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Anatomic capillarization is elevated in the medial gastrocnemius muscle of mighty mini mice

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Wong LE, Garland T Jr., Rowan SL, Hepple RT. Anatomic capillarization is elevated in the medial gastrocnemius muscle of mighty mini mice. *J Appl Physiol* 106: 1660–1667, 2009. First published March 12, 2009; doi:10.1152/jappphysiol.91233.2008.—House mice selectively bred for high voluntary wheel running display a mini-muscle (MM) phenotype wherein mass-specific mitochondrial enzyme activities are double that of normal, but muscle mass is reduced by half. In addition, mini-muscles are characterized by small muscle fibers in the superficial region of the plantaris and medial gastrocnemius muscles. To determine the structural alterations facilitating aerobic metabolism in these mini-muscles, cross-sections of the medial gastrocnemius muscle of normal (N; $n = 6$) and mini-muscle (MM; $n = 6$) mice were histo- and immunochemically labeled and analyzed for fiber size, capillarization, and fiber type. On the basis of the higher mitochondrial enzyme activities in muscles of MM mice, we hypothesized that they would have greater fiber capillarization in the medial gastrocnemius than N mice. Furthermore, we hypothesized that augmented capillarization in MM would principally be a function of the smaller fibers in the superficial aspect of this muscle. On average, MM had higher capillary-to-fiber ratio and higher capillary density. Binning fibers according to size revealed that it was primarily the normal-sized fibers of the MM that had higher capillarity. The small fibers seen in the superficial region of MM were distinct from N mice in that they had heterogeneous myofibrillar ATPase staining and patchy succinate dehydrogenase staining in the interior of the fibers. These results support the hypothesis that the MM have higher indexes of capillarity, caused primarily by greater capillary number around normally sized fibers. These alterations are consistent with the superior mass-specific aerobic function of these muscles.

aerobic metabolism; capillary; experimental evolution; selective breeding; skeletal muscle

THE MAXIMAL CAPACITY for aerobic production of ATP in locomotor muscles is dependent in part on the body's ability to deliver oxygen to working muscles (4, 19, 37). It is thought that variations in capillary number and fiber size in muscles across a wide spectrum of aerobic capacity generally represent optimization of oxygen delivery to meet skeletal muscle oxygen demand (30), a view based on the observation that the size of the capillary network is regulated principally in relation to the aerobic capacity of skeletal muscles (21, 25, 50). Historically, a denser capillary network per volume of muscle tissue was thought to facilitate greater oxygen delivery to the mitochondria by minimizing diffusion distance (27), but more recent data documenting low intracellular PO_2 in myocytes during exercise (43) suggest that changes in capillarity may occur to facilitate an increased proportion of fiber surface that

is adjacent to capillaries (32). This may occur by an elevated number of capillaries per fiber and/or small fiber size (30). Small fiber size has been observed in skeletal muscles from animals adapted for high aerobic capacity, such as hummingbirds (35) and bats (36). Rats bred for high treadmill-running capacity also have a smaller fiber size and greater capillary density compared with rats bred for low running capacity, characteristics that are considered important to the superior endurance capacity of the high-capacity running rats (24). Finally, an increase in capillary number per fiber is one of the hallmark features of the response to endurance training (1), including voluntary wheel running in mice (52), and this increase is thought to play an important role in the improvement in skeletal muscle aerobic capacity following endurance training (51). Specifically, an increase in capillary number per fiber increases the surface area available for blood-tissue exchange (30).

To investigate the evolution of locomotor behavior and physiological capacities for exercise in house mice (*Mus domesticus*), artificial selection for increased wheel-running behavior was initiated in the early 1990s (46). Running by the four replicate selected lines increased for ~16 generations until they ran ~2.7-fold more than the four nonselected control lines (6). By generation 22, two of the four replicate selected lines exhibited a substantial frequency of a “mini-muscle” phenotype, in which the “triceps surae” complex (gastrocnemius, plantaris, and soleus muscles) had a 50% smaller mass (7). Although the gene that determines this phenotype has not yet been identified, it is known to behave as a Mendelian recessive (7, 12) and to reside in a 2.6335-Mb interval on MMU11 (13) that harbors ~100 expressed or predicted genes, many with known roles in muscle development and/or function. Mini-muscle individuals routinely run faster on wheels than their normal-muscled counterparts, and in some samples they also run more total revolutions/day (8, 26, 49). Interestingly, the mass-specific enzyme activity is doubled in “mini-muscle” (MM) mice compared with normal (N) mice (23). Two lines of these mice bred for high voluntary running activity have continued to display the mini-muscle phenotype in conjunction with both elevated mitochondrial enzyme activity and decreased fiber size in later generations (9, 10, 23). At the whole animal level, MM individuals tend to run faster on wheels and, in some samples, more total revolutions/day, although not for more minutes per day (8, 12, 26, 49).

Studies in later generations of the selection experiment have also observed an abundance of small fibers in the superficial region of the gastrocnemius muscle of MM mice, previously suggested to be incompletely differentiated type IIb fibers (9). Although some biochemical and morphological analyses have been completed in the muscles of MM mice (8–10, 23, 42), no

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prior studies have looked at capillarity. The purpose of this study, therefore, was to evaluate the morphological characteristics that comprise the anatomic determinants of capillarity insofar as they may contribute to the increased aerobic capacity of the muscles of the MM mice. Given the importance of capillarity to muscle oxygen supply (32) and the higher mitochondrial enzyme activities previously observed in the medial gastrocnemius muscle of MM mice (23, 42), it was hypothesized that the medial gastrocnemius muscle of MM mice would have increased capillarization compared with N mice. Furthermore, due to the aforementioned reports of small fibers along the superficial region of the gastrocnemius muscle (9, 10), we hypothesized that the increase in capillarization would be primarily a function of these smaller fibers rather than an increase in number of capillaries per fiber.

METHODS

Animals. Animals selectively bred for high voluntary running capacity were obtained from the University of California, Riverside. Swallow et al. (46) provide complete details regarding the selection experiment. In brief, voluntary wheel running was quantified in every generation in eight independent lines on days 5–6 of a 6-day wheel-exposure period. Four of the eight lines were assigned to the selection protocol, while the remaining four were used as controls. For selected lines, the highest-running male and female from each family within each line were bred (but not with siblings). After 22 generations, two of the four selected lines showed a substantial frequency of a MM phenotype, in which the mice exhibited a decreased overall body mass and relative triceps surae mass, among other organ-mass differences (7). Because the gastrocnemius muscle exhibits a marked increase in blood flow (and thus oxygen delivery) as a result of its recruitment during locomotion in rodents (2, 28) and because this muscle exhibits both a reduced mass (7) and a higher mitochondrial enzyme activity

in MM mice (23), we obtained medial gastrocnemius muscle samples from male mice of both MM mice ($n = 6$) and N mice ($n = 6$) from two separate generations of selected mice, for a total of 12 animals. Half of the samples (3 N mice and 3 MM mice) at generations 36–38 were part of a study previously published by Syme et al. (49). The second group of samples, also comprising 3 N mice and 3 MM mice from generation 36, were part of a study by Rezende and colleagues (41). During dissection, the medial and lateral heads of the gastrocnemius muscle of one leg were separated and mounted transversely on cork and frozen in liquid isopentane cooled in liquid nitrogen before being stored at -80°C until processing. All procedures that involved animals were approved by the University of California, Riverside, Institutional Animal Care and Use Committee.

In situ labeling. Cork-mounted muscle samples were equilibrated to the temperature of the cryostat (-18°C) and then cut into 10- μm -thick sections. The sections were stored at -80°C until processed for histochemistry. Capillaries were labeled via a lead-ATPase staining procedure first developed by Rosenblatt et al. (44). This method permits identification of a similar number of capillaries as vascular perfusion fixation (17). To establish fiber type, myofibrillar ATPase staining (pH 4.6) (11) was used and results were verified by antibody labeling for developmental myosin heavy chain (dMHC) and fast myosin heavy chain (MHCf). Tissue samples were incubated with the primary antibody (1:10 dilution, anti-dMHC and anti-MHCf, Novocastra) for 1 h at room temperature. Antibodies were visualized with the Vectastain ABC kit (Vector Laboratories) and counterstained with hematoxylin. Fiber oxidative capacity was visualized via succinate dehydrogenase (SDH) staining, as done previously (18).

Morphometry. Morphometric analysis was completed by a single observer who was blind to the identity of the samples. Images of the samples were obtained in three systematically selected regions (as depicted in Fig. 1) and were captured by a digital camera (Nikon CoolPix 990) from the microscope stage (Nikon Eclipse E400; magnification of 200 \times) before being downloaded onto a computer for image analysis (Sigmascan Pro 5.0, SPSS Science). This sampling

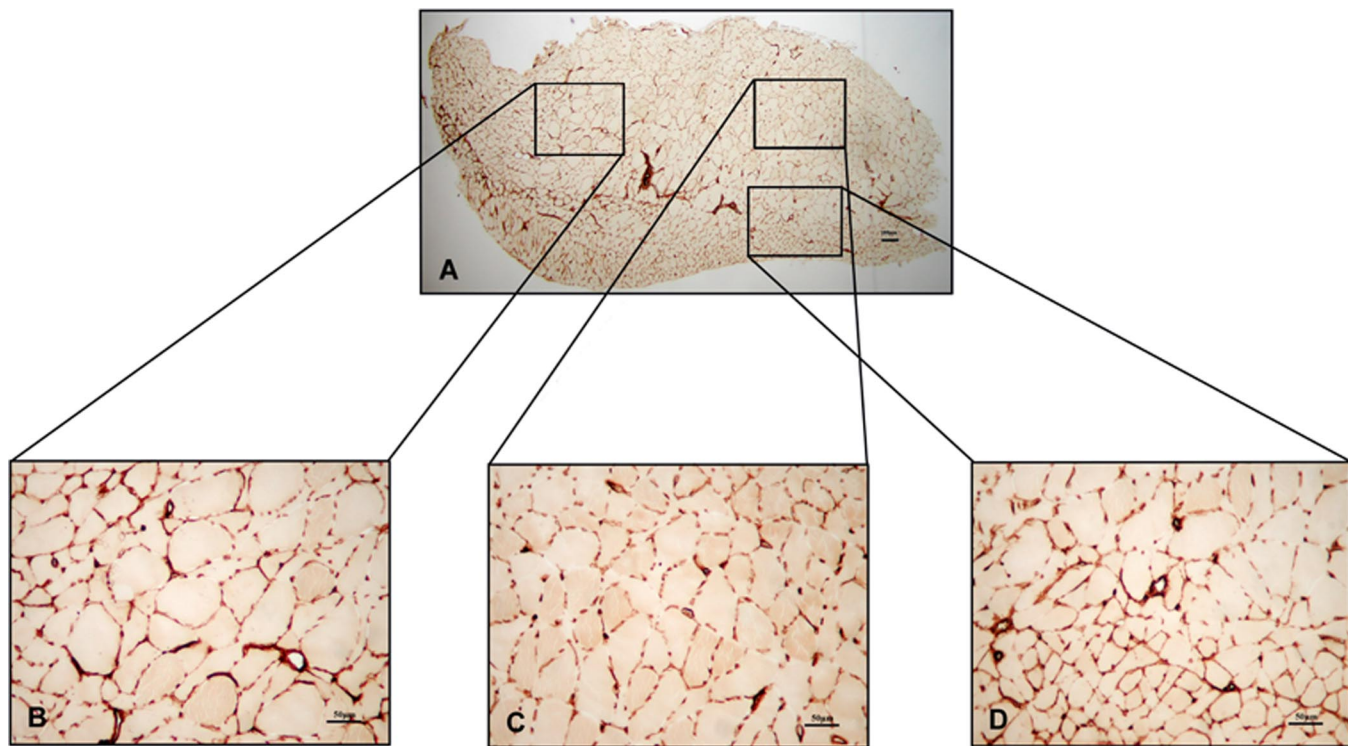


Fig. 1. Photomicrographs of lead ATPase-stained cross sections of the medial head of gastrocnemius muscle in mini-muscle (MM) mice demonstrating the heterogeneity of fiber size within their muscles and the prevalence of very small muscle fibers along the superficial border of this muscle.

approach permitted us to obtain representation of the distinct heterogeneity in fiber size evident in this muscle (Fig. 1). A total of 179 ± 24 fibers per sample were analyzed to obtain a representative sampling of all the fiber types present. For each image, a sampling frame was created within the image to ensure unbiased sampling of all fibers regardless of size, as done previously (17). The image analysis included analyzing fiber cross-sectional area (CSA), the number of capillaries around a fiber (Ncaf), individual capillary-to-fiber ratio (C/F), sharing factor [representing the quotient of Ncaf and C/F (38)], and capillary density at $250\times$ magnification, as done previously for histochemically labeled muscle cross sections (16, 17).

Statistical analysis. Data are presented as means \pm SE. For comparisons between the N and MM muscles, unpaired *t*-tests were used to assess differences between the CSA, C/F, Ncaf, and capillary density. As the distribution deviated from normal for the sharing factor, a Mann-Whitney rank sum test was used to compare this aspect of capillarization (SigmaStat 3.0, SPSS Science).

RESULTS

Although body mass did not significantly differ in MM (34.5 ± 1.2 g) vs. N (37.9 ± 2.2 g), the mass of the medial head of the gastrocnemius muscle was significantly less in MM (33.0 ± 4.2 mg) vs. N (79.8 ± 3.0 mg), consistent with previous studies (41, 49). Fiber CSA varied greatly within individual medial gastrocnemius muscle cross sections from MM. Figure 1 shows the variability in fiber size. While some areas had normally sized and shaped muscle fibers (Fig. 1B), other areas within the muscle had very small, irregularly shaped fibers adjacent to very large, round fibers (Fig. 1C). In addition, the superficial region of the samples in MM mice contained very small fibers (Fig. 1D). Overall, MM fiber CSA ($1,861 \pm 85 \mu\text{m}^2$) was 27% smaller compared with N ($2,545 \pm 245 \mu\text{m}^2$) (Fig. 2). Consistent with this observation, the MM group displayed a shift to the left in distribution of fiber size, indicating a greater number of fibers with smaller CSA (Fig. 2A).

Phenotyping of small fibers in superficial region of medial gastrocnemius muscle. Figure 3 depicts the phenotypes evident in the superficial (Fig. 3, left) vs. deep (Fig. 3, right) regions of the medial gastrocnemius muscle of MM mice, where fibers that share a number represent the same fibers in serial sections. MM mice for the most part exhibited the expected phenotypes in the deep region of this muscle, with fibers exhibiting staining patterns largely consistent with those expected for type I, type IIa and type IIb fibers. However, MM mice exhibited several features that were distinct from N mice with respect to the phenotypes of the fibers in the superficial region of this muscle, a region normally dominated by type II fibers in N mice (Fig. 4A). These distinct features included a heterogeneous myosin ATPase (mATPase) staining pattern (Fig. 3A) and abnormal SDH staining pattern marked by an inconsistent and sometimes complete lack of SDH staining in the interior of the fibers (Fig. 3D). It was noteworthy, however, that none of the fibers in the superficial region of MM mice were positive for developmental myosin heavy chain, which does not support the previous suggestion that these cells are incompletely differentiated fibers (9). These issues are elaborated on in DISCUSSION.

Capillary data. As shown in Fig. 5, the sharing factor was 8% lower in MM (2.29 ± 0.16) than N (2.64 ± 0.04) ($P = 0.004$) (Fig. 5A), whereas there was no difference in Ncaf between groups (6.44 ± 0.46 for N and 7.26 ± 0.39 for MM;

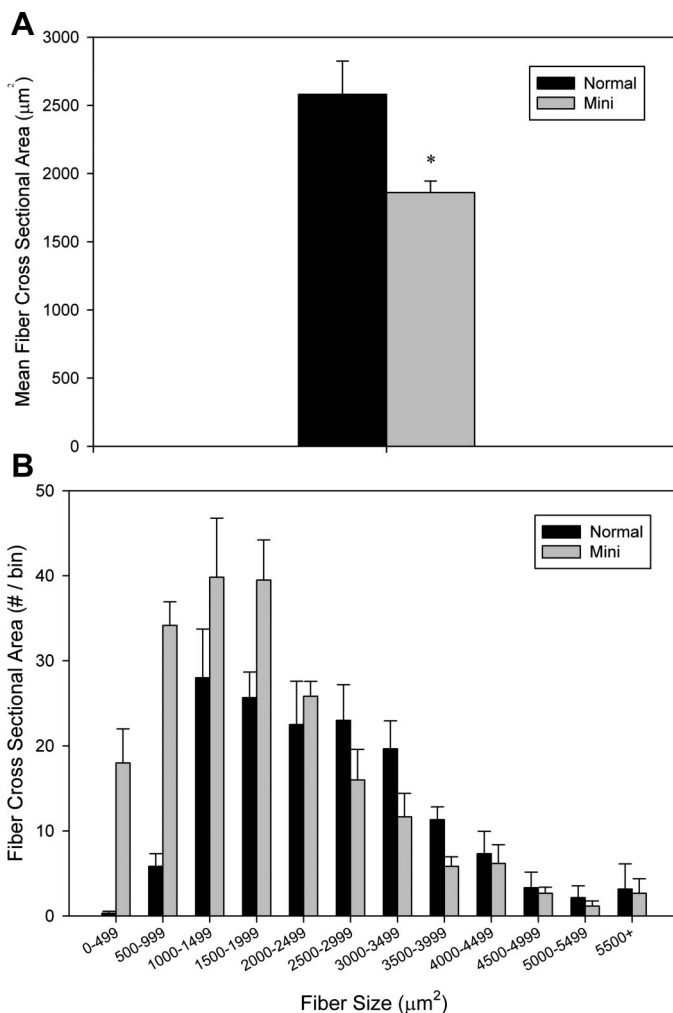


Fig. 2. Mean fiber size (A) and fiber size distribution (B) in cross sections of medial gastrocnemius muscle from normal (N; black bars) and MM (gray bars) mice. * $P < 0.05$ vs. N mice.

$P = 0.204$) (Fig. 5B). C/F was 32% greater ($P = 0.029$) in MM (3.24 ± 0.22) vs. N (2.45 ± 0.21) (Fig. 5C). Capillary density was also 76% greater ($P = 0.010$) in MM ($1,772 \pm 191$ capillaries/ mm^2) than N ($1,007 \pm 115$ capillaries/ mm^2) (Fig. 5D). When indexes of capillarization were examined on the basis of fiber size, it was observed that in MM, Ncaf increased with fiber size whereas N displayed relatively consistent Ncaf throughout the fiber size distribution (Fig. 6A). C/F was also greater in larger fibers in MM, and in the case of both Ncaf and C/F, the larger fibers had more capillaries in MM than fibers of the same size in N (Fig. 6B).

DISCUSSION

Artificial selection for high voluntary wheel-running behavior in house mice (*Mus domesticus*) resulted in expression of a MM polymorphism in mice with high voluntary activity (7). The MM phenotype is characterized by a mass-specific increase in aerobic capacity and a 50% decrease in triceps surae mass (7, 23, 42). The purpose of the present study was to assess what structural adaptations contribute to the previously reported (9, 10, 23, 42) increased mass-specific aerobic capacity in medial gastrocnemius muscle from MM mice compared

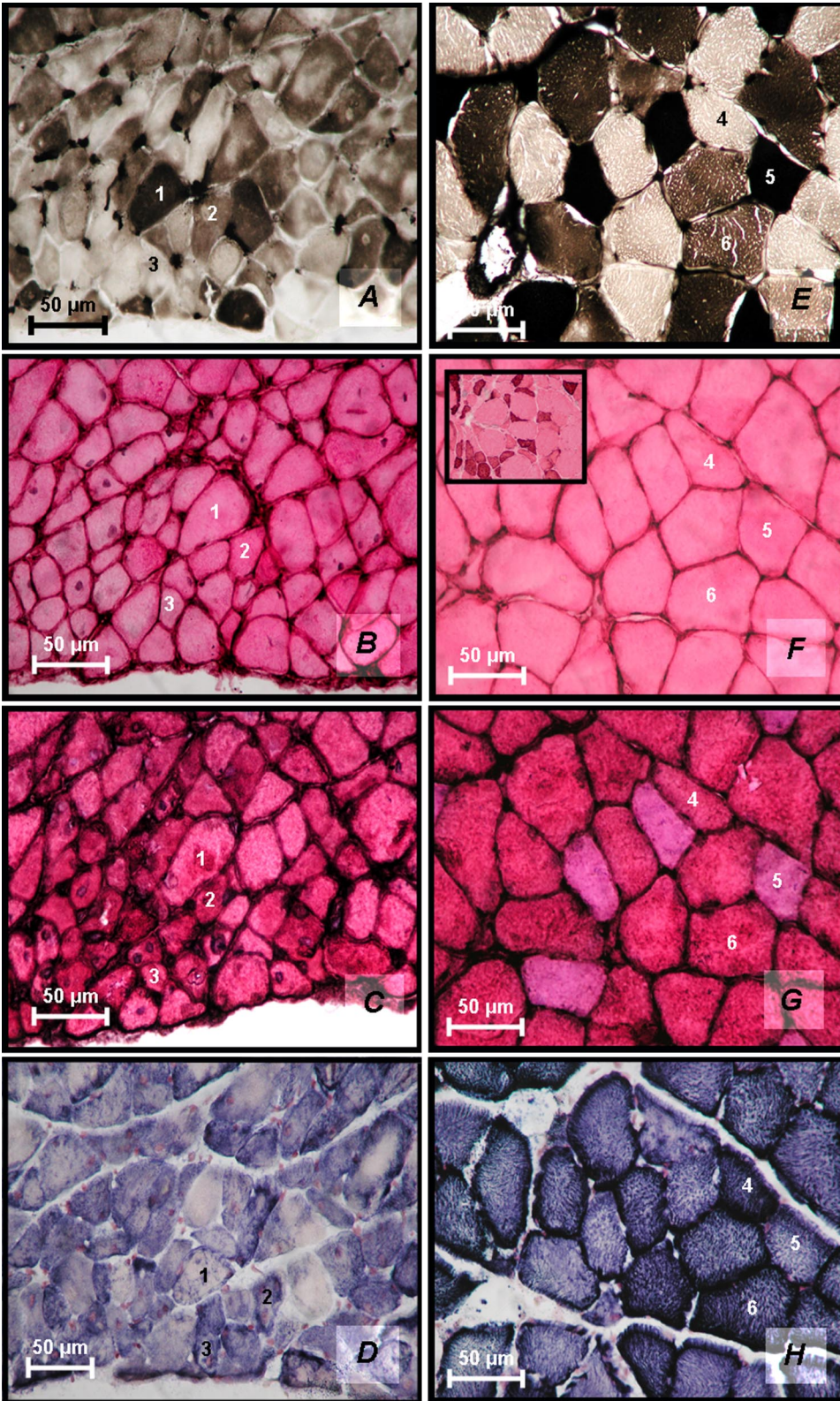


Fig. 3. Photomicrographs of serial sections of the medial gastrocnemius muscle of a mini-muscled mouse in the superficial (A–D) and deep (E–H) aspects of this muscle. Serial sections are labeled for myosin ATPase (pH = 4.6; A, E), developmental myosin heavy chain (B, F), fast myosin heavy chain (C, G), and succinate dehydrogenase (D, H). *Inset* in F represents a positive control for the developmental myosin heavy chain antibody in senescent rat gastrocnemius muscle. Numbers identify the same fibers in serial sections.

with N mice. It was hypothesized that the MM medial gastrocnemius muscle would have increased capillarization due to either an elevated capillary number per fiber or a decreased fiber size (or both), features commonly found in animals adapted for high aerobic capacity, either evolutionarily (30) or

via endurance training (15). Consistent with this hypothesis, results from our analyses indicated that MM mice have greater capillary density, both as a function of a subpopulation of very small fibers (area < 1,000 μm^2), as well as greater capillary number around larger fibers. Most of the very small fibers were

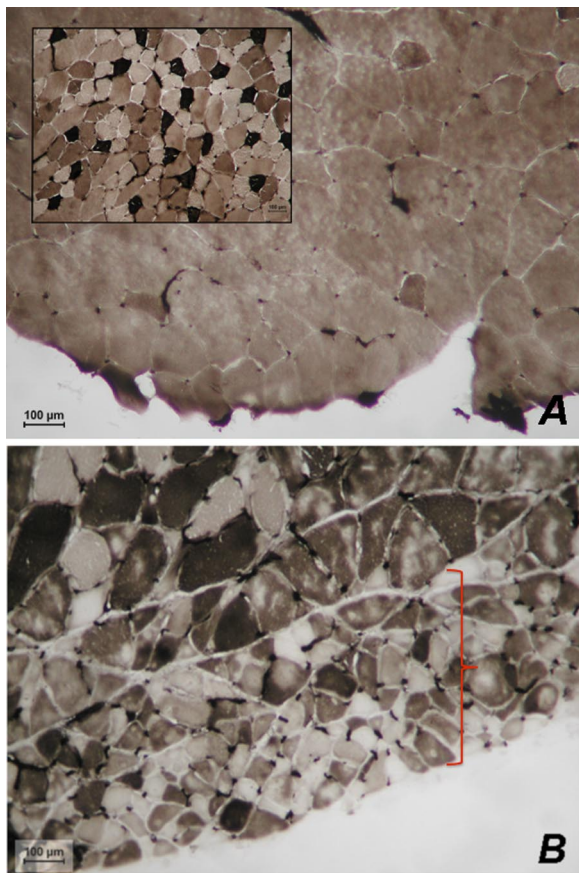


Fig. 4. Myosin ATPase stained sections (pH = 4.6) of the superficial aspect of the medial gastrocnemius muscle from a normal mouse (A) and a mini-muscled mouse (B). A, inset, illustrates the typical mosaic staining pattern in the normal mouse in the deeper portion of the muscle. Red bracket in B indicates the region of the medial gastrocnemius muscle that is dominated by type IIb (intermediate staining intensity) in fiber type composition in a normal mouse.

located along the superficial aspect of the muscle, a region where type IIb fibers dominate in N mice, but by the criteria we used these small fibers could not be distinctly fiber typed (ambiguous mATPase staining, positive for MHCf, negative for dMHC, low and often absent SDH staining). Thus, while an increased capillary density is caused in part by accumulation of small muscle fibers in the superficial region of the muscle of MM mice, there was also a markedly greater number of capillaries per fiber in fibers of normal size, showing that the higher mass-specific aerobic capacity of the medial gastrocnemius muscle in MM mice is associated with distinct alterations in different muscle regions that increase anatomic capillarization.

MM muscles. After 13 generations in this breeding experiment, lines selected for high voluntary activity ran approximately double the daily distance compared with control lines, mainly by running faster rather than for more minutes per day (47). Body mass of mice from the high voluntary activity lines was also significantly lower than control lines, due in part to lower fat mass (47) and lower muscle mass (22), characteristics that have also been demonstrated in rats following 12 wk of voluntary wheel running (45). In contrast, although body fat was reduced after 8 wk of voluntary wheel access in high voluntary activity mice from our lab previously

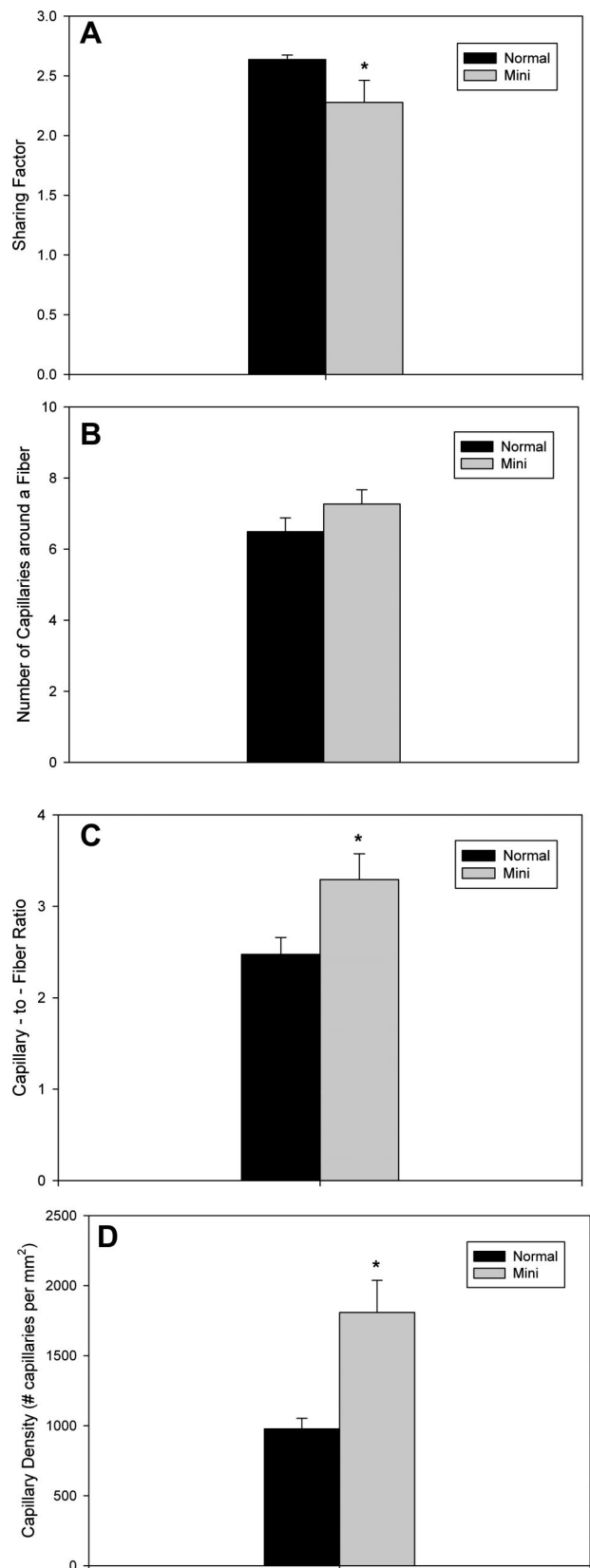


Fig. 5. Morphological indexes of capillarization in cross sections of the medial gastrocnemius muscle of N (black bars) and MM (gray bars) mice. A: sharing factor. B: no. of capillaries around a fiber. C: capillary-to-fiber ratio. D: capillary density. * $P < 0.05$ vs. N mice.

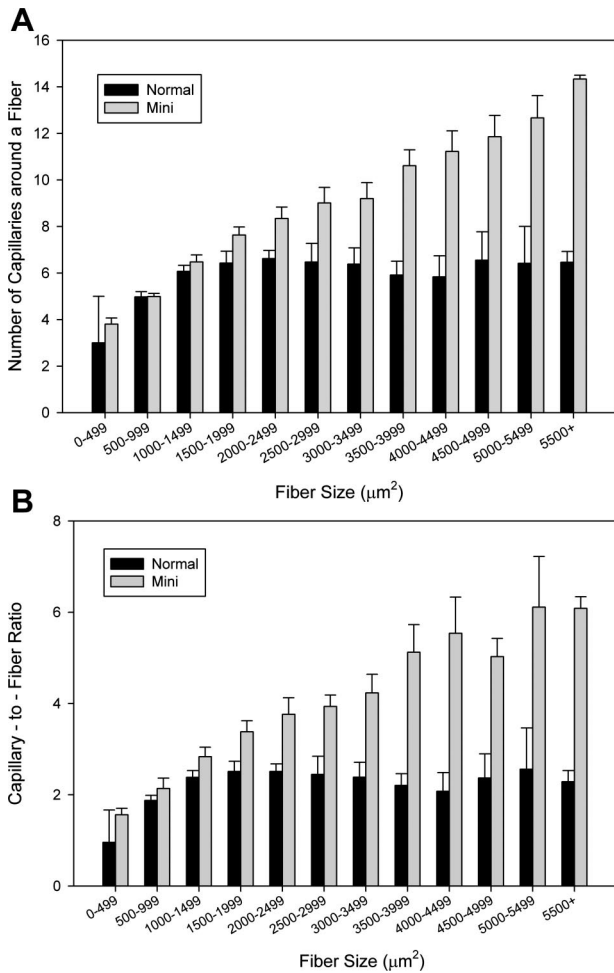


Fig. 6. Morphological indexes of capillarization plotted as a function of fiber size in cross sections of medial gastrocnemius muscle in N (black bars) and MM (gray bars) mice.

(47), voluntary wheel access per se was not associated with a reduction in triceps surae mass (48).

The appearance of the MM polymorphism was first reported in two of four of the lines selected for high voluntary activity, and in one of the four nonselected control lines (7), which eventually lost the phenotype. Subsequent analysis of MM mice found not only that the medial gastrocnemius muscle smaller, but that it also had higher mass-specific aerobic capacity (10) and slower twitch properties (49). Our current morphometric analysis in the medial gastrocnemius muscle showed that the decrease in overall muscle size in MM is a function of both a greater number of small fibers (<1,000 μm^2) and fewer fibers of a larger size (between 2,500 and 4,000 μm^2), whereas there was similar distribution of fibers > 4,000 μm^2 in both groups. Note also that the location of the fibers less than 1,000 μm^2 in MM was largely in the superficial region of the muscle, a location in N mice that is comprised of type IIB fibers (Fig. 4). Both the left-shifted fiber size distribution and location of the small muscle fibers in medial gastrocnemius muscle is similar to the first report documenting small fibers in the superficial fiber layers of the plantaris muscle (9). Previously it was suggested that these small fibers may be satellite cells or type IIB fibers that did not fully

complete differentiation (9). This idea was based on the increase in specific activities of mitochondrial enzymes and reduced type IIB myosin heavy chain expression (9) in the plantaris muscle. This idea may also apply to the medial gastrocnemius muscle, where it was previously reported that there is a 50% reduction in type IIB fiber proportion (10). However, no prior studies have directly assessed whether these small fibers were undifferentiated in either medial gastrocnemius or plantaris muscles. Our present analysis has shown that these small fibers in medial gastrocnemius muscle are not expressing dMHC, which is an isoform typically only seen during embryogenic myogenesis (3) or during muscle regeneration following injury (14). Thus, on the basis of this criterion, the fibers have not stalled in the myogenic differentiation program. With respect to other criteria for describing fiber phenotype, this superficial region in MM mice has other characteristics that distinguish it from the superficial region in N mice. Specifically, we observed a markedly heterogeneous mATPase staining pattern (Fig. 3A), with some fibers exhibiting a staining pattern consistent with type I fibers (*fiber 1*), and other fibers exhibiting a staining pattern consistent with type IIa fibers (*fiber 3*), in addition to the expected type IIB fibers (*fiber 2*). In addition, the morphological features of the fibers in this region were also distinctive, with the fibers being much smaller than normal, and generally having a much poorer defined staining pattern with respect to the mATPase and SDH stains. Notably, the SDH staining pattern was considerably less intense than any fiber types found in the deep region of this muscle in MM mice. In addition, the SDH staining pattern was characterized by a very patchy distribution and in many cases a complete lack of staining in the interior. Thus, although the small fibers typified in this region of the muscle were negative for the developmental myosin heavy chain, the patchy SDH staining pattern could be taken as evidence of incomplete differentiation of these fibers. Clearly, a more definitive explanation for the nature and basis of the alterations in fiber phenotypes within this region of the medial gastrocnemius muscle in MM mice remains to be provided.

Changes in muscle structure to facilitate high aerobic capacity. After 36 generations of selective breeding, MM mice displayed higher maximal oxygen uptake ($\dot{V}O_{2\text{max}}$) during forced treadmill running in hypoxia but not normoxia or hyperoxia (41). Further to this, several previous studies have documented heavier heart ventricles, after adjusting statistically for body mass, in MM individuals (7, 12, 42, 48). It is also worth noting that mass-specific ventricle myoglobin content is marginally significantly higher in MM mice (42). At the enzymatic level, the medial gastrocnemius muscle of MM demonstrated a 71% higher citrate synthase activity and 165% higher cytochrome oxidase activity (10) at generation 14. These differences are similar to what is seen in animals adapted to high aerobic demand either through evolutionary selection or in response to exercise training. Specifically, differences in mitochondrial content (greater), capillary number (greater), and muscle fiber size (smaller) are similar in many species with very high aerobic demand compared with less aerobic animals of similar size (21, 30). Endurance exercise training also increases mitochondrial enzyme activities (20) and capillary number (1), whereas a reduction in muscle fiber size in this context is not consistently seen (15, 48). Similarly, we have observed previously that 8 wk of voluntary wheel access leads

to increased skeletal muscle mitochondrial enzyme activities in our lines of mice (22) but does not change triceps surae mass (meaning a reduction in fiber size is unlikely) (48). In other studies it was shown that 12 wk of voluntary wheel running in rats leads to increased skeletal muscle citrate synthase activity and enhanced capillary diffusing capacity (45), and 4 wk of voluntary wheel running in mice leads to an increased capillary-to-fiber ratio and capillary density in skeletal muscle (52). In this respect, the increases in fiber mitochondrial content and fiber capillarization are proportional to one another after exercise training (39), and also after more extreme increases in muscle activation via chronic electrical stimulation (31). Thus, given the aforementioned higher levels of mitochondrial enzyme activities seen in the medial gastrocnemius muscle of MM mice (9, 10, 23, 42), it was hypothesized that these muscles would also demonstrate greater fiber capillarization.

In the present study, the differences in capillarity between N and MM are consistent with the greater mass-specific aerobic capacity in medial gastrocnemius muscle of MM mice. Smaller mean fiber CSA, as observed in the superficial region of medial gastrocnemius muscle in MM, is also consistent with comparative studies showing that animals adapted to high aerobic demand, like bats and hummingbirds, typically have very small muscle fibers relative to less aerobic animals of similar size (30). Small fiber size is thought to help to minimize diffusion distances from the vascular bed to the mitochondria by increasing capillary density (27). However, it is interesting to note that in MM mice the more normal-sized fibers ($\geq 2,000 \mu\text{m}^2$) had greater numbers of capillaries around them relative to fibers of the same size in N mice. This shows that the factors accounting for the greater capillary density in MM mice include not only smaller fiber size in the superficial region of the muscle, but also augmented capillary number per fiber throughout the remaining part of the muscle where more normal-sized fibers occur. These larger fibers also have greater mitochondrial content, as indicated by greater SDH staining intensity (see Fig. 3, *H* vs. *D*), showing that the previously observed doubling of mitochondrial enzyme activities (23, 42) and mass-specific myoglobin content in MM (42) are matched by proportionally greater capillary number.

Although not assessed in the present study, capillary tortuosity also affects the amount of capillary surface area (32). Previously, it was shown that tortuosity varies largely as a function of sarcomere length rather than muscle aerobic capacity, and that tortuosity increases as sarcomeres shorten with muscle contractions (29, 33, 34). Furthermore, although there is some variability in capillary tortuosity between different species (30), it appears that neither endurance running training (40) nor chronic electrical stimulation (31) (both of which increase capillary number) affect capillary tortuosity. Thus it seems unlikely on this basis that capillary tortuosity would differ between N and MM mice.

Evolutionary adaptation to high aerobic demand has led to similar characteristics in a broad range of species and has exemplified the "plasticity" of skeletal muscle. Use of an experimental evolution approach (5) led to discovery of the "mini-muscle" phenotype, and in the context of the present results, demonstrated a novel pattern of adaptation to facilitate an increased muscle capillarization. Specifically, it is usually observed that high muscle capillarization is facilitated by either a reduced fiber size (e.g., as in hummingbirds) or an increased

capillary number per fiber (e.g., as seen following endurance training), but not both within distinct regions of the same muscle. In contrast to this typical pattern, the present results show that the medial gastrocnemius muscle of MM mice is associated with region-specific alterations within the same muscle where the superficial region achieves a higher capillary density via a reduced fiber size, and the remaining part of the muscle achieves a higher capillary density by increasing the number of capillaries surrounding individual fibers. As such, our results further demonstrate the considerable evolutionary plasticity of the structures that facilitate aerobic performance. Understanding these morphological characteristics and other adaptations in the mighty "mini-muscle" mouse will help illustrate how locomotor behavior coadapts with physiological capacities for exercise and will broaden our understanding of evolutionary processes.

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