grouped type IIB together with IID. Previous authors have also experienced difficulties in separating types IIB and IID by the myosin ATPase stain (Rivero and Serrano, 1999; Talmadge and Roy, 1993). Finally, our histochemical methods did not permit identification of fibres that express two or more myosin isoforms (Caiozzo et al., 1993). Nonetheless, our histochemical data revealed marked changes in fibre types in red and white muscle samples and support the hypothesis that the decrease in size with the mini-phenotype is due to a marked reduction of type IIB fibres.

4.2. Effects of selective breeding separate from the mini-phenotype

After 10–14 generations of selection for voluntary wheel running, enzymatic activities and fibre type composition of hind limb muscles did not significantly differ between control and selected lines unless mice were given wheel access after the selection of the future breeders (Houle-Leroy et al., 2000; Zhan et al., 1999). Only the mini-phenotype provided a clear signature of a direct effect of the selection protocol on muscle characteristics. However, additional generations of selective breeding have brought about changes in muscle structure: We found that the mean surface area of type I fibres and the % of type IIA fibres were greater in red muscle samples from selected (e.g. lines 3,6, 7 and 8) than control mice. In the lateral gastrocnemius, CS activity was significantly greater in muscles from selected mice (without body mass as a covariable). While the response of wheel running to selection levelled off after approximately 16 generations of selective breeding (Garland, 2003; Swallow et al., 1999), maintenance of selective breeding has apparently caused additional changes in fibre structure of red muscle and in the enzymatic profile of lateral gastrocnemius. Overall, selection for high levels of voluntary wheel running has favoured an increased aerobic capacity in red muscle, in both mini- and normal phenotypes, although white muscle fibre types were only changed by the mini-phenotype.

4.3. Line-specific manifestations of the mini-phenotype

The specific manifestations of the mini-phenotype in the two selected lines indicate that its expression is modified by genetic background. Earlier during the selection protocol, normal and mini-phenotypes could be compared within both lines, and these comparisons showed that the expression of the mini-phenotype differed between lines 3 and 6 (lab designations). In line 3 but not in line 6, mitochondrial volume density was greater in the plantaris of “mini-mice”. The proportions of type IIA myosin heavy chain and of myosin light chain 1 s in the gastrocnemius were increased more by the mini-phenotype in line 3 than line 6 (Guderley et al., 2006). After 35 generations of selective breeding, only line 6 contains both phenotypes, limiting the use of such within-line comparisons. Nonetheless, although the extreme muscle atrophy seen in the white muscle samples (Fig. 2) occurred in both lines, some cellular manifestations of the mini-phenotype differed between lines 3 and 6. In red muscle samples, type IIA fibres appeared larger in mini-muscles in line 3 than in line 6. The mini-phenotype favoured different types of oxidative fibres in red muscle of lines 3 and 6. In line 3, the % type IIA in red muscle samples was considerably higher than in line 6, whereas line 6 showed the highest % type I of all lines (Table 4). Type IIA fibres are the most oxidative in rodents (14), and are the smallest of the fibre types. In line 6, the mini-phenotype actually reduced the proportion of type IIA fibres in red muscle below that in the normal phenotype of selected lines. These line-specific manifestations of the mini-phenotype suggest alternative responses of fibre types to the selection protocol. Both lines increased the percentage and space used for oxidative “red” fibres, but favoured different fibre types. The mini-phenotype seems to favour skeletal muscle oxidative capacities, without specifying exactly how to increase them.

4.4. Potential mechanisms underlying the mini-phenotype

Although the actual cause of the “mini-fibres” in white muscle samples from the mini-phenotype remains unknown, other studies have shown similar patterns. For example, Reisz-Porszasz et al. (2003) observed that mice with increased myostatin expression (a negative regulator of skeletal muscle fibre growth during embryological development) have lower muscle mass. Degeneration (or apoptosis) may have occurred in the white muscle sections of the mini-muscles. Enhanced expression by mice with the mini-phenotype of the transcrip-

tional co-activator PGC 1b could enhance muscle oxidative capacities (Arany et al., 2007). The "mini-muscles" we are studying may also lack protection against free radicals (reactive oxygen species). After 14 generations of selective breeding, the antioxidant enzyme, superoxide dismutase, was significantly less active in livers of selected than control females, regardless of training history (Thomson et al., 2002). If this effect was more pronounced in type IIB fibres, then it could accentuate apoptosis. Accordingly, Anderson and Neufer (2007) have recently shown that type II muscle fibres from rats, and particularly the IIB fibres, possess properties that enhance mitochondrial peroxide production. Finally, the unusual cells we observed in white gastrocnemius of mice with the mini-phenotype are reminiscent of atrophied cells found in some forms of spinal muscle atrophy (e.g. Soubrouillard et al., 1995). This suggests that mice with the mini-phenotype might suffer some damage or loss of function at the level of specific motor neurons. These hypotheses remain to be tested.

Our results show that the mini-phenotype is associated with an increased proportion of oxidative fibres (either type I or type IIA) in red muscle samples and an abundance of atypical "mini-fibres" in white muscle samples. The reduced mass and high levels of mitochondrial enzymes in "mini-muscles" can be explained by these changes. Although the altered white muscle structure and its atrophy have not been quantified, these observations show that the "mini" allele not only changes fibre type expression but also causes an atypical cellular distribution in white muscle. That the oxidative fibre type enhanced varied between the two lines expressing the mini-phenotype shows that the specific manifestation of the phenotype depends upon the genetic environment. Our results also demonstrate that extension of the selection protocol (which includes an unavoidable component of random genetic drift) beyond the time (approximately generation 16) when the criterion upon which selection is based (i.e. distance run) has apparently stopped responding to selection (Garland, 2003) may still modify the mechanistic underpinnings of the selected behaviour.

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