Experimental Physiology – *Research Paper*

Relative proportions of hybrid fibres are unaffected by 6 weeks of running exercise in mouse skeletal muscles

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Hybrid muscle fibres co-expressing two or more myosin heavy chain (MHC) isoforms represent a significant proportion of fibres in many muscles, but the prevalence and precise composition of these fibres varies significantly among muscles and animal species. In the present study, we used a forced running protocol for 6 weeks to determine the effects of running exercise on the relative proportion of hybrid muscle fibre types in mouse muscles. In the course of this experiment, we also determined the relative proportions of these fibres in several different skeletal muscles, since data about hybrid fibres in the mouse are sparse. We found that the proportions of hybrid fibres in mouse muscles varied significantly among specific muscles (2–25%), but these proportions were unaffected by 6 weeks of forced running exercise. In contrast, weight-bearing muscles significantly increased in mass in response to running. These data suggest that hybrid muscle fibres are relatively refractory to the effects of moderate exercise and represent a stable phenotype in normal mouse muscles. The precise nature and physiological function of these fibres remain incompletely understood, but it is clear that hybrid fibres represent a common phenotype in many muscles.

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Functional differences in skeletal muscle performance are derived from the existence of different fibre types within an organism. Many of the functional differences in fibre type are an outcome of the specific myosin heavy chain (MHC) isoform expressed within a specific muscle fibre (Pette & Staron, 2001). Since the advent of techniques that allow for the determination of the precise MHC isoform content within single muscle fibres, it has been recognized that some 'hybrid' or 'polymorphic' fibres express a combination of two or more isoforms (Stephenson, 2001; Caiozzo *et al.* 2003). Over the past several years, it has become increasingly clear that these hybrid fibres are a common occurrence, frequently representing the predominant fibre type in certain skeletal muscles (Stephenson, 2001; Caiozzo *et al.* 2003).

The existence of hybrid muscle fibres raises a number of important questions about the basic nature of skeletal muscle phenotype and muscle plasticity (Stephenson, 2001; Caiozzo *et al.* 2003). One prevailing view has been that hybrid fibres are evidence of the plastic nature of skeletal muscles, with the presumption that fibres expressing multiple isoforms are transitional between one 'pure' fibre type and another (Pette & Staron, 2001). Although a number of experimental models support the idea of hybrid fibres being transitional, other studies have shown that hybrid fibres are also present in normal control muscles (Stephenson, 2001; Caiozzo *et al.* 2003). Most of the data about hybrid fibres come from studies of rat muscles, and the relative proportions of hybrid fibres vary significantly from muscle to muscle. These range from only about 3% hybrid fibres within the vastus lateralis to more than 75% in the rectus femoris (Caiozzo *et al.* 2003). Human studies collectively indicate that skeletal muscles possess ~25% hybrid fibres, with some variability possibly being related to the functional demands imposed on the muscles (Andersen *et al.* 1999; Andersen & Aagaard, 2000; Williamson *et al.* 2001; Parcell *et al.* 2005; Kohn *et al.* 2007*a*,*b*; Malisoux *et al.* 2007; Kesidis *et al.* 2008). Based on these data, hybrid muscle fibre types clearly represent a significant population of fibres in many muscles, but the relative stability of these intermediate phenotypes is still not well established.

Several experimental models have shown that the proportion of hybrid fibres can change dramatically.

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Spinal cord transection or denervation leads to a significant increase in the relative numbers of hybrid fibres, since these events cause a dramatic shift towards faster MHC isoform expression (Talmadge *et al.* 1995, 1999; Talmadge, 2000; Patterson *et al.* 2006). Chronic low-frequency electrical stimulation (CLFS) drives a fast-to-slow fibre type transition that is accompanied by an increase in the proportion of hybrid fibres (Pette & Staron, 2001). Mechanical load and thyroid hormone concentration manipulated in combination can produce very dramatic changes in the relative proportions of hybrid fibres (Caiozzo *et al.* 1998, 2000). Collectively, these studies demonstrate that muscle fibres possess the cellular and molecular potential for dramatic shifts in fibre phenotype. However, the extent to which hybrid fibres change in more normal physiological conditions is less clear. Exercise generally causes a shift in fibre type similar to that elicited by CLFS, but to a lesser degree (Pette & Staron, 2001). It is likely that fibres are limited in their capability to transform completely into alternative fibre types in normal physiological conditions (Schiaffino *et al.* 2007).

The extent to which exercise causes physiological shifts in the relative proportion of hybrid fibres is incompletely understood, because the results from available studies are often equivocal. Resistance training can cause a significant reduction in hybrid fibre types (Williamson *et al.* 2001), yet the relative proportion of hybrid fibres is no different in muscles of long-term bodybuilders from that in age-matched control muscles (Kesidis *et al.* 2008). Running exercise causes a decline in the proportion of hybrid fibres in human muscles, but only when a threshold level of training is reached (Kohn *et al.* 2007*b*). Sprint training has no effect on the relative proportion of hybrid fibres (Parcell *et al.* 2005). The currently available data do not definitively reveal how susceptible hybrid fibres are to changes in MHC expression in response to exercise, or what role these fibres may play in functional adaptation.

In the present study, we had two primary objectives. Our first goal was to define the relative proportion of hybrid fibres in several limb muscles, including specific anatomical regions of the muscles where multiple fibre types were present. Despite the fact that mouse models of physiological function are probably the most extensively used in research today, data about the relative proportion of hybrid fibres in mouse muscles remain quite limited. Our second objective was to examine whether a moderate exercise intervention would elicit a shift in the relative proportion of these fibre types. Although previous studies have documented significant shifts in MHC isoform content following running exercise in mouse muscles (Allen *et al.* 2001; Pellegrino *et al.* 2005), none has tracked changes in the relative proportion of hybrid fibres. If hybrid fibres represent transient phenotypes intermediate to pure fibre types, then running exercise might be expected to reduce the relative proportion of these fibres.

Methods

Animals and experimental protocol

Male mice (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in accordance with an approved IACUC protocol (BIO12075N) at the University at Buffalo. They were housed in cages with 12 h–12 h light–dark cycle and were provided with food and water *ad libitum*.

Animals were 6 weeks old at the start of the experiment and were randomly assigned to control or treatment groups. Animals from the control group (C) were not exercised during the experiment ($n = 9$); animals from one running group were exercised on a flat incline (0 deg; $n = 8$); and animals from a second running group were exercised on an uphill incline of 15 deg ($n = 8$). Animals were exercised on a multi-laned treadmill at speeds of 20–30 cm s^{-1} for 30 min, twice per week for 6 weeks. This training appeared to be fairly rigorous, since mice could not consistently maintain running at speeds greater than this for longer than a few seconds. In addition, mice appeared to be taxed by the end of a training period, as indicated by rapid respiration and a flushed appearance in the feet and ears. At the end of the experimental period, animals were killed by exposure to CO_2 and muscle tissues were collected. Multiple muscles were sampled, and wet weights were determined for the shank flexors (including the soleus, plantaris and gastrocnemius muscles), the quadriceps (vastus medialis, vastus intermedius, vastus lateralis and rectus femoris), the tibialis anterior and the triceps brachii. Muscles were either mounted in optimal cutting temperature (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) and frozen in isopentane cooled in liquid N_2 for sectioning, or placed in relaxing solution (50% glycerol, 100 mM KCl, 2 mM EGTA, 1 mM $MgCl_2$, 4 mM ATP and 10 mM imidazole, pH 7.0) for dissection of single fibres.

In a subsequent experiment, mice were exercised using the same protocol with the uphill incline (15 deg) for 30 min, two to five times per week (averaging 3.5 times per week). Pairs of mice were killed by exposure to CO_2 at 2 week intervals until 12 weeks of running exercise. The soleus muscles were harvested from the mice and processed for histochemistry as described in the previous paragraph. In addition, some sections were processed to stain capillaries surrounding individual muscle fibres, and the number of capillary contacts per fibre was determined (see '*Determination of capillary densities*' below).

Histochemistry for ATPase

Frozen muscles were cut into 8 μm serial sections and mounted on microscope slides (Leica Microsystems, Bannockburn, IL, USA). Sections were kept at −20°C

until used for ATPase histochemistry, which followed the methods of Dubowitz & Sewry (2007). Sections were air dried for 30 min and then incubated in a saline solution buffered with glycine (50 mM glycine, 50 mM NaCl and 100 mM $CaCl_2$, pH 9.4) containing 1 mM ATP for 30 min at 37°C. Following this incubation, slides were rinsed for 6 min with three changes of 1% $CaCl_2$ followed by 2 min with two changes of $CoCl_2$. They were then rinsed several times in deionized water and developed by immersing the sections in 2% ammonium sulphide solution. Slides were then rinsed several times in deionized water, dehydrated through a graded ethanol series, cleared with xylenes and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA). This procedure stains all fast fibres a dark brown to black, while slow fibres remain unstained. To identify multiple fast fibre types, sections were pre-incubated for 30 min in an acetic acid solution (pH 4.6) prior to the above staining procedure. This acid pretreatment reverses the staining of fast and slow fibres, and produces a range of staining intensities of the fast fibres.

Determination of capillary densities

We used the ATPase method of Sillau & Banchero (1977) to visualize capillaries in the soleus muscles of mice. Briefly, pre-incubation of muscle sections for 30 min at pH 4.20–4.35 results in activation of phospatases present in the capillary endothelium. In the soleus muscle, the type I fibres stain dark (although less so than at pH 4.6) and the type IIA fibres remain unstained, allowing the simultaneous determination of capillary density and muscle fibre type. High-resolution digital images of stained muscles were analysed at final magnifications of ×250 or greater, and the number of capillaries per fibre was determined. Random samples of between 20 and 113 fibres for each fibre type were used from each muscle, and the average number of capillary contacts was determined.

Identification of MHC isoforms in single fibres

Muscles were stored at −20°C in a relaxing solution (50% glycerol, 2 mM EGTA, 1 mM $MgCl_2$, 4 mM ATP, 10 mM imidazole and 100 mM KCl) until they were used for single fibre dissection. Small bundles of fibres were dissected out from whole muscles and placed in a glass Petri dish positioned under a stereomicroscope. For muscles with significant regional variation in fibre type, specific anatomical regions of the muscles were sampled from the middle (proximal–distal) of the muscle. These included the deep region of the medial gastrocnemius, the lateral region of the vastus intermedius and the central part of the triceps brachii muscles. The bulk of these muscles consisted of homogeneous regions of type IIB fibres, and we avoided these areas in our sampling. A few drops of mineral oil were placed on top of the muscle sample and relaxing solution, to prevent drying and improve the contrast of individual fibres. Single fibres were separated from larger bundles with fine forceps and placed into a 1.5 ml microcentrifuge tube, and 30 μl of sample buffer was added. The sample buffer contained 8 M urea, 2 M thiourea, 50 mM Tris base, 75 mM dithiothreitol, 3% sodium dodecyl sulphate (SDS) and 0.004% Bromophenol Blue, pH 6.8. This buffer was prepared as described by Blough *et al.* (1996) and stored in 1 ml aliquots at −20°C until ready to use. Samples were heated to 65°C for 15 min and vortexed prior to loading, and 10 μl of each sample was applied to the gel. Samples taken from the soleus muscle containing both type IIA and type I isoforms were used as standards on each gel with samples from predominantly fast muscles.

Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) were used with a Hoeffer SE 600 to resolve individual isoforms of MHC. Resolving gels consisted of 12% acrylamide (200:1 acrylamide:methylene-*bis*-acrylamide), 12% glycerol, 0.675 M Tris base (pH 8.8) and 0.1% SDS. Stacking gels consisted of 4% acrylamide (20:1 acrylamide:methylene-*bis*-acrylamide), 0.125 M Tris base (pH 6.8) and 0.1% SDS. Running buffer contained 0.192 M glycine, 25 mM Tris base, 0.1% SDS and 0.08% 2-mercaptoethanol. Gels were run at a constant current of 20 mA until the samples entered the resolving gels, and then the current was increased to 35 mA. Total running time for the gels was ∼24 h. At the end of the run, gels were fixed in 50% methanol with a trace of formaldehyde (0.037%) added to increase the sensitivity of the silver staining procedure. Gels were allowed to fix overnight and were then washed for 1 h in deionized water and stained with silver according to the procedure of Wray *et al.* (1981).

Statistical analyses

Differences in muscle mass were compared between control and treatment groups using an analysis of covariance (ANCOVA) model, where the main effect was running exercise group (control, 0 or 15 deg) and animal body mass was used as a covariate. Using body mass as a covariate helped to account for interindividual variability and increased the power of the test statistics. When significant differences were found, a Bonferroni *post hoc* test was used to determine which groups were significantly different from one another (experiment-wise error rate, $\alpha = 0.05$). Linear regression was used to determine whether changes in capillary density as a function of training duration were significant.

For identification of muscle fibre types in single fibres, a minimum of 200 fibres were sampled for each muscle or specific muscle region analysed. For each individual mouse, approximately 20 fibres were used to estimate the fibre composition of that animal. All data are reported as

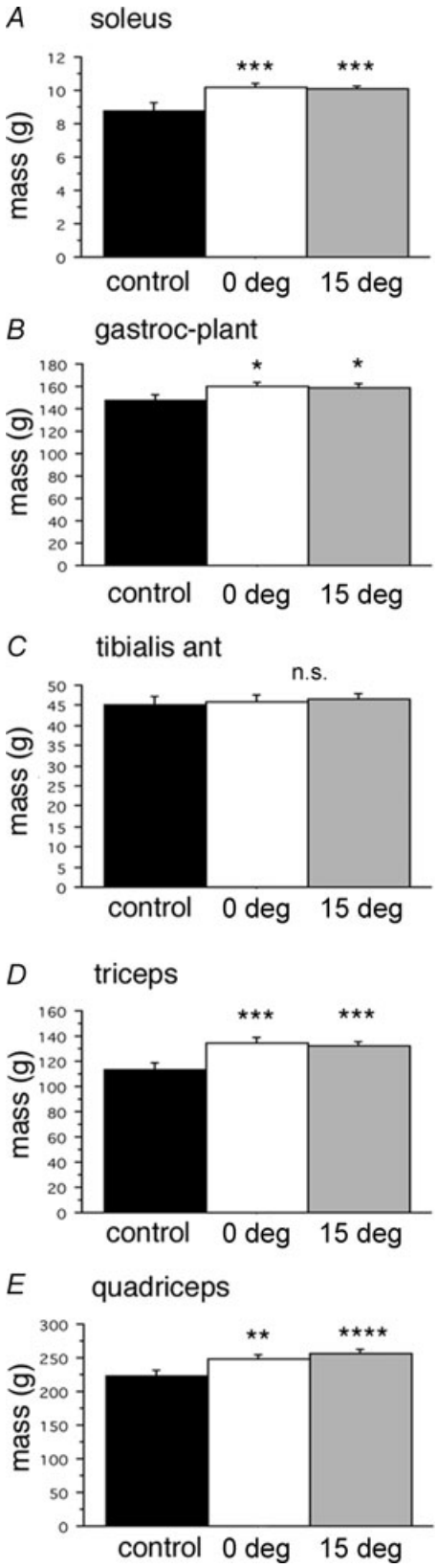


Figure 1. Muscle mass of limb muscles was significantly greater following 6 weeks of running exercise compared with muscles from non-exercised control mice

Muscle mass was greater in the soleus (*A*), gastrocnemius–plantaris (*B*), triceps brachii (*D*) and the quadriceps complex (*E*) following exercise compared with control muscles. The tibialis anterior mass (*C*) in running animals was not significantly different from that in control animals. The muscles from animals exercised on a flat treadmill (0 deg) were not significantly different in mass from animals run at an uphill incline (15 deg). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ and ****$P < 0.001$ in comparison to control; control, $n = 9$; 0 deg, $n = 8$; and 15 deg, $n = 8$).

means ± S.E.M. of fibre type proportion for a particular treatment group. Comparisons of the relative proportions of muscle fibre types between exercised and control muscles were made using a Mann–Whitney *U* test because of lack of normality within the data. A Kruskal–Wallis test was used to determine whether significant differences existed in the proportion of hybrid fibres among specific muscles. Statistical significance was accepted at $P < 0.05$. Statview (version 5.0.1, SAS Institute, Cary, NC, USA) was used for all statistical computations.

Results

General effects of running exercise on mouse muscles

Six weeks of running exercise led to a significant increase in the mass of the weight-bearing muscles examined (soleus, gastrocnemius–plantaris complex, quadriceps complex and triceps brachii; Fig. 1). By contrast, the tibialis anterior did not show a significant difference in mass when compared with control muscles. The effects of running on a flat incline (0 deg) *versus* uphill running (15 deg) were not different with respect to changes in whole muscle mass, and in subsequent analyses these two groups were combined into a single exercise group. The magnitude of change in muscle wet mass was 7.6% in the gastrocnemius–plantaris complex, 13.7% in the quadriceps complex, 15.3% for the soleus and 18.4% in the triceps brachii. There was no significant difference in overall body mass between exercised and control animals ($P > 0.92$).

Running exercise caused a significant increase in capillary density as a function of the number of weeks of training. This increase was linear in the type IIA fibres (no. of contacts, $4.72 + 0.165 \times$ week; $r^2 = 0.85$; $P < 0.0001$), but was curvilinear in the type I fibres (no. of contacts, $5.53 + 0.4 \ln(\text{week})$; $r^2 = 0.94$; $P < 0.0001$; Fig. 2). The number of capillary contacts became significantly greater in exercised animals compared with non-exercised control animals after between 2 and 6 weeks of training for both fibre types. The average number of capillary contacts increased by 44% in the type IIA fibres and by 37% in the type I fibres after 12 weeks of training. The same training did not result in any significant change in the proportion of muscle fibre types.

Muscle fibre type regionalization

Using standard myofibrillar ATPase histochemistry, we assessed the relative proportions and distribution of fibre types in several different limb muscles. We found a significant degree of inter- and intramuscular heterogeneity in the distribution of muscle fibre types, a pattern commonly referred to as fibre type regionalization (Fig. 3; see review by Kernell, 1998). For most muscles examined, there was a clear gradation in fibre type, with slower fibre types (I, IIA and IIX) being concentrated towards the deep regions of the muscles and with an increasing proportion of type IIB fibres more superficially (Fig. 3). In many of these muscles, including the gastrocnemius and quadriceps complexes, the bulk of the muscle mass in the peripheral regions of the muscle was composed of a nearly uniform population of type IIB fibres. For single fibre analyses, we avoided these uniform populations and focused on areas possessing two or more fibre types.

We subsequently focused on five specific muscle regions composed of mixed fibre types (Fig. 4). Using ATPase histochemistry, we observed histochemical staining patterns consistent with those previously reported for mouse muscles pre-incubated at pH 4.6 (Hamalainen & Pette, 1993). Using this technique, we found that the soleus was composed of ∼70% type IIA fibres and 30% type I fibres (Fig. 4*A*). The deep region of the medial gastrocnemius muscle was primarily composed of type IIX

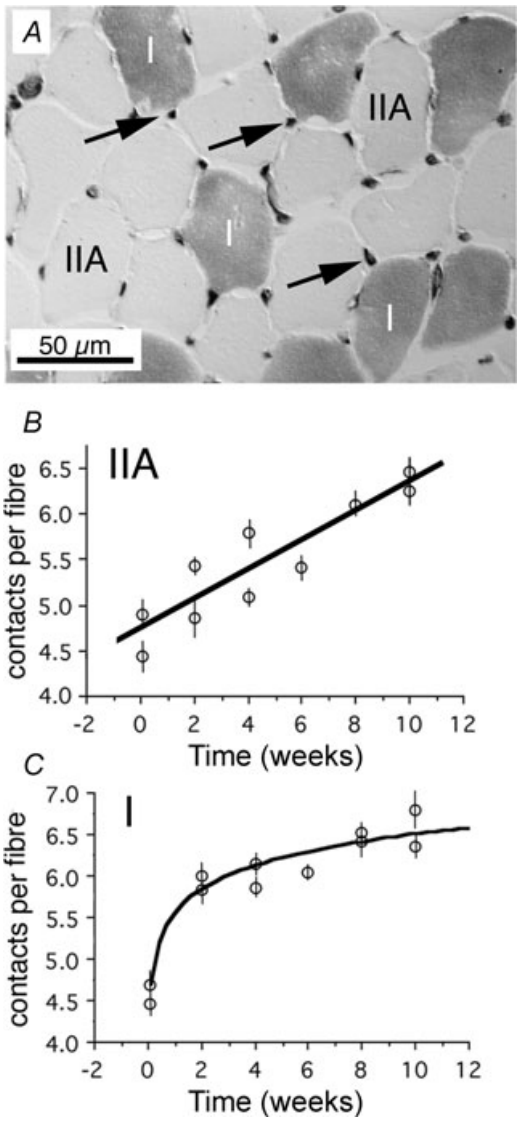


Figure 2. Capillary density significantly increased during exercise training
A, capillaries (arrows) were stained in the soleus muscles to determine the number of capillaries around type IIA and type I fibres. *B* and *C*, the number of capillaries per fibre increased significantly as a function of training duration (weeks) in both type IIA (*B*) and type I fibres (*C*). This increase was linear in the type IIA fibres ($r^2 = 0.85$; $P < 0.0001$), but was curvilinear in the type I fibres ($r^2 = 0.94$; $P < 0.0001$). Each point is the mean value ± S.E.M. for an individual mouse.

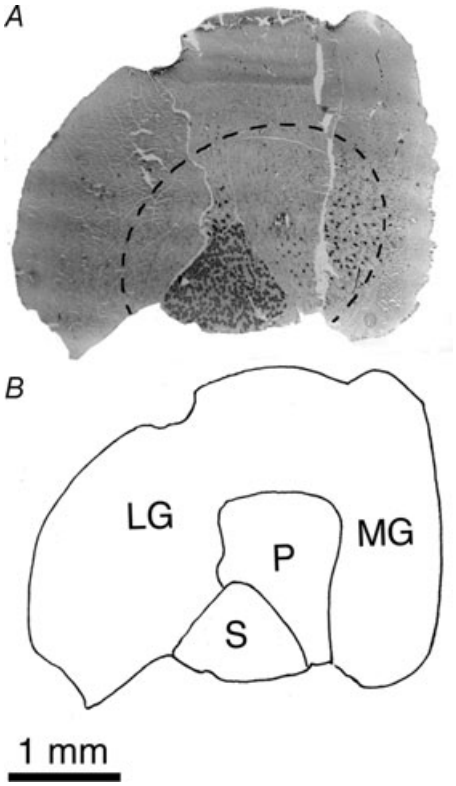
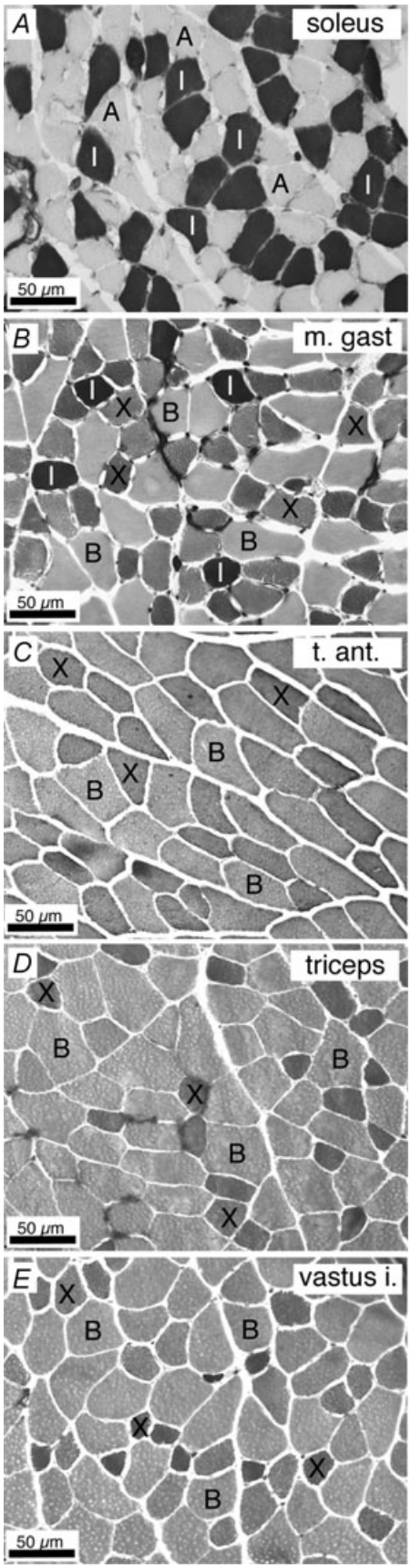


Figure 3. Muscle fibre types vary regionally
A, muscles were stained for myofibrillar ATPase (pH 4.6 pre-incubation) to differentiate fibre types. Slow (I) fibres are visible as black points and are found primarily in the soleus and deep regions of the medial gastrocnemius. Most of fibres in these muscles are type IIB, particularly in the more superficial regions of the muscles. The dashed line encloses the deep regions of the muscles where different fibre types are found. The fibres outside this region are uniformly type IIB (∼98%). *B*, diagram of the section in *A* to identify specific muscles: LG, lateral gastrocnemius; S, soleus; P, plantaris; and MG, medial gastrocnemius.



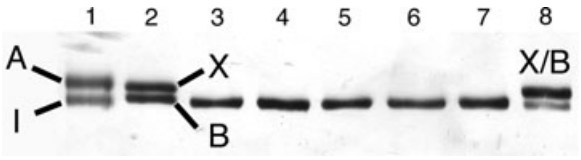


Figure 5. Representative SDS-PAGE gel showing the migration pattern of the different MHC isoforms
Lane 1 contains a homogenate of the soleus muscle, showing type IIA and I MHC isoforms. Lanes 2–8 are from single fibres from the tibialis anterior. Lanes 2 and 8 are from hybrid X/B fibres, while proteins in lanes 3–7 are from pure type IIB fibres.

and IIB fibres, with a minority of type I and IIA fibres (Fig. 4*B*). The tibialis anterior was comprised almost exclusively of type IIX and IIB fibres in nearly equal proportions (Fig. 4*C*). In many cases, it was difficult to distinguish between these two fibre types in this muscle. The triceps brachii (Fig. 4*D*) and vastus intermedius (Fig. 4*E*) were both comprised mainly of type IIB fibres, with varying numbers of type IIX fibres scattered among them. These type IIX fibres were concentrated at higher densities in the deep regions of the muscles and were rarely seen in the more peripheral regions.

Myosin heavy chain expression in single muscle fibres

We used SDS-PAGE to separate MHC isoforms from fragments of single muscle fibres (Fig. 5). Based on a lack of consistent resolution of IIA and IIX isoforms, we cannot rule out the possibility that we overlooked some hybrids containing both IIA and IIX isoforms. We sampled a total of 1077 fibres, with >200 fibres being collected from each of the muscle regions shown in Fig. 4. The relative proportions of fibre types from the different muscles are shown in Fig. 6. Overall, we found that the specific type and proportion of hybrid fibres differed significantly among muscle regions ($P < 0.0001$; Fig. 7). The soleus muscle possessed $4.9 \pm 1.4\%$ type I/IIA hybrids (Figs 6*A*, 7). The medial gastrocnemius muscle had $9.4 \pm 3.0\%$ hybrid fibres, primarily of the IIX/IIB type (Figs 6*B* and 7). The tibialis anterior possessed the highest proportion of

Figure 4. Muscle fibre types in specific muscle regions
Muscles were stained for myofibrillar ATPase (pH 4.6 pre-incubation). Staining intensity (dark → light) using this method is I > IIX > IIB > IIA, consistent with the pattern demonstrated by Hamalainen & Pette (1993). *A*, soleus muscle is composed of 65–70% type IIA fibres, with 30–35% being type I fibres. *B*, medial gastrocnemius (deep region) is highly mixed in fibre type, frequently containing each of the four adult fibre types. *C*, tibialis anterior is composed of an approximately equal mixture of type IIX and IIB fibres. The distinction between these two fibre types is often obscure. *D*, triceps brachii muscle (mid region) is primarily composed of type IIB fibres, with scattered type IIX fibres being found particularly in deeper muscle regions. *E*, vastus intermedius muscle is similar in composition to the triceps brachii.

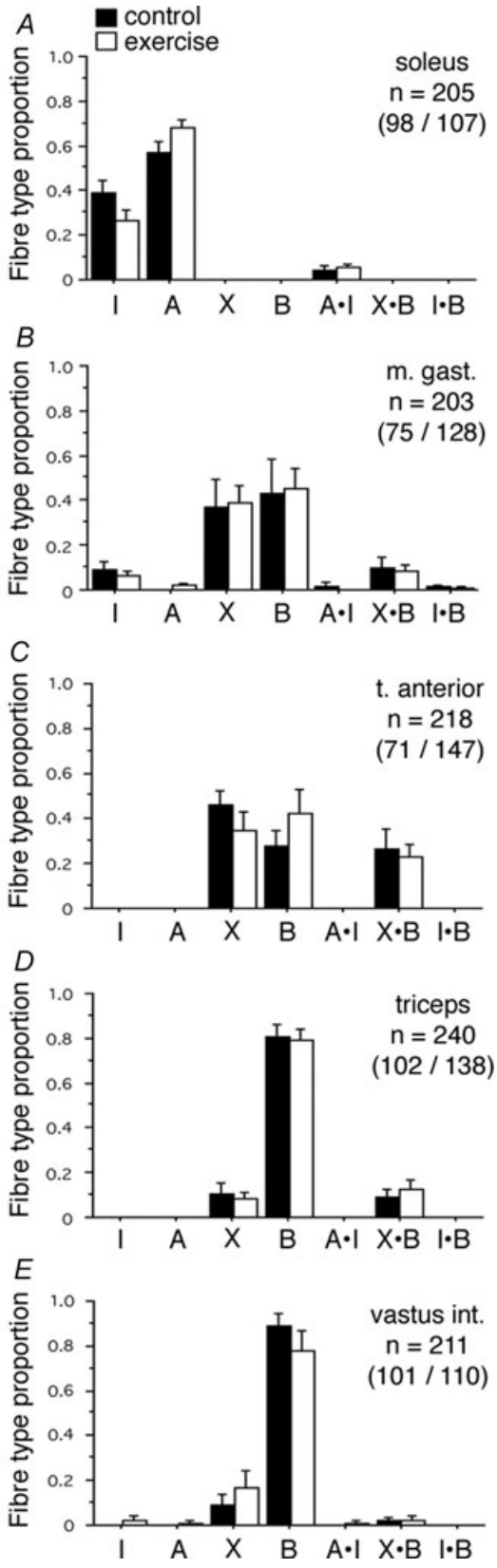


Figure 6. Relative proportion of fibre types in several muscles as determined from single fibre SDS-PAGE

Proportions in control fibres (black) where not significantly different from those in exercised muscles (white). *A*, soleus muscle. *B*, medial gastrocnemius. *C*, tibialis anterior. *D*, triceps brachii. *E*, vastus intermedius. The total number of fibres sampled for each muscle is indicated, and numbers in parentheses are the number of control/number of exercised fibres sampled.

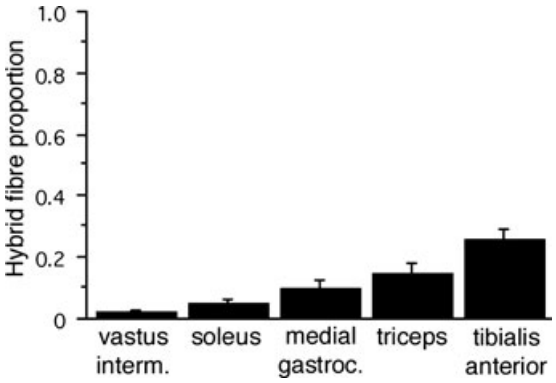


Figure 7. Relative proportion of hybrid fibres in limb muscles sampled

Hybrid fibres represented only about 2% of fibres sampled from the vastus intermedius, but nearly 25% of the fibres in the tibialis anterior. Other muscles were intermediate to these values.

hybrid fibres (25.4 ± 3.9%), all of which were type IIX/IIB (Figs 6*C* and 7). The triceps brachii and vastus intermedius were both composed of a preponderance of type IIB fibres, with a minority of type IIX fibres (Fig. 6*D* and *E*). The triceps brachii had a significant proportion of IIX/IIB hybrids (14.4 ± 3.7%), but these were a small minority in the vastus intermedius (1.9 ± 1.1%; Figs 6*D* and *E* and 7). Since we specifically sampled regions containing mixed fibre types in the gastrocnemius, triceps brachii and vastus intermedius, the overall average proportion of hybrid fibres within the whole muscles is probably much lower than these values. There were no significant differences in fibre type proportions between exercised muscles and control muscles in any of the muscles examined. The results from the single fibre analyses were consistent with the general patterns of fibre type proportions determined from ATPase histochemistry (Fig. 6). Single fibre analysis provides a more precise determination of the prevalence in hybrid fibre types that may be missed with histochemistry.

Discussion

Although other studies have reported changes in rodent muscles in response to running exercise (Allen *et al.* 2001; Pellegrino *et al.* 2005), this is the first to examine the effects of running exercise on the relative proportion of hybrid fibres. Moreover, this is the first study to define the nature and extent of hybrid single fibres from several

limb muscles of the mouse. We found that the hybrid fibre population comprises from 2 to 25% of different muscles or specific regions within muscles, but this proportion was unaffected by 6 weeks of running exercise. By comparison, the same level of running exercise significantly increased the muscle mass from 8 to 18% in weight-bearing muscles, while the tibialis anterior was not affected (Fig. 1). In a subsequent experiment, the same intensity of running exercise caused significant increases in capillary density within the soleus muscle as a function of training duration (Fig. 2). This type of increase in capillary density may precede fibre type changes in exercised muscle (Waters *et al.* 2004), and we intend to follow this process more closely in future studies. Together, these data indicate that the imposed exercise was sufficient to elicit physiological adaptations, but that the hybrid fibre population was not affected. Hybrid muscle fibres in mouse muscles seem to represent stable phenotypes that vary significantly in their proportions among specific muscles.

Based on our observations, we believe that our treadmill running protocol is similar in intensity to a loaded wheel running protocol used in mice (Konhilas *et al.* 2005). Like mice performing loaded wheel running, our animals were made to move their body weight (∼30 g) across some distance. By comparison, mice running on an unloaded wheel are essentially spinning an unloaded wheel around their bodies, without performing nearly as much work in the process. Consistent with this interpretation, the increases in muscle mass in the present study (∼8–20%) are comparable to those observed in the soleus muscles of mice running on a wheel loaded with 9–12 g of resistance (∼20–25%; Konhilas *et al.* 2005). Similar patterns have also recently been observed in rats following several weeks of voluntary resistance wheel running, where several limb muscles increased in mass following training (Legerlotz *et al.* 2008). These patterns contrast with unloaded wheel running in rodents, which results in less pronounced, or no change, in muscle wet mass (Allen *et al.* 2001; Konhilas *et al.* 2005; Legerlotz *et al.* 2008). The total volume of treadmill or loaded wheel training is significantly less than that observed in voluntary unloaded wheel running, but is higher in intensity and may be viewed as intermediate between resistance and endurance training (Konhilas *et al.* 2005). Further work is needed to directly compare the effects of forced treadmill running with voluntary unloaded wheel running on skeletal muscle changes in rodents.

The general effects of exercise training on hybrid fibre proportions in skeletal muscles are currently unclear. If hybrid fibres are viewed as being transient phenotypes in the process of shifting from one fibre type to another, then we might expect training to elicit a shift in their proportions (Pette & Staron, 2001). Indeed, Williamson *et al.* (2001) reported significant reductions in hybrid fibre proportions following several weeks of resistance training, suggesting that exercise may elicit a shift from hybrid fibres towards pure phenotypes. However, bodybuilders with several years of training do not possess significantly different proportions of total hybrid fibres from untrained control subjects, although they do have a larger proportion of the type I/IIA hybrids (Kesidis *et al.* 2008). Similarly, elite female track athletes do not possess different hybrid proportions from untrained subjects (Parcell *et al.* 2003), and several weeks of sprint cycle training failed to cause a change in the relative proportion of hybrid fibres (Parcell *et al.* 2005). These results indicate that the specific type or duration of resistance training may influence the relative proportion of hybrid fibres. An observational study of runners and non-runners found that training volume has a significant influence on the relative proportion of hybrid fibres (Kohn *et al.* 2007*b*). In that study, the relative proportion of type I/IIA hybrids was positively correlated with weekly training distance, while the proportion of the faster type IIA/IIX fibres was negatively correlated with training (Kohn *et al.* 2007*b*). Overall, these findings suggest that a threshold may exist for training volume or intensity, beyond which significant shifts in fibre type can occur. We observed significant changes in muscle mass and capillary density following several weeks of exercise training, but no shifts in muscle fibre type, indicating that this level of exercise was insufficient to elicit changes in muscle fibre type.

Staining patterns for ATPase across different regions within individual muscles revealed a strong tendency for specific fibre types to be organized anatomically (Fig. 3). This type of non-random fibre type distribution, known as fibre type regionalization, is well known in many muscles (Kernell, 1998). A general trend in mammalian muscles is for deeper muscle regions to possess slower fibre types, while the more peripheral muscles are fast (Kernell, 1998). This kind of regionalization seems to be particularly pronounced in rodent muscles (Wang & Kernell, 2000, 2001*a*,*b*; Kohn & Myburgh, 2007), and we found this pattern in the mouse muscles studied here, particularly within muscles with a predominantly fast phenotype (Fig. 3). In the gastrocnemius, the quadriceps complex and the triceps brachii, the outermost regions of the muscles were composed almost entirely (∼98%) of type IIB fibres. In these muscles, a gradation existed from the deepest regions of the muscles outwards, with much of the intermediate regions being comprised of type IIX and IIB fibres. Although the functional significance of fibre type regionalization is still debated, a non-random distribution of fibre types can present challenges for studies based on single fibre samples. The potential problem arises if fibres used for comparison are collected from different anatomical regions, leading to significant differences that result from regionalization, rather than from experimental treatment. In the present study, we avoided this pitfall by targeting specific anatomical regions within muscles

that exhibited significant fibre type regionalization. We also used comparisons between our fibre type proportions made from single fibre analysis with those from standard histochemical staining to ensure that general trends were consistent (Figs 4 and 6).

When examined across several different muscle regions, hybrid single fibres in the mouse muscles appear to represent a stable minority. The relative proportion of hybrid fibres ranged from about 2% up to more than 25% depending on the muscle and the specific anatomical region of the muscle sampled (Fig. 7). These values are significantly lower than the values reported for a number of different rat muscles, which are comprised of as much as 75% hybrid fibres. We found that the majority of these hybrid fibres (∼88%) were of the X/B fibre type, with the tibialis anterior being composed of ∼25% of these fibres. Examination of the data presented by Caiozzo *et al.* (2003) indicates that the majority of the hybrid fibres in rat muscles (∼65%) are also represented by X/B hybrids. Moreover, these X/B hybrids were the predominant fibre type in more than half of the limb muscles examined in that study (Caiozzo *et al.* 2003). The X/B hybrid fibre type thus appears to be one of the major fibre types frequently present in normal rodent muscles.

Overall, mouse muscles are composed of significantly more fast fibres than those of the rat (Wang & Kernell, 2001*b*). Furthermore, mouse MHC isoforms tend to be faster in their shortening velocities than orthologous proteins from the rat (Pellegrino *et al.* 2003; Marx *et al.* 2006). These differences are likely to stem from the 10-fold difference in body size between these two species, because body size significantly affects the kinetics of muscle contraction, with smaller animals possessing faster shortening velocities (Reggiani *et al.* 2000; Marx *et al.* 2006). These differences are related to the operational frequency of the limbs during locomotion, which are about 5 and 8 Hz in the rat and mouse, respectively (Medler & Hulme, 2009). Whatever the precise mechanisms involved, the proportion of fast fibre types is clearly greater in mouse than in rat muscles. We found the soleus muscle to be composed of ∼65% type IIA fibres and ∼30% slow type I fibres in the mouse (∼5% are type I/IIA hybrids; Fig. 6*A*). By comparison, these proportions in the rat are skewed towards slower fibre types, with the type IIA fibres representing only ∼10% of the fibres, while the slow type I fibres represent ∼80% (∼10% are type I/IIA hybrids; Caiozzo *et al.* 2003). These differences are potentially of practical importance to researchers, since both the rat and mouse are common laboratory species, but clearly have significant differences in fibre composition.

It is becoming increasingly clear that hybrid muscle fibres represent a common phenotype in normal skeletal muscles (Stephenson, 2001; Caiozzo *et al.* 2003). Although a comprehensive survey of hybrid fibres in different species is beyond the scope of the present study, hybrid fibres have been identified in a number of different mammalian species (Rivero *et al.* 1996*a*,*b*; Graziotti *et al.* 2001; Smerdu *et al.* 2005, 2009; Toniolo *et al.* 2007), birds (Bartnik *et al.* 1999; Rosser *et al.* 2000), amphibians (Lutz *et al.* 2001, 2002; Stephenson, 2001) and invertebrates (Medler *et al.* 2004; Perry *et al.* 2009). The relative proportions of these fibres vary significantly among species and among specific muscles within species, consistent with our findings in mouse muscles. The stable presence of hybrid fibres has raised a number of basic questions about skeletal muscle organization and function (Stephenson, 2001; Caiozzo *et al.* 2003). One of the most basic questions is whether hybrid muscle fibres have a functional role. Since the contractile properties of hybrid fibres are intermediate to those of pure fibre types, one interpretation is that these fibres provide a continuum of contractile properties to 'fine-tune' the mechanical requirements of the muscles. An alternative hypothesis is that hybrid fibres are simply a consequence of the intermediate properties of the motor neurons that control muscle contraction. If this is the case, then fibres belonging to 'hybrid' motor units should be identifiable within whole muscles. These and other important questions relating hybrid fibres to basic muscle function remain to be investigated, but should provide a rich context for further research (Stephenson, 2001).

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