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# Changes in efficiency and myosin expression in the small-muscle phenotype of mice selectively bred for high voluntary running activity

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## SUMMARY

**Mice from lines selectively bred for high levels of voluntary wheel running express a high incidence of a small muscle phenotype (‘mini-muscles’) that may confer an adaptive advantage with respect to endurance-running capacity. Plantar flexors in the mini-muscle phenotype exhibit a high capacity for aerobic activity, including altered enzyme activities, loss of expression of type $II_b$ myosin heavy chain (MHC), increased expression of type I, $II_x$ and $II_a$ MHC, and mechanical performance consistent with slower, more fatigue-resistant muscles. We hypothesized that these changes may accompany enhanced efficiency of contraction, perhaps in support of the enhanced capacity for endurance running. To assess efficiency, we measured work and associated oxygen consumption from isolated soleus and medial gastrocnemius muscles from mice with mini-muscle and normal phenotypes. We also measured the MHC expression of the plantar flexor muscles to better understand the physiological basis of any differences in efficiency. The proportion of the various MHC isoforms in the soleus was shifted toward a slightly faster phenotype in the mini-muscle mice, whereas in the gastrocnemius and plantaris it was shifted toward a markedly slower phenotype, with large reductions in type $II_b$ MHC and large increases in type I, $II_a$, and $II_x$ MHC. Soleus muscles from normal and mini-muscle mice showed no statistical differences in efficiency, but medial gastrocnemius from mini-muscle mice were significantly less efficient than those from normal mice, despite the distinctly slower MHC phenotype in mini-muscle mice. Thus, based on measures of efficiency from isolated muscles under conditions near optimal for power output, the shift toward a slower phenotype in ‘mini’ gastrocnemius muscles does not appear to confer advantages directly through increased efficiency. Rather, the slower phenotype may reduce energy used by the muscles and be permissive to enhanced running ability, perhaps by reducing reliance on anaerobic metabolism.**

Key words: artificial selection, efficiency, energetics, experimental evolution, multiple solutions, muscle, myosin heavy chain, oxygen, work.

## INTRODUCTION

Beginning in 1993 with a sample of 224 outbred Hsd:ICR house mice (*Mus domesticus*), selective breeding for high voluntary activity, measured by distance travelled on exercise wheels, was implemented (Swallow et al., 1998). In total, this selection experiment is composed of four replicate control lines (lab designated lines 1,2,4,5), which are bred without regard to wheel running, and four replicate selected lines (lab designated lines 3,6,7,8), which are subjected to the selection protocol. Over the first 16 generations of such breeding, mice in the selected lines ran increasingly more revolutions per day than controls, eventually reaching an apparent plateau at which they run 2.5- to 3-fold more revolutions per day at nearly twice the average speed of control mice (e.g. Swallow et al., 1999; Girard et al., 2001; Garland, 2003; Belter et al., 2004; Rezende et al., 2006c; Gomes et al., 2009).

Of particular interest to the present study, two of the selected lines (lab designations 3 and 6) express a high incidence of a ‘mini-muscle’ phenotype (Garland et al., 2002), in which the plantar flexor muscle group is 44–50% the mass of mice with normal (wild-type) muscles (Garland et al., 2002; Houle-Leroy et al., 2003; Belter et al., 2004; Syme et al., 2005; Guderley et al., 2006). The mini-muscle phenotype results from a Mendelian recessive allele (Hannon et al., 2008) that occurred at a frequency of ~7% in the founder population (Garland et al., 2002). The dramatic increase in frequency of the mini-muscle phenotype only in two of the selected lines, leading to fixation in line 3 by generation 36 (Syme et al., 2005), is best explained by a statistical model in which the phenotype was favoured by the selection protocol (Garland et al., 2002). That the phenotype was lost in the other two selected lines (7 and 8) and in all four control lines is explainable by random genetic drift (Garland et al., 2002). Efforts to identify the mini-muscle gene are underway (Hartmann et al., 2008).

Aside from the mini-muscle phenotype, which is restricted to two of the four selected lines, all four selected lines exhibit a number of phenotypes that appear to represent adaptations to support sustained, aerobically maintained endurance running. These phenotypes include elevated maximal oxygen consumption (Rezende et al., 2006a; Rezende et al., 2006b; Rezende et al., 2006c) and running capacity (Meek et al., 2007) during forced treadmill exercise, increased insulin-stimulated glucose uptake in extensor digitorum longus (Dumke et al., 2001), and larger femoral heads and increased symmetry of hindlimb bones (Garland and Freeman, 2005; Kelly et al., 2006). A number of other traits that evolved in the selected lines may or may not represent adaptations that facilitate increased endurance running, including reduced body mass (Swallow et al., 1999), elevated circulating corticosterone levels (Girard and Garland, 2002; Malisch et al., 2009), and elevated

circulating adiponectin levels [Vaanholt et al. (Vaanholt et al., 2008) and references therein)].

The mini-muscle phenotype has been associated with characteristics other than just changes in muscle mass. Some reports indicate that mice possessing the mini-muscle phenotype have reduced body mass (Garland et al., 2002; Guderley et al., 2006; Guderley et al., 2008), while others do not (Houle-Leroy et al., 2003; Guderley et al., 2008). Mice that possess mini-muscles generally do not differ from those with normal muscles in time spent on exercise wheels, although they tend to run significantly faster and, in some samples, further (Garland et al., 2002; Kelly et al., 2006; Gomes et al., 2009). Mice with mini-muscles exhibit larger heart ventricles (Garland et al., 2002; Swallow et al., 2005) and higher $\dot{V}_{O_2}$ max (maximum rates of oxygen uptake) in hypoxia (Rezende et al., 2006a; Rezende et al., 2006b), and their triceps surae muscles have greater mitochondrial volume densities and higher mass-specific hexokinase and cytochrome C oxidase activities (Guderley et al., 2006) (see also Houle-Leroy et al., 2003). Also, their medial gastrocnemius muscles contain nearly twice the mass-specific myoglobin concentration and mass-specific citrate synthase activity as normal muscles (Rezende et al., 2006b), and their gastrocnemius muscles have greatly reduced amounts of myosin heavy chain type $II_b$ and a large number of very small, unidentifiable muscle fibres (Guderley et al., 2006; Guderley et al., 2008) (L. E. Wong, T.G. and R. T. Hepple, manuscript submitted).

Analysis of contractile characteristics reveals no differences between soleus muscles in normal and mini-muscle mice, but a shift toward a markedly slower and more fatigue-resistant medial gastrocnemius in the mini-muscle phenotype, with slower twitches, a 50–80% reduction in mass-specific cyclic power production, and a 50% reduction in mass-specific isotonic power production (Syme et al., 2005). Collectively, these findings indicate a shift toward a slower phenotype in some limb muscles. As mice with these mini-muscles may be better suited to sustained, aerobic activity than their normal counterparts, this constitutes further evidence that the increased incidence of the mini-muscle phenotype in the selected lines is an adaptive response to the selection regimen (see also Garland et al., 2002; Gomes et al., 2009).

Slower, more aerobic muscles are commonly held to be more efficient at producing mechanical work than faster, glycolytic muscles (e.g. Crow and Kushmerick, 1982; Curtin and Woledge, 1993; Barclay, 1994; Barclay, 1996). Therefore, we hypothesized that the increase in oxidative capacity and shift to a slower, more fatigue-resistant phenotype in medial gastrocnemius muscles of mini-muscle mice would be associated with an increase in efficiency of contraction compared with the normal medial gastrocnemius. The soleus, showing no notable difference in contractile properties between mini-muscle and normal mice (Syme et al., 2005), is conversely not expected to exhibit a change in fibre type or contraction efficiency. Such changes, or lack thereof, in these muscles will have important consequences for both running ability and economy of movement, as these plantar flexors are key producers of power for both slow and fast locomotion (Walmsley et al., 1978; Gregor et al., 1988; Prilutsky et al., 1996).

We measured the efficiency of performing cyclic work in soleus and medial gastrocnemius muscles from normal mice (selected lines 7 and 8) and from mini-muscle mice (selected line 3). In addition, we measured the proportion of myosin heavy chain isoforms in the plantar flexor muscles (soleus, medial and lateral gastrocnemius, plantaris) using SDS-PAGE, allowing comparison of potential changes in efficiency with changes in myosin expression. The masses of the ventricles and several limb muscles were also measured to asses differences between the selected lines and relationships with efficiency and myosin expression.

## MATERIALS AND METHODS

All procedures were approved and followed animal care guidelines of the Canadian Council on Animal Care and the University of Calgary. Experiments were conducted on muscles of adult, female mice (*Mus musculus* Linnaeus) from generation 46 of the above-mentioned selection experiment. Ten mice from each of lab designated lines 3 (fixed for the mini-muscle phenotype) and lines 7 and 8 (lacking the mini-muscle phenotype) were studied, all three lines being selected for high levels of running activity. Henceforth, mini-muscle mice from line 3 will be referred to as M3, and normal mice from lines 7 and 8 will be referred to as N7 and N8, respectively. For efficiency measurements, $N$=6 (M3), $N$=9 (N7), and $N$=10 (N8) were successful for the medial gastrocnemius, whereas $N$=9 (M3 and N7) and $N$=10 (N8) were successful for the soleus.

### Muscle preparation and apparatus

Mice were weighed and then killed by $CO_2$ inhalation with subsequent cervical dislocation. The left hindlimb was removed and placed in a dish containing chilled, physiological saline [in mmol $l^{-1}$: 144 NaCl, 10 glucose, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 $NaH_2PO_4$, 1 $MgSO_4$, 10 Hepes, pH 7.4 at 20°C using Tris base: based on Daut and Elzinga (Daut and Elzinga, 1989)]. Both the soleus and the medial head of the gastrocnemius were isolated intact with tendon on either end. The soleus of all mice and the medial gastrocnemius of M3 mice were relatively small and used intact. The medial gastrocnemius of N7 and N8 mice were relatively large (normal sized) and so were dissected to approximately one half of their original width and then muscle fibres from the proximal end were cleared from the tendon to produce preparations comparable in mass to those from M3 (see Results). Great care was taken to minimize damage to the remaining muscle bundle, and to ensure the location of the segment of muscle that remained was consistent across preparations. Further efforts to assess consistency are described in the section discussing myosin heavy chain sampling.

Muscles were secured by their tendons in a glass chamber for measurement of work and oxygen consumption; see Trinh and Syme (Trinh and Syme, 2007) for details of the measuring apparatus and calculation of energy use from oxygen consumed by the muscles. One end of the muscle was secured to a rigid pin and the other end to an ergometer (model 350, Cambridge Technology, Cambridge, MA, USA), which controlled muscle length and measured force production. The length of the muscle was adjusted to remove visible slack. A bipolar stimulus pulse, 1 ms duration, was applied to the muscle *via* the attachment pins, and the stimulus voltage was set to 150% of that required to elicit maximal twitch force. Muscle fibre length was then varied systematically until the length giving maximal, isometric force (using double stimulus pulses, 10 ms spacing) was found. This length was measured using a calibrated ocular micrometer on a stereomicroscope. The temperature of the chamber was maintained at 20°C.

### Measurement of work and oxygen consumption

Different measurement protocols were employed for the soleus and medial gastrocnemius muscles, each designed to result in the muscles producing near maximal power and consuming oxygen in support of aerobic work without causing fatigue. Work and power output were assessed using the work-loop technique (Josephson, 1985). For the soleus, muscle length was cycled in a sinusoidal

trajectory at a frequency of 2 Hz. For the medial gastrocnemius, muscle length was cycled at a frequency of 4 Hz. The amplitude of muscle strain was 10% peak-to-peak. These frequencies and strains were selected to approximately maximize power output from the muscles at 20°C based on preliminary experiments and predictions from previous studies on mouse muscles at 35°C (James et al., 1995) and 27°C (Syme et al., 2005). The muscles were stimulated phasically during the length cycles with a stimulus pulse frequency of 100 Hz using stimulus durations and phases that maximized net work production for each preparation: typically 140 ms duration (28% duty cycle) and 15% phase for the soleus, and 70–90 ms duration (32% duty cycle) and 10% phase for the medial gastrocnemius.

To assess efficiency of work production, the muscles were made to work under the conditions described above while their oxygen consumption was simultaneously measured. Before measurements began, the muscle chamber was flushed with saline with a partial pressure of oxygen ($P_{O_2}$) of about 70 kPa. The $P_{O_2}$ of the saline in the chamber then declined gradually over the course of the experiment as the muscle consumed oxygen, typically ending near 35 kPa. The $P_{O_2}$ of the saline bathing the muscle was measured every second (PSt3 fibre-optic oxygen probe connected to a Fibox 3 oxygen metre, PreSens Precision Sensing GmbH, Regensburg, Germany) and logged to a computer.

At the beginning of each experiment, the decline in $P_{O_2}$ of the saline due to resting muscle metabolism was measured for about 20 min to obtain a reliable baseline. The muscle was then activated to perform work, causing an increase in the rate of decline of $P_{O_2}$ in the chamber. For the medial gastrocnemius of N7 and N8 mice, the muscle was subjected to three cycles of work in sequence followed by a 90 s rest; this was repeated 10 times for a total of 30 work cycles. For the soleus muscles of all lines and the medial gastrocnemius of M3 mice, the muscle was subjected to three cycles of work in sequence followed by a 60 s rest; this was repeated 20 times for a total of 60 work cycles. These protocols were devised to ensure deflections of the oxygen trace that could be resolved reliably, yet not result in fatigue. After the bouts of work were completed and the muscle was again resting, the rate of decline of $P_{O_2}$ recovered back to the baseline level over a period of approximately 10 min for the soleus muscle and about 20 min for the medial gastrocnemius, and was measured for an additional 10–20 min to ensure a resting rate had been re-attained.

The change in $P_{O_2}$ in the saline as a result of the muscle being active and performing work was then calculated from the oxygen records, and converted to joule equivalents of energy released by oxidizeable substrates in a mixed diet, assuming 450 kJ mol$^{-1}$ $O_2$ consumed [see Syme and Trinh (Syme and Trinh, 2007) for details and rationale]. Muscle efficiency was then calculated by dividing the net work done during the entire series of contractions by the energy released *via* oxidation of substrates, as assessed from oxygen consumption.

Adequate diffusion of oxygen into the muscle is important to ensure that oxidative metabolism supports muscle contraction and that measures of oxygen consumption are a faithful representation of energy used by the muscle during contraction. A number of precautions were taken to ensure this was the case. (1) The muscles were activated for only three cycles in succession, followed by 60–90 s of rest before the next contractions. Based on previous experience with these preparations, this is a very modest work rate that could be sustained for hours without signs of fatigue suggestive of anaerobic metabolism. (2) Measures of oxygen consumption following termination of stimulation continued for about 30 min, considerably longer than required to attain rates of oxygen consumption similar to those in resting muscle before stimulation. This ensured that recovery processes were complete and that any potential glycolytic activity during contractions would be accounted for in oxidative recovery. (3) The temperature at which the experiments were conducted was reduced to 20°C, which will markedly reduce metabolic demand, both resting and active. Although a reduced temperature may alter the power from what might occur under normal body temperatures in these mice (Rhodes et al., 2000), evidence suggest that such temperature changes will have little, if any, effect on efficiency (Smith et al., 2005), and only a relative comparison of efficiencies between the normal and mini-muscle phenotype was required for the purposes of this study. (4) Finally, the sizes of the muscle preparations were relatively small, and the gastrocnemius preparations of normal mice were further reduced to approximately half their original mass (mini-muscles were already relatively small). Barclay (Barclay, 2005) estimated the maximal radii of muscles that could sustain aerobic activity to the core relying on diffusion alone when bathed in saline perfused with 95% oxygen at 20°C and subjected to an activation duty cycle of 30% (as in the present study): for mouse soleus, this radius was about 0.4 mm, and for mouse EDL (gastrocnemius in our study being not quite as fast and thus allowing a larger maximal radius) it was about 0.25 mm. Based on the masses and lengths of the preparations used in the present study (see Results), and assuming a cylindrical cross-section as did Barclay (Barclay, 2005), we estimate the radii to be M3 soleus=0.15 mm, N7 and N8 soleus=0.11 mm for both, M3 medial gastrocnemius=0.35 mm, and experimental segments of N7 and N8 medial gastrocnemius=0.39 mm for both. Thus, the soleus samples were easily small enough to support oxidative metabolism through diffusion alone, whereas the gastrocnemius muscles were slightly large. However, the muscles were exposed to a stimulation duty cycle of about 30% only during three consecutive cycles and then were allowed to rest for 60–90 s before subsequent contractions. Therefore, the functional activation duty cycles were about 7% for the soleus and 3% for the medial gastrocnemius, placing the radii of the experimental samples well below those required to satisfy aerobic activity based on diffusion alone.

At the end of the experiments, muscles were removed from the chamber, the tendons were cut from the muscle, and the muscles were cleared of visible fatty tissue. To obtain consistent masses of these small preparations, they were stored in centrifuge tubes at –70°C, then upon completion of all experiments the muscles were freeze dried, weighed using a Mettler MT5 microbalance, and associated wet masses of the muscles were calculated using an experimentally determined wet/dry mass ratio measured from samples of larger muscles, where measurements of wet mass are more consistent. Work is expressed relative to this muscle wet mass (J kg$^{-1}$).

### Myosin heavy chain isoform analysis

To provide further insight into potential differences in running ability and muscle efficiency among lines and between mini-muscle and normal phenotypes, the proportions of myosin heavy chain (MHC) isoforms in the plantaris, soleus and the medial and lateral heads of the gastrocnemius were determined using SDS-PAGE. Furthermore, to determine if the segments of medial gastrocnemius used for measurement of muscle efficiency were representative of the entire medial head of the gastrocnemius, the MHC isoforms were analysed in both the segments and intact medial head.

MHC was isolated and purified based on established procedures (Talmadge and Roy, 1993), with all homogenization and purification

Table 1. Analysis of variance or covariance of $\log_{10}$ body and $\log_{10}$ organ masses

| | N | P for $\log_{10}$ | Three-group comparison | | Contrast of Line 3 *vs* 7 and 8 | | Least squares means ± s.e.m. | | |
|---|---|---|---|---|---|---|---|---|---|
| | (M3, N7, N8) | body mass | $F_{df}$ | P | $F_{df}$ | P | Mini line 3 | Normal line 7 | Normal line 8 |
| Body mass | 10,10,10 | n.a. | $F_{2,27}$=0.95 | 0.3977 | $F_{1,27}$=0.20 | 0.6561 | 1.5597±0.02809 | 1.5183±0.02809 | 1.5183±0.02809 |
| Organ mass | | | | | | | | | |
| Soleus | 9,10,10 | 0.1178 | $F_{2,25}$=12.43 | 0.0002 | $F_{1,25}$=24.68 | <0.0001 | –2.1243±0.01819 | –2.2431±0.01765 | –2.2248±0.01705 |
| Medial gastrocnemius | 10,10,10 | n.a. | $F_{2,27}$=15.37 | <0.0001 | $F_{1,27}$=30.32 | <0.0001 | –1.8448±0.04956 | –1.5330±0.04956 | –1.4882±0.04956 |
| Lateral gastrocnemius | 10,10,10 | n.a. | $F_{2,27}$=10.32 | 0.0005 | $F_{1,27}$=19.42 | 0.0001 | –1.7062±0.06909 | –1.3874±0.06909 | –1.2793±0.06909 |
| Plantaris | 10,10,10 | n.a. | $F_{2,27}$=1.87 | 0.1729 | $F_{1,27}$=0.01 | 0.9038 | –2.1543±0.05904 | –2.2261±0.05904 | –2.0648±0.05904 |
| Medial and lateral quadriceps | 10,10,10 | 0.8175 | $F_{2,26}$=22.36 | <0.0001 | $F_{1,26}$=43.40 | <0.0001 | –1.1203±0.04230 | –0.8097±0.04315 | –0.7475±0.04262 |
| Upper forelimb | 9,10,10 | 0.2987 | $F_{2,25}$=3.96 | 0.0320 | $F_{1,25}$=7.47 | 0.0113 | –1.0749±0.06968 | –0.8039±0.06762 | –0.8835±0.06532 |
| Lower forelimb | 10,10,10 | n.a. | $F_{2,27}$=6.29 | 0.0057 | $F_{1,27}$=1.61 | 0.2151 | –1.0719±0.04079 | –1.2309±0.04079 | –1.0398±0.04079 |
| Heart ventricles | 10,10,10 | 0.7198 | $F_{2,26}$=0.76 | 0.4787 | $F_{1,26}$=1.52 | 0.2293 | –0.9575±0.03369 | –1.0090±0.03436 | –1.0077±0.03395 |

Least squares means and s.e.m. are from SAS procedure mixed.
All masses were measured in grams.

steps being performed on ice. The muscles were homogenized using a motor-driven mortar and pestle in a solution of 250 mmol l$^{-1}$ sucrose, 100 mmol l$^{-1}$ KCl, 5 mmol l$^{-1}$ EDTA and 10 mmol l$^{-1}$ Tris base, pH 6.8. The homogenate was centrifuged at 3000 ***g*** for 10 min at 3°C and the supernatant was discarded. The pellet was resusupended in a solution of 150 mmol l$^{-1}$ KCl, 10 mmol l$^{-1}$ Tris base and 0.5% Triton X-100, pH 6.8, homogenized, centrifuged as above, and the supernatant discarded. The pellet was resususpended in a solution of 150 mmol l$^{-1}$ KCl and 10 mmol l$^{-1}$ Tris base, pH 7.0, homogenized, centrifuged as above, and this final procedure repeated three more times. Upon final centrifugation, the supernatant was discarded and the pellet was suspended in enough 150 mmol l$^{-1}$ KCl and 10 mmol l$^{-1}$ Tris base pH 7.0 to cover the pellet, along with approximately 300 µl of protein sample buffer. The sample was boiled for 5 min, and 25 µl was loaded into the wells of the gel [prepared as per Talmadge and Roy (Talmadge and Roy, 1993)]. Gel electrophoresis was performed using a Bio-Rad Mini-Protean III gel unit in a refrigerator at 4°C for 48 h at a constant 100 V (Accu Power model 500, VWR Scientific Products, West Chester, PA, USA). Gels were stained using Coomassie Blue; some gels with a low protein concentration were silver stained following directions in the kit (Bio-Rad silver stain kit, Hercules, CA, USA). Gels were photographed using Bio-Rad Gel doc 2000, and images stored as tiff files using Quantity One (Bio-Rad). Band (MHC isoform) density was quantified using Scion Image for Windows (based on NIH Image, National Institute of Health, Frederick, MA, USA). Band densities were then expressed as a percentage of the sum of all MHC bands in a lane. In some instances it was not possible to clearly resolve the separation between MHC types $II_a$ and $II_x$, which run in very close proximity on the gel. Thus, results are also presented as type $II_{a+x}$ for all samples, where the two bands were treated as one. Where it was possible to resolve these bands reliably, the data for type $II_a$ and $II_x$ MHC are also presented separately.

### Statistical analyses

Data were transformed when necessary to satisfy the requirement of normal residuals from the statistical model (e.g. arcsine for the MHC data). Most comparisons among lines employed one-way analysis of variance followed by Holm–Sidak tests. However, for organ masses, which would be expected to correlate positively with overall body mass, we used analysis of covariance (ANCOVA) with $\log_{10}$ body mass as the covariate and a planned contrast of line 3 mini-muscle *versus* lines 7 and 8 normal (using SAS Procedure Mixed). For some of the organ mass ANCOVAs, the partial regression coefficient for body mass was negative, apparently because of one or two heavy mice with a large amount of body fat noted at dissection. In these cases, we re-ran the analysis without body mass as the covariate. We also re-ran analyses without the single heaviest individual to verify that results did not change in any appreciable way. As they did not, all individuals were included in statistical analyses.

Differences were considered significant at $P<0.05$. As the Holm–Sidak procedure adjusts the level of significance for repeated comparisons, and because we stress only differences that are large and clear and are not attempting to tease out small or marginal effects, we did not apply further adjustments to the level of significance for multiple comparisons. All data are presented as means with s.e.m.

## RESULTS

### Body and muscle mass

The three lines of mice did not differ statistically for body mass or mass-corrected ventricle mass (Table 1). However, there were variable differences in limb muscles among the lines (Table 1). The

mass of the quadriceps of M3 mice (medial and lateral combined) was about 50% that of these muscles in N7 and N8 mice, with no difference between the masses of the quadriceps in N7 and N8 mice. Likewise, the masses of both the medial and lateral gastrocnemius muscles of M3 mice were approximately 50% of those of N7 and N8 mice, with no difference between N7 and N8. In contrast, the soleus of M3 mice was about 140% the mass of soleus muscles from N7 and N8 mice, with no significant difference between N7 and N8 mice. Plantaris mass did not vary significantly among lines (Table 1). Upper forelimb muscles were significantly reduced in M3 as compared with N7 and N8. Interestingly, the mass of lower forelimb muscles in N7 mice were 61% and 69% of those from N8 and M3 mice, respectively; the mass of lower forelimb muscles in M3 and N8 mice were not significantly different.

Masses of the experimental samples used for measurement of efficiency were: M3 medial gastrocnemius 13.9±1.46 mg, M3 soleus 8.91±0.960 mg, N7 medial gastrocnemius 16.1±2.21 mg, N7 soleus 5.64±0.191 mg, N8 medial gastrocnemius 17.0±0.767 mg, N8 soleus 6.03±0.203 mg.

### Muscle efficiency

Examples of work loops as recorded during measures of efficiency for soleus and medial gastrocnemius muscles from each line are shown in Fig. 1. There were no notable differences in the shapes of the loops between soleus preparations from the different lines, suggesting similar abilities to produce force and rates of contraction and relaxation. While the shapes of the loops between medial gastrocnemius preparations from the different lines did not differ substantially, again suggesting similar rates of contraction and relaxation, muscles from line 3 tended to produce less force than muscles from lines 7 and 8. The net work done per cycle by the medial gastrocnemius averaged 2.32±0.291 J kg$^{-1}$ for M3 ($N$=6), 7.48±1.24 J kg$^{-1}$ for N7 ($N$=9) and 9.17±0.933 J kg$^{-1}$ for N8 ($N$=10). The mass-specific work output of the M3 gastrocnemius was significantly less ($F_{2,25}$=9.455, $P$<0.001) than that done by N7 and N8, with no difference between N7 and N8. The equivalent power output of the medial gastrocnemius with a cycle frequency of 4 Hz was 9.3 W kg$^{-1}$ for M3, 29.9 W kg$^{-1}$ for N7 and 36.7 W kg$^{-1}$ for N8. The mass-specific net work done per cycle by the soleus averaged 4.42±0.436 J kg$^{-1}$ for M3 ($N$=9), 4.69±0.441 J kg$^{-1}$ for N7 ($N$=9) and 4.52±0.477 J kg$^{-1}$ for N8 ($N$=10). There were no statistically significant differences among lines ($F_{2,28}$=0.092, $P$=0.912). The equivalent power output for the soleus with a cycle frequency of 2 Hz was 8.8 W kg$^{-1}$ for M3, 9.4 W kg$^{-1}$ for N7 and 9.0 W kg$^{-1}$ for N8.

Efficiency was calculated as the ratio of net work performed by the muscle to energy released by oxidizeable substrates based on the amount of oxygen consumed. The efficiency of soleus muscles from M3 (15.2±1.03%), N7 (14.4±1.08%) and N8 mice (14.3±1.29%) did not differ among the three lines studied ($P$=0.843; Fig. 2). Perhaps surprisingly, the efficiency of medial gastrocnemius from M3 mice (13.0±1.67%) was significantly lower than the efficiency of medial gastrocnemius from N7 (19.3±0.97%) and N8 mice (19.5±0.83%; $P$<0.001; Fig. 2).

### Myosin heavy chain isoforms

SDS-PAGE of extracts from soleus and medial gastrocnemius muscles had four bands, representing the mammalian type $II_a$, $II_x$, $II_b$ and I MHC isoforms (Fig. 3). The proportions of the MHC isoforms in soleus muscles from mini-muscle and normal mice were not similar, there being a shift toward faster MHC isoforms in mini-muscle mice (Fig. 4). Compared with N7 and N8, soleus muscles of M3 mice showed a significant decrease in the proportion of type I MHC ($P$<0.01) and a significant increase in the proportion of type $II_{a+x}$ MHC ($P$<0.01). This increase appeared to be the result of an approximately equal increase in type $II_a$ and $II_x$ MHC. N8 and N7 mice did not show any significant differences in the proportions of MHC isoforms in the soleus.

The medial gastrocnemius of M3 mice exhibited a significant shift in MHC isoform content toward a generally slower profile (Fig. 5). The proportion of fast type $II_b$ MHC in M3 mice was approximately 7% ($P$<0.001) that of N7 and N8 mice, whereas the proportions of type $II_a$, $II_x$ and I MHC in M3 mice were significantly greater ($P$<0.001) than in N7 and N8 mice. The increase in type $II_a$ and $II_x$ was approximately equal. There were no differences between N7 and N8 mice.

The proportions of the MHC isoforms in the lateral gastrocnemius were very similar to those of the medial gastrocnemius (Fig. 6), with

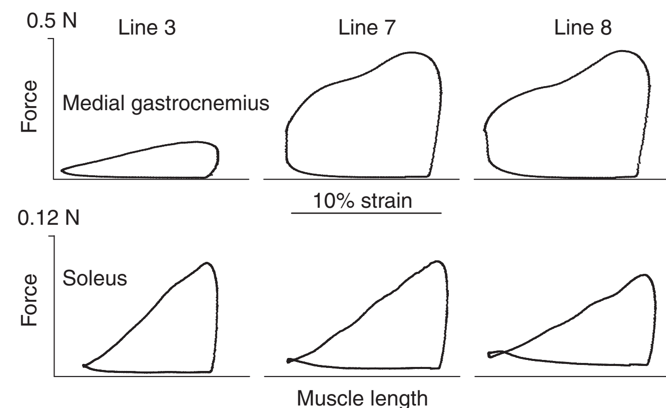


Fig. 1. Examples of work loops generated by medial gastrocnemius muscles (upper panels) and soleus muscles (lower panels) from mini-muscle mice (line 3; M3) and normal mice (lines 7 and 8; N7, N8). Cycle frequency was 4 Hz for medial gastrocnemius and 2 Hz for soleus. Strain was 10% peak-to-peak. Stimulus phase and duration were adjusted to maximize net work output for each preparation. Temperature was 20°C. All loops are traversed in a counter-clockwise direction, indicating net positive work output.

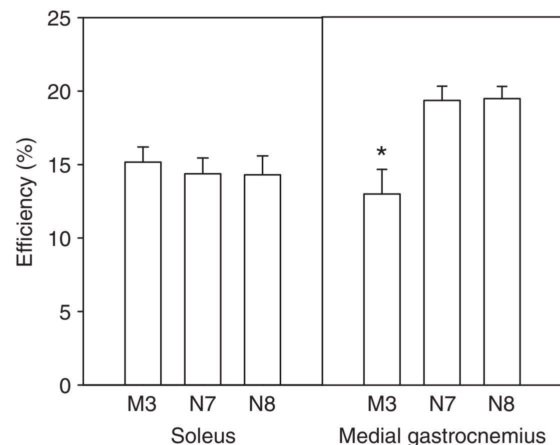


Fig. 2. Efficiency of soleus and medial gastrocnemius muscles from mini-muscle mice (line 3; M3) and normal (lines 7 and 8; N7, N8) mice. Values are means and s.e.m. *Significantly different ($P$<0.001) from other groups for that type of muscle.

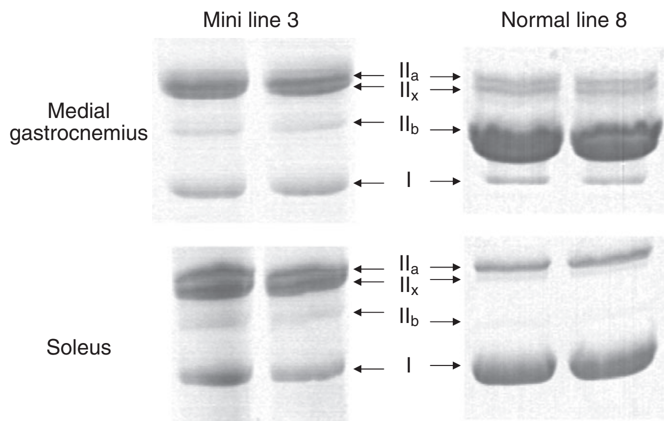


Fig. 3. SDS-PAGE showing myosin heavy chain isoforms in medial gastrocnemius muscles (upper panels) and soleus muscles (lower panels) from mini-muscle mice (line 3; M3) and normal (line 8; N8) mice. Results from normal line 7 were similar to those from line 8.

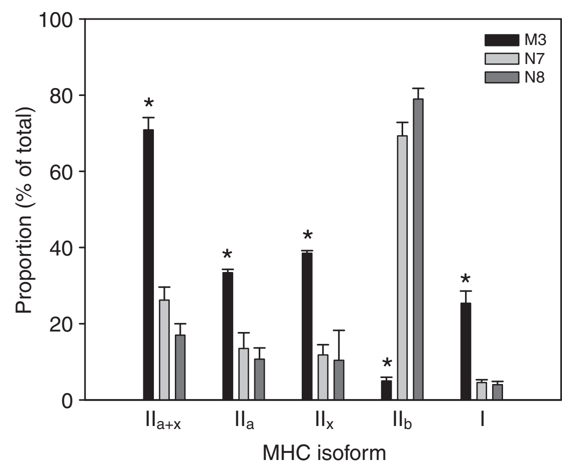


Fig. 5. Myosin heavy chain isoforms in medial gastrocnemius muscles of mini-muscle (line 3; M3) and normal (lines 7 and 8; N7, N8) mice. Myosin heavy chain isoforms are expressed as a percentage (mean+s.e.m.) of the total in each muscle sampled. *Significantly different ($P$<0.001) from all other groups.

M3 mice expressing much smaller proportions of the faster type $II_b$ MHC ($P$<0.001) and greater proportions of the slower types $II_a$, $II_x$ and I ($P$<0.001) compared with N7 and N8 mice. Again, the increase in the proportion of type $II_{a+x}$ MHC in M3 lateral gastrocnemius appeared to be equally divided between the $II_a$ and $II_x$ isoforms. The distribution of MHCs in the lateral gastrocnemius did not differ between N7 and N8 mice.

The MHC isoform profile of the plantaris muscle in the different lines was similar to that of the medial and lateral heads of the gastrocnemius, with a shift toward a slower profile in M3 mice (Fig. 7). M3 mice showed a large decrease in type $II_b$ MHC ($P$<0.001) and increases in type I ($P$<0.001) and types $II_{a+x}$ ($P$<0.001). The increases in type $II_x$ and $II_a$ MHC in M3 mice were more modest than observed in the gastrocnemius, being statistically significant only from N8 mice. Of interest, N7 mice had significantly less type $II_b$ MHC and more type $II_{a+x}$ MHC than N8 mice ($P$<0.001).

**Comparison of experimental samples with whole muscle**

As discussed above, it was necessary to use segments of the larger medial gastrocnemius muscles from N7 and N8 mice rather than the entire muscle when measuring efficiency. Although care was taken to ensure that samples were removed from the same anatomical region of the muscle for each experiment, this did not ensure that the dissected segments were representative of the whole muscle. To assess this, MHC analysis was performed on both the experimental segments and whole medial gastrocnemius muscles taken from the contralateral leg of N7 and N8 mice. No significant differences in MHC content were found for any comparisons between experimental and whole muscles for type I, $II_{a+x}$, $II_a$, $II_x$ or $II_b$ isoforms from N7 mice ($P$>0.37–0.74) or N8 mice ($P$>0.15–0.84). This demonstrates that the experimental segments of medial gastrocnemius used in the efficiency experiments were representative of the whole muscles.

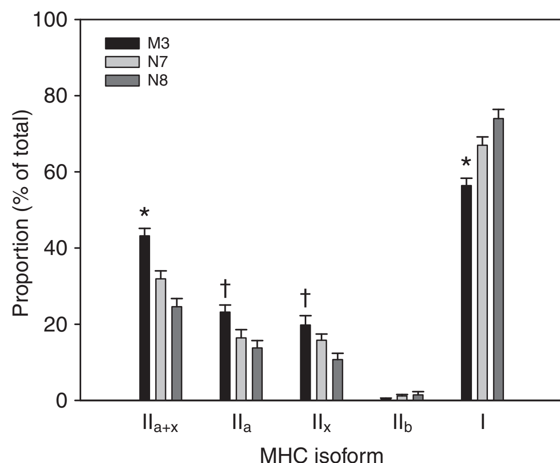


Fig. 4. Myosin heavy chain isoforms in soleus muscles of mini-muscle (line 3; M3) and normal (lines 7 and 8; N7, N8) mice. Myosin heavy chain isoforms are expressed as a percentage (mean+s.e.m.) of the total in each muscle sampled. *Significantly different ($P$<0.01) from all other groups. †Significantly different ($P$<0.01) from N8.

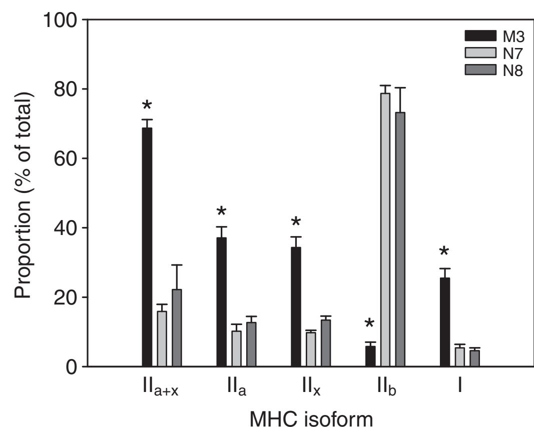


Fig. 6. Myosin heavy chain isoforms in lateral gastrocnemius muscles of mini-muscle (line 3; M3) and normal (lines 7 and 8; N7, N8) mice. Myosin heavy chain isoforms are expressed as a percentage (mean+s.e.m.) of the total in each muscle sampled. *Significantly different ($P$<0.001) from all other groups.

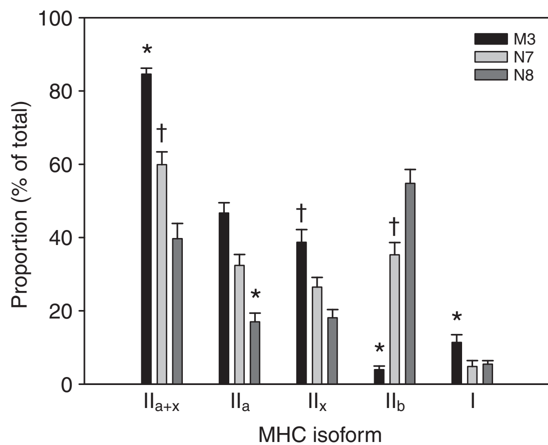


Fig. 7. Myosin heavy chain isoforms in plantaris muscles of mini-muscle (line 3; M3) and normal (lines 7 and 8; N7, N8) mice. Myosin heavy chain isoforms are expressed as a percentage (mean s.e.m.) of the total in each muscle sampled. *Significantly different ($P$<0.001) from all other groups. †Significantly different ($P$<0.001) from N8.

## DISCUSSION

Despite many generations of selective breeding for enhanced voluntary wheel running in these mice, the normal gastrocnemius has retained a distinctly fast phenotype and the soleus a slow phenotype (Figs 4, 5 and 6). By contrast, based on contractile performance of muscles from mice expressing the mini-muscle phenotype, previous studies have shown no difference in the soleus but an apparent shift toward a slower phenotype in the medial gastrocnemius (Syme et al., 2005). The lack of change in the soleus probably reflects the very small change in myosin expression toward faster isoforms (Fig. 4), while the slowing of contractile performance in the gastrocnemius muscles of mini-muscle mice probably reflects the loss of type $II_b$ myosin and increased type $II_{a,x}$ and I myosin (Guderley et al., 2006; Guderley et al., 2008) (Figs 5 and 6). The shift toward a marginally faster phenotype (and larger mass) in soleus muscles of mini-muscle mice may endow an enhanced ability to run faster on the wheels than normal mice (Kelly et al., 2006; Gomes et al., 2009), while still retaining the putative benefits of a slower phenotype (discussed below). The shift toward a much slower phenotype in the gastrocnemius and plantaris muscles of mini-muscle mice may reflect selection for sustained, but not necessarily high-speed, running. Of interest, lines 7 and 8, which lack the mini-muscle phenotype, have responded similarly to the selection protocol as has line 3 in terms of their amount of wheel running. This indicates that high voluntary running can be accomplished in more than one way with respect to muscle anatomy and physiology, which is an example of multiple adaptive solutions in response to selection (see also Garland, 2003; Houle-Leroy et al., 2003; Swallow et al., 2009).

As the mini-muscle phenotype appears to be favoured by selection for high, sustained activity (Garland et al., 2002; Gomes et al., 2009), we hypothesized that the slower, mini-muscle phenotype of gastrocnemius may be accompanied by greater muscle efficiency and favour more economical running as compared to the normal muscle condition. Based on the lack of change in contractile performance in the soleus of mini-muscle mice (Syme et al., 2005) and given the modest differences in the proportions of the MHC isoforms in the mini and normal soleus (Fig. 4), we did not anticipate a notable change in efficiency of the soleus. In support of the latter, the efficiency of soleus muscle was not statistically different between the normal and mini-muscle phenotypes (Fig. 2). However, in contrast to expectations for the medial gastrocnemius, we noted a reduced efficiency of medial gastrocnemius from M3 mice compared with that from N7 and N8 mice (Fig. 2), despite the considerably slower contractile phenotype (Syme et al., 2005) and differences in MHC isoforms (Fig. 5) in M3 compared with N7 and N8 gastrocnemius (see also Guderley et al., 2006; Guderley et al., 2008).

Other observations comparing efficiency of fast and slow muscles also lead to questions about the functional and adaptive basis for the slower mini-muscle phenotype. Muscle efficiencies measured from the slower soleus and faster medial gastrocnemius of mice expressing the normal phenotype (N7 and N8) were likewise not consistent with a notion that slower muscles are more efficient than faster muscles (Fig. 2). Our observations were conspicuously similar to those from studies using slow and fast rat muscle during shortening contractions and using oxygen analysis to assess energy use; 19% efficient for the relatively fast EDL shortening at 1.0 muscle length $s^{-1}$, and 15% efficient for the slow soleus shortening at 0.5 muscle lengths $s^{-1}$ (Heglund and Cavagna, 1987). Barclay and Weber (Barclay and Weber, 2004) also argue, based on initial and recovery processes in mouse muscle, that net efficiencies of fast and slow muscles do not appear to be different. Furthermore, Smith et al. (Smith et al., 2005) provide a preliminary rationale for why an unfailing relationship between efficiency and fibre types may not exist. Thus, despite well-established expectations that slow muscles are inherently more efficient than fast (see Introduction), there are clearly either exceptions or conditions under which this is not the case.

One potential complication in interpreting and comparing measures of efficiency between fast and slow muscle (soleus *versus* gastrocnemius and/or normal *versus* mini-muscle) is that each possesses a different (at the time unknown) MHC profile, and thus has different force–velocity properties and capacities for contraction kinetics. Yet the cycle frequency for work measurements (i.e. rate of shortening and extension) was maintained constant across all gastrocnemius preparations (4 Hz) and all soleus preparations (2 Hz) to facilitate direct comparisons between the muscles. Thus, faster and slower preparations would be operating at different relative velocities of shortening and load, and with different abilities to contract and relax at the rates imposed. As efficiency is affected by load and velocity (Smith et al., 2005), these relative and absolute differences between conditions under which efficiency was measured may confound our ability to make direct comparisons. However, when muscles are operating at the loads and velocities near which they produce maximal power, efficiency is relatively insensitive to either load or velocity (Smith et al., 2005). Our protocols were designed to produce near-maximal power, so we expect efficiency to be near maximal for each preparation. Furthermore, the modest changes in MHC composition between normal and mini soleus would probably minimize the effect of this complication. The larger changes in MHC composition of the medial gastrocnemius would result in the M3 muscles working at relatively faster velocities than the N7 and N8 muscles, but efficiency is maximized over a much broader range of velocities in faster than in slow muscles (Smith et al., 2005; Reggiani et al., 1997), so the impact will be lessened. Activation phase and duration were optimized individually for each preparation, and so changes in MHC composition between preparations was accounted for. However, it cannot be argued with certainty that differences in efficiency between normal and mini gastrocnemius preparations are due entirely to inherent differences in the physiology of the muscle itself.

Furthermore, the present study only describes the efficiency of the muscles when operating under conditions for near maximal power output, which in an absolute sense will probably be different from the efficiency when operating under conditions as experienced *in vivo*.

Mice from the selected lines primarily run faster than control lines, and only in males is the increase in amount of time spent running statistically significant (Swallow et al., 1998; Girard et al., 2001; Garland, 2003; Belter et al., 2004; Swallow et al., 2005; Rezende et al., 2006c). Likewise, mini-muscle individuals run faster than normal mice (Kelly et al., 2006; Hannon et al., 2008; Gomes et al., 2009). In the context of selective breeding for higher sustained running activity, the evolution of a slower, more economical muscle phenotype would seem to be beneficial. Yet the apparent lack of an increase in efficiency of the functionally and physiologically slower gastrocnemius muscle of mini-muscle mice lends support to the notion that the benefit of slower muscle may not be an increase in efficiency *per se*. In mice with *ad-libitum* access to food and water, living in a controlled and hospitable caged environment, and where enhanced fecundity is not a trait under direct selection (Girard et al., 2002), increased muscle efficiency for routine running might not be an important selective advantage (Swallow et al., 2001) [see also Vaanholt et al. and references therein (Vaanholt et al., 2007)], particularly given that selected mice with normal muscles show similar enhancements in running behaviour (wheel revolutions per day).

Rather, perhaps the advantage of slower muscle is a reduced overall rate of energy use (Barclay and Weber, 2004), making them more suitable for sustained tasks or for completing a task with less reliance on anaerobic metabolism. Reduced energy use may have permissive effects on the behaviour of high voluntary wheel running. For example, a slower, more oxidative mini-muscle phenotype may allow the mice to run faster without significant accumulation of anaerobic by-products, which appear to promote discomfort (Pan et al., 1999; Immke and McCleskey, 2001; Yagi et al., 2006), and may inhibit volition to exercise (see also Li et al., 2004; Keeney et al., 2008). In other words, the mini-muscle phenotype may confer an advantage by enhancing the ability of the mice to run on wheels through reduced reliance on glycolytic metabolism. If the mini-muscle phenotype confers such an advantage in wheel-running ability, then mice with the normal phenotype in the selected lines, which also show enhanced running ability over control mice, must have adopted an alternate adaptive response. Perhaps normal mice in the selected lines have overcome inhibitory effects related to the use of faster, more glycolytic muscles through increased behavioural tolerance of discomforts associated with endurance running. Although Li et al. (Li et al., 2004) did not find significant differences in pain tolerance between the control lines and those selected for high activity, it is not known if such differences might exist among the four selected lines (see also Keeney et al., 2008). In addition, the observation that selected mice with the normal phenotype run slower than those with the mini-muscle phenotype (Kelly et al., 2006; Hannon et al., 2008; Gomes et al., 2009) may be of consequence in this context.

With regard to masses of the muscles (Table 1), previous studies (Garland et al., 2002; Houle-Leroy et al., 2003; Syme et al., 2005; Guderley et al., 2006; Guderley et al., 2008; Gomes et al., 2009) have reported differences in masses of the plantar flexor muscles between mini-muscle and normal mice. We further note that the mini-muscle phenotype is not restricted to the plantar flexors, with muscles of the upper forelimb being reduced by about one third in mass in the M3 mice compared with normal, and the quadriceps of M3 mice being approximately half the mass of N7 and N8 mice. Unexpectedly, in N7 mice the mass of the lower forelimb muscles was less compared with both N8 and M3 (Table 1). Such observations of variability not clearly associated with selective breeding or the normal/mini phenotypes point to the importance of random genetic drift and/or alternate responses to uniform and well-defined selection, especially with such complex traits as voluntary activity levels (Swallow et al., 2005; Swallow et al., 2009; Garland and Kelly, 2006). Future studies will address whether the identified differences in mass, efficiency (this study) and contractile properties (Syme et al., 2005) of mini-muscles are reflected in whole-animal cost of transport (Rezende et al., 2006c), maximal sprint-running speed (Djawdan and Garland, 1988) or endurance capacity.

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