

Phenotypic Effects of the “Mini-Muscle” Allele in a Large HR × C57BL/6J Mouse Backcross

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Abstract

From outbred Hsd:ICR mice, we selectively bred 4 replicate lines for high running (High-Runner [HR] lines) on wheels while maintaining 4 nonselected lines as controls (C lines). An apparent Mendelian recessive, the “mini-muscle” (MM) allele, whose main phenotypic effect is to reduce hindlimb muscle mass by 50%, was discovered in 2 HR lines and 1 C line. This gene of major effect has gone to fixation in one selected line, remains polymorphic in another, and is now undetectable in the one C line. Homozygotes exhibit various pleiotropic effects, including a doubling of mass-specific muscle aerobic capacity, and larger hearts, livers, and spleens. To create a population suitable for mapping the genomic location of the MM allele and to better characterize its pleiotropic effects, we crossed females fixed for the MM allele with male C57BL/6J. F₁ males were then backcrossed to the MM parent females. Backcross (BC) mice ($N = 404$) were dissected, and a 50:50 ratio of normal to MM phenotype was observed with no overlap in relative muscle mass. In the BC, analysis of covariance revealed that MM individuals ran significantly more on days 5 and 6 of a 6-day exposure to running wheels (as in the routine selective-breeding protocol), were smaller in body mass, and had larger ventricles and spleens.

A basic assumption of quantitative genetics is that most aspects of the phenotype are affected by alleles segregating at multiple loci, each having a small effect on the trait (Fisher 1930). However, recent studies suggest that genes of major effect (GOMEs) are important during the adaptive evolution of many traits and that these genes are almost always associated with a large number of pleiotropic effects (Orr and Coyne 1992; Bradshaw et al. 1998; Orr 1998; Agrawal et al. 2001). GOMEs have been identified in both wild and captive populations. Some important examples in domestic livestock are the muscle-doubling gene in cattle (Rollins et al. 1972), dwarfing gene in poultry (Merat and Ricard 1974), *DGAT1* gene in dairy cattle (Grisart et al. 2002), and *IGF2* gene in pigs (Van Laere et al. 2003). Notable examples in natural populations are the *PITX1* gene, which alters the pelvis in stickleback fishes (Shapiro et al. 2004), and the *Mt1r* gene, which affects coat color in beach mice (Hoekstra et al. 2006). GOMEs also play a critical role in genetic research surrounding several major human health problems. For example, gene expression

studies of GOMEs have provided valuable insight concerning treatments for both leukemia (Mullighan et al. 2007) and autism (Muhle et al. 2004).

We identified a GOME, termed the “mini-muscle” (MM) allele (Garland et al. 2002), within some of the replicate lines of a long-term selection experiment for high locomotor activity in house mice. Selection began in 1993 from a base population of outbred Hsd:ICR mice (Swallow et al. 1998; Garland 2003). After initial generations of random mating, the base population was divided randomly into 8 lines, 4 of which serve as controls (C lines), whereas the other 4 are selectively bred for high voluntary wheel running (High-Runner [HR] lines). After 16 generations, mice from the HR lines ran an average 170% more revolutions/day than C-line mice. These mice have been the subjects of many behavioral and physiological studies, which are summarized elsewhere (e.g., Garland 2003; Rhodes et al. 2005; Swallow et al. 2005; Rezende, Gomes et al. 2006; Rezende, Kelly et al. 2006; Belke and Garland 2007; Girard et al. 2007; Malisch et al. 2007).

The putative MM allele was discovered via dissections of early generations that revealed a subset of individuals with an approximate 50% reduction in “triceps surae” (gastrocnemius, soleus, and plantaris) mass relative to wild-type mice (Garland et al. 2002). Further analyses indicated that the allele operated as a simple Mendelian recessive. The phenotype was only observed in 3 of the 8 lines (1 C, 2 HR). In the one C line (laboratory designated line 5), the phenotype was apparently lost sometime after generation 22. In one HR line (laboratory designated line 6), the phenotype remains polymorphic as of generation 50. In the other HR line (laboratory designated line 3), the mutation apparently had gone to fixation by generation 36 (Syme et al. 2005). Model fitting and statistical analysis indicated that the MM allele must have been favored by the selection protocol in the HR lines (Garland et al. 2002). Loss of the allele from 2 of the HR lines and from all 4 C lines can be explained by random genetic drift. Whether the MM allele occurs at an appreciable frequency in any wild population of house mice is presently unknown.

The most obvious phenotype associated with the MM allele in homozygotes is a 50% reduction in mass of the triceps surae muscle complex (Garland et al. 2002) as well as in mass of mixed hindlimb muscle exclusive of the triceps surae (Houle-Leroy et al. 2003). However, the MM allele has many pleiotropic effects. For example, homozygous individuals exhibit a doubling of mass-specific aerobic capacity as compared with normal muscle (Houle-Leroy et al. 2003), along with altered mitochondrial density and myosin heavy chain composition (Guderley et al. 2006); altered fiber type composition in the gastrocnemius (Guderley et al. 2008); elevated HSP72 expression in triceps surae (Belter et al. 2004); altered muscle contractile performance (Syme et al. 2005); an increase in size of their ventricles, liver, and spleen (Garland et al. 2002; Swallow et al. 2005); and longer and thinner hindlimb bones (Kelly et al. 2006). Many of these pleiotropic effects would appear conducive to the support of sustained aerobic exercise (Garland 2003; Guderley et al. 2006; Rezende, Gomes et al. 2006).

The aim of the present study was to determine whether the Mendelian recessive nature of the mutation is unique to Hsd:ICR mice or robust against a different genetic background and whether some of the pleiotropic effects segregate with the mutation and display a similar expression pattern within a different genetic background. Finally, if the mutation breeds true, then the resulting population would be ideal for marker analysis to map the genomic position of the mutation and identify the underlying gene.

Materials and Methods

Animals

Full details of the selection experiment have been provided elsewhere (see Swallow et al. 1998), but we provide a brief overview here. The original progenitors of the selection experiment were 224 individuals of the outbred, genetically variable house mice (*Mus domesticus*; Hsd:ICR; Harlan

Sprague Dawley, Indianapolis, IN). Mice were randomly mated for 2 generations and randomly assigned to 8 closed lines (10 mating pairs in each). In successive generations, mice from each of the 8 lines did not mix. In each line for each generation, offspring were weaned at 21 days of age and at 6–8 weeks of age were individually housed with access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days. Food (Harlan Teklad, Madison, WI, Rodent Diet [W] 8604) and water were available ad libitum. Daily wheel-running activity was monitored with a computer-automated system. Wheel running was quantified as the total number of revolutions run on days 5 and 6 of the 6-day test.

In the 4 HR lines, the highest-running males and females from each family (highest number of revolutions on days 5 and 6) were chosen to propagate the lines into the next generation (i.e., within family selection). In the 4 C lines, breeders were randomly chosen from each family. Within all lines, males and females were randomly paired, excluding sibling mating.

Females for the current experiment originated from generations 42 ($n = 60$) and 43 ($n = 17$) from the HR line (laboratory designated line 3) that is fixed for the MM allele (Syme et al. 2005). Mice were weaned at 21 days of age and then wheel tested at 6–8 weeks of age following the routine selection protocol (as described above). After wheel testing, a portion of the females were used as breeders to supply animals for the next generation. Therefore, some of the females had given birth to one litter prior to being used in the current study.

Adult males ($n = 24$; C57BL/6J strain) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J was the source of DNA for the first draft sequence of the mouse genome (Mouse Genome Sequencing Consortium 2002), thus making it ideal for future genetic mapping studies.

Breeding Design

We harem-mated 20 (of 24 available) C57BL/6J males with 3 HR females. When a dam appeared to be pregnant, it was removed from the cage and housed separately. From the 60 mating pairs, 33 litters were produced, yielding a total of 316 F₁ individuals at weaning (21 days of age). Ninety males from these F₁s were randomly chosen and represented all 33 sibships (1–3 males were chosen from each sibship).

At 8 weeks of age, F₁ males were backcrossed to the original 60 HR line 3 parent females to produce the backcross (BC) generation. To ensure enough BC animals for the eventual mapping of the MM allele, 17 additional HR line 3 females from generation 43 were also mated to F₁ males (13 of 90 F₁ males were kept as backups). All breeding pairs were randomly assigned with mother–son and aunt–nephew mating disallowed. When the females appeared pregnant, the F₁ males were removed. Of 77 mating pairs, 53 litters were produced, yielding a total of 553 BC individuals at weaning. The total number of BC individuals was reduced from 553 to 404 by randomly choosing a maximum of 4 males and 4 females from each litter, when available. The 404 individuals represented all 53 families that produced a successful litter.

Wheel Access and Dissection

When BC individuals reached 43 ± 3 (\pm standard deviation [SD]) days of age, 384 (due to space constraints) were singly housed and given access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days as in the routine selection regime (see above and Swallow et al. [1998] for details). Food (Harlan Teklad, Rodent Diet [W] 8604) and water were always available ad libitum. Rooms were controlled for temperature (~ 22 °C) and photoperiod 12:12 h light:dark cycle (lights on 0700). Wheels were checked daily to ensure freedom of rotation. Wheel running was monitored with a computer-automated system, and revolutions were recorded in 1-min bins (intervals). Wheel running was quantified as means for days 5 and 6 of the 6-day test. We analyzed means for total revolutions/day, the number of intervals/day with at least one revolution, the mean speed when running (revolutions/intervals), and the highest single 1-min interval/day.

After wheel testing, mice were sacrificed by CO₂ inhalation in batches to allow for the harvesting of organs and muscle tissue. Mean age at sacrifice was 234 ± 41 (\pm SD) for MM parent females (generations 42 and 43), 94 for C57BL/6J males, 74 ± 9 for F₁ animals, and 57 ± 8 for BC individuals. After sacrifice, mice were weighed and dissected. The heart was detached, and ventricles were removed from the atria and connecting blood vessels. Ventricles were blotted to remove any excess blood prior to weighing. The spleen was excised followed by the right and left triceps surae muscles (which include the lateral and medial heads of the gastrocnemius, soleus, and the plantaris, as described in Carter et al. 1999). Wet masses of all tissues were recorded to the nearest 0.001 g on an electronic balance (Denver Instruments, Denver, CO, model M-220).

Statistical Analysis

The MIXED procedure in SAS (version 9.1; SAS Institute, Cary, NC) was used to apply analysis of covariance (ANCOVA) models. A 2-way ANCOVA was used to test for the effects of MM (normal phenotype vs. MM phenotype) and sex (male vs. female) on wheel running, ventricle mass, and spleen mass in the BC generation of mice. Effects of the MM \times sex interaction were also examined. Age, body mass, and wheel freeness were used as additional covariates when applicable. Family was a random effect in all analyses. Body mass and organ masses were log₁₀ transformed because this was expected to achieve linearity of allometric relations. Wheel-running traits were transformed as necessary to achieve normality of residuals.

Results

Phenotype Observation and Characterization

The relation of triceps surae muscle mass to body mass in mice from the parent, F₁, and BC generations is shown in Figure 1. Muscles from MM individuals could be identified visually in such graphs regardless of their body mass, sex, or

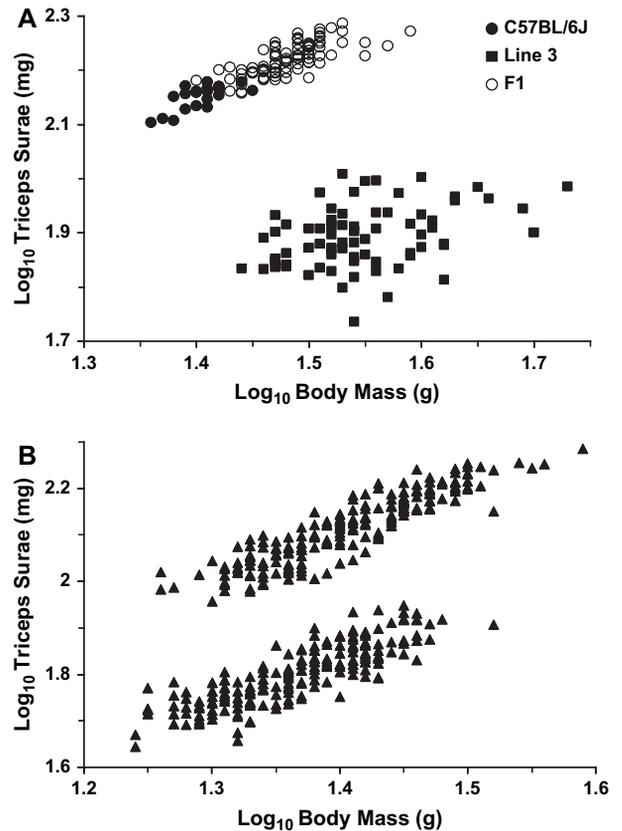


Figure 1. Relation between log₁₀ triceps surae mass and log₁₀ body mass for line 3 HR females, C57BL/6J males, their F₁ offspring, and BC individuals. As shown in panel (A), the MM phenotype was observed in all line 3 parent females, no C57BL/6J parent males, and no F₁ individuals, thus indicating its recessive nature. As shown in panel (B), the BC generation of mice exhibited a 1:1 ratio of normal ($N = 203$) to MM ($N = 201$) phenotypes, with no intermediates. See text for statistical analyses.

lineage. As expected, all line 3 HR females expressed the MM phenotype, whereas none of the C57 or F₁ individuals did. For the 74 HR females (3 excluded before dissection), average age at dissection was 234 ± 41 (\pm SD) days, range = 146–261. Mean triceps surae mass was 0.0788 ± 0.0102 g (\pm SD), range = 0.0544–0.1020 g, whereas mean body mass was 35.64 ± 5.080 g (\pm SD), range = 27.54–53.65 g.

For 21 C57BL/6J males (20 of which were part of the actual breeding design), the mean age at dissection was 94 days, range = 91–97. Mean triceps surae mass was 0.1416 ± 0.0073 g (\pm SD), range = 0.1269–0.1507 g, with a mean body mass of 25.34 ± 1.235 g (\pm SD), range = 22.94–28.01 g.

Mean age at dissection for F₁ individuals ($n = 89$, one died before dissection) was 74 ± 9 (\pm SD) days, range = 61–106. Mean triceps surae mass was 0.1663 ± 0.0116 g (\pm SD), range = 0.1435–0.1934 g, with mean body mass of 30.44 ± 2.313 g (\pm SD), range = 25.32–39.34 g.

Table 1. ANCOVA for effects of the MM phenotype in the BC generation

Trait	N	MM	Sex	Mini × sex	Age	log ₁₀ body mass
log body mass (g)	403 ^a	$F(1,397) = 129.6,$ $P < \mathbf{0.0001}$ (–)	$F(1,397) = 587.0,$ $P < \mathbf{0.0001}$ (+)	$F(1,397) = 1.1,$ $P = 0.2964$	$F(1,397) = 60.4,$ $P < \mathbf{0.0001}$ (+)	
log triceps surae mass (g)	404	$F(1,397) = 6686.6,$ $P < \mathbf{0.0001}$ (–)	$F(1,397) = 69.6,$ $P < \mathbf{0.0001}$ (+)	$F(1,397) = 7.5^b,$ $P = \mathbf{0.0066}$	$F(1,397) = 19.0,$ $P < \mathbf{0.0001}$ (+)	$F(1,397) = 373.8,$ $P < \mathbf{0.0001}$ (+)
log ventricle mass (g)	402 ^a	$F(1,395) = 157.5,$ $P < \mathbf{0.0001}$ (+)	$F(1,395) = 2.9,$ $P = 0.0890$ (+)	$F(1,395) = 0.1,$ $P = 0.7544$	$F(1,395) = 17.9,$ $P < \mathbf{0.0001}$ (–)	$F(1,395) = 479.4,$ $P < \mathbf{0.0001}$ (+)
log spleen mass (g)	403 ^a	$F(1,396) = 85.6,$ $P < \mathbf{0.0001}$ (+)	$F(1,396) = 44.2,$ $P < \mathbf{0.0001}$ (–)	$F(1,396) = 1.3,$ $P = 0.2646$	$F(1,396) = 0.5,$ $P = 0.4716$ (–)	$F(1,396) = 192.7,$ $P < \mathbf{0.0001}$ (+)
(Revolutions/day) ^{0.5}	384	$F(1,328) = 25.4,$ $P < \mathbf{0.0001}$ (+)	$F(1,328) = 14.6,$ $P = \mathbf{0.0002}$ (–)	$F(1,328) = 2.2,$ $P = 0.1395$	$F(1,328) = 3.4,$ $P = 0.0648$ (–)	$F(1,328) = 2.3^c,$ $P = 0.1332$ (+)
Intervals/day	384	$F(1,328) = 0.5,$ $P = 0.4634$ (+)	$F(1,328) = 15.1,$ $P < \mathbf{0.0001}$ (–)	$F(1,328) = 0.9,$ $P = 0.3481$	$F(1,328) = 4.5,$ $P = \mathbf{0.0357}$ (–)	$F(1,328) = 2.5^c,$ $P = 0.1128$ (+)
Mean speed (rpm)	384	$F(1,328) = 35.0,$ $P < \mathbf{0.0001}$ (+)	$F(1,328) = 5.5,$ $P = \mathbf{0.0200}$ (–)	$F(1,328) = 1.7,$ $P = 0.1898$	$F(1,328) = 1.0,$ $P = 0.3315$ (–)	$F(1,328) = 0.9^c,$ $P = 0.3531$ (+)
Maximum speed (rpm)	384	$F(1,328) = 41.6,$ $P < \mathbf{0.0001}$ (+)	$F(1,328) = 12.7,$ $P = \mathbf{0.0004}$ (–)	$F(1,328) = 0.9,$ $P = 0.3449$	$F(1,328) = 2.0,$ $P = 0.1613$ (–)	$F(1,328) = 3.9^c,$ $P = \mathbf{0.0490}$ (+)

For *P* values, bold indicates $P < 0.05$, 2-tailed, unadjusted for multiple comparisons. Signs following *P* values indicate direction of effect: +, MM mice > normal; +, male > female.

^a One or two individuals removed as statistical outliers.

^b Least squares means ± standard errors are normal females 2.0785 ± 0.003156, normal males 2.1263 ± 0.004087, mini females 1.8021 ± 0.004115, mini males 1.8332 ± 0.003043; back-transformed mean values (mg) are 119.8, 133.8, 63.4, and 68.1, respectively.

^c Square root of wheel freeness rather than body mass was used as a covariate for the wheel-running traits.

At the time of dissection, the 404 BC individuals shown in Figure 1B were an average age of 57 ± 8 (±SD) days, range = 50–70. Two hundred and one individuals expressed the MM phenotype and 203 did not. This obviously does not differ from the 1:1 expectation given inheritance as a simple Mendelian recessive ($\chi^2 = 0.0099$, $P = 0.95$). Mean triceps surae mass of the BC individuals expressing the MM phenotype was 0.0643 ± 0.0095 g (±SD), range = 0.0442–0.0887 g, with a mean body mass of 23.50 ± 3.079 g (±SD), range = 17.55–33.26 g. Mean triceps surae mass of individuals not expressing the phenotype (i.e., normal muscles) was 0.1327 ± 0.0224 g (±SD), range = 0.0907–0.1932 g, with a mean body mass of 25.79 ± 3.730 g (±SD), range = 18.11–39.19 g.

Analysis of BC Generation

Adjusting for variation in age, ANCOVA (Table 1) indicated that MM individuals were significantly lighter than normal mice ($P < 0.0001$) and that female mice were lighter than males ($P < 0.0001$), with no statistically significant MM × sex interaction ($P = 0.2964$).

After adjusting for variation in age and body mass, MM individuals had significantly smaller triceps surae muscles ($P < 0.0001$), males had significantly larger muscles than females ($P < 0.0001$), and the MM × sex interaction was significant ($P = 0.0066$, see footnote to Table 1 for adjusted means). MM individuals had larger ventricles relative to normal individuals ($P < 0.0001$), with no statistical effect of sex or a MM × sex interaction (Table 1). MM individuals had significantly larger spleens, and spleens were larger in females than in males ($P < 0.0001$) with no MM × sex interaction.

Analysis of wheel-running data revealed that MM individuals ran significantly more revolutions because they

ran at higher average and maximum speeds, with no statistical difference in the amount of time spent running/day (Table 1). Females ran significantly more than males by all 4 measures. The MM × sex interaction was not statistically significant for any measure of wheel running (Table 1).

Discussion

In the BC generation, the numbers of animals exhibiting the MM phenotype did not significantly differ from the expected 1:1 ratio under Mendelian laws. No affected mice were found in the F₁ generation, providing strong additional evidence that this phenotype is the result of a gene that segregates as a simple Mendelian recessive within this mouse lineage (see also Garland et al. 2002).

Body Size and Organ Measurements

The reduced body mass of BC individuals expressing the MM phenotype follows the same pattern as has been reported for MM individuals within the selection experiment (Garland et al. 2002; see also Syme et al. 2005). This reduction can be accounted for in part by the reduction in triceps surae mass as well as thigh muscle mass (Houle-Leroy et al. 2003). All else being equal, a reduction in body mass would reduce the absolute amount of energy needed during exercise, and it is conceivable that this is advantageous for high wheel running (see also Rezende, Kelly et al. 2006).

The 51.9% reduction in mass of the triceps surae muscle complex in affected BC mice is similar to that shown by MM individuals within the selection experiment (Garland et al. 2002; Belter et al. 2004; Swallow et al. 2005; Kelly et al. 2006; Rezende, Gomes et al. 2006). BC males had larger

triceps surae than females regardless of their mini status, but males showed a relatively greater reduction in mass of the triceps surae when expressing the mini phenotype (see Table 1). Many factors could account for a sex difference in the effects of the MM allele, one being testosterone, which affects muscle differentiation and growth in mammals.

BC individuals expressing the MM phenotype had significantly larger ventricles and spleens than their non-MM counterparts. Female BC mice had significantly larger spleens than male mice regardless of mini status, as was also reported within the selection experiment (Garland et al. 2002; but see Swallow et al. 2005). Both of these effects may be advantageous to HR mice. Increased ventricle size would be expected to increase stroke volume and hence cardiac output (Rezende, Gomes et al. 2006), whereas increased spleen size might indicate enhanced red blood cell production (or immune function).

The foregoing pleiotropic effects of the MM mutation can be explained in 3 possible ways. First, the mutation itself may affect the sizes of other organs. Second, the presence of the smaller muscle may cause indirect pleiotropic effects on masses of other organs. For example, if smaller muscles cause increased peripheral resistance in the cardiovascular system, then blood pressure would increase and could lead to cardiac hypertrophy. Third, genes that affect organ mass could be tightly linked to the locus of the MM mutation. If other genes that affect ventricle or spleen mass were relatively close to the MM gene on the chromosome, then deviation from the observed segregation pattern would be rare (Falconer and Mackay 1996). Given that organ mass is a complex trait (Deschepper et al. 2002), a quantitative genetic approach could be helpful in determining which of these hypotheses is correct.

Wheel Running

BC individuals expressing the MM phenotype showed a significant increase in wheel revolutions/day compared with non-MM individuals. However, within the selection experiment, individuals with the MM mutation exhibit revolutions/day similar to other HR individuals (Garland et al. 2002; Houle-Leroy et al. 2003; Swallow et al. 2005; Kelly et al. 2006; but see Syme et al. 2005 who only studied lines 3 and 6). Thus, the MM allele may have a stronger positive effect on wheel revolutions in the BC population than in the context of the selection experiment. Alternatively, statistical power may be higher in the BC population, with a large sample size and a 1:1 ratio of normal to affected individuals. Aside from total daily running distance, the increased average running speed of MM individuals (Table 1) has been reported previously in the context of the selection experiment, at least for some samples (Kelly et al. 2006; see also Syme et al. 2005).

Females ran significantly more revolutions/day, at higher speeds, and for more minutes/day than males regardless of MM phenotype. These results are consistent with previous reports on the selection experiment (Swallow et al. 1998; Koteja and Garland 2001; Garland et al. 2002; Swallow et al. 2005).

Future Directions

The putative MM allele exhibits “classic” properties of a GOME, including its dramatic effect on muscle mass, its Mendelian recessive nature, and its many other pleiotropic effects. Nondeleterious genes that have all the above properties are rare, and so this mutation provides an excellent model in which to study the quantitative genetics of major-effect genes (Orr 1998).

Typically, phenotypes affected by GOMEs are also affected by so-called modifier genes that have smaller phenotypic effects (Futuyma 1998). These modifier genes may either magnify or mask some of the properties of the major gene (Lynch and Walsh 1998). Consistent with the presence of modifier genes, there are minor differences between the phenotypes of mice within the line where the mutation is fixed (line 3, used for the present crosses) and the line in which it is still polymorphic (6). For example, in line 3 there is a >50% reduction of myosin heavy chain (IIB) fibers in the gastrocnemius, whereas in line 6 the reduction is only 30%. Also, mitochondrial volume density in the plantaris muscle was significantly higher in MM individuals of line 3 but not so for line 6 (Guderley et al. 2006). Breeding experiments between lines could discern the direct effects of the mutation, and variance measures of muscle and organ mass could identify the presence of background genes of small effect that contribute additive genetic variance to the phenotype.

Future studies will include mapping the MM gene. The BC population produced for the present study is highly suitable for mapping of the chromosomal position of the MM allele and eventually detection of the underlying gene and the nature of variation causing MM. Other proposed research into this mutation includes examining effects on other muscles. Within the triceps surae, it has already been shown that the reduction in mass is greater for the gastrocnemius than for the plantaris and that the soleus is actually enlarged in MM individuals (Syme et al. 2005; Guderley et al. 2006). If the effects of the mutation are not limited to the triceps surae, then this GOME could prove useful in several biological areas of study that deal with muscle mutation/dysfunction. With the growing base of information linking GOMES with common health disorders, this mutation could provide important insight into muscle degenerative diseases. For example, blockade of myostatin has been proposed as a treatment for muscle-wasting disorders (e.g., see Amthor et al. 2007). Importantly, MM individuals actually show enhancement of some muscle functional properties, that is, increased fatigue resistance (Syme et al. 2005).

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