



Myostatin dysfunction impairs force generation in extensor digitorum longus muscle and increases exercise-induced protein efflux from soleus muscle

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1 **Myostatin dysfunction impairs force generation in *extensor digitorum longus* muscle and**
2 **increases exercise-induced protein efflux from *extensor digitorum longus* and *soleus***
3 **muscles**

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26 **ABSTRACT**

27 Myostatin dysfunction promotes muscle hypertrophy which can complicate assessment of
28 muscle properties. We examined force generating capacity and creatine kinase (CK) efflux
29 from skeletal muscles of young mice before they reach adult body and muscle size. Isolated
30 *soleus* (SOL) and *extensor digitorum longus* (EDL) muscles of Berlin high (BEH) mice with
31 dysfunctional myostatin, i.e. homozygous for inactivating myostatin mutation, and with a
32 wild type myostatin (BEH+/+) were studied. The muscles of BEH mice showed faster ($P <$
33 0.01) twitch and tetanus contraction times compared to BEH+/+ mice, but only EDL
34 displayed lower ($P < 0.05$) specific force. SOL and EDL of age matched, but not younger
35 BEH mice showed greater exercise-induced CK efflux compared to BEH+/+ mice. In
36 summary, myostatin dysfunction leads to impairment in muscle force generating capacity in
37 EDL and increases susceptibility of SOL and EDL to protein loss after exercise.

38

39 **Keywords:** lengthening contractions, muscle force, muscle damage, myostatin.

40

41 **INTRODUCTION**

42 Skeletal muscles play an important role in health and disease (Wolfe 2006). Unaccustomed
43 exercise and some diseases can lead to injury and efflux of proteins from the affected muscles
44 (Armstrong 1984). An increase in total plasma CK activity has been used as evidence of
45 muscle damage after exercise in humans (Brancaccio et al. 2007; Skurvydas et al. 2011).
46 However, swelling and infiltration of skeletal muscles by immune cells can occur without
47 signs of structural damage after exercise (Pizza et al. 2002, Yu et al. 2013). It is believed that
48 exercise increases permeability of sarcolemma and can trigger the secondary events
49 associated with actions of immune system (Tidball 1995; McHugh 2003, Yu et al. 2013).
50 Isolated skeletal muscle model permits studying the primary effects of exercise by limiting
51 the complex influence of the immune and hormonal systems (Jackson et al. 1987; Suzuki et
52 al. 1999).

53
54 Various hormones and growth factors can affect functional properties and susceptibility to
55 damage of the skeletal muscles (Amelink et al. 1990). There has been a considerable interest
56 in effects of myostatin (Smith and Lin 2013). Myostatin knockout is associated with a
57 significant increase in muscle mass due to muscle fiber hypertrophy and hyperplasia
58 (McPherron et al. 1997). Myostatin blockade can improve muscle function in Duchenne
59 muscular dystrophy (Bogdanovich et al. 2002), and has been proposed as a promising
60 treatment strategy against muscle wasting in chronic diseases (Grossmann et al. 2014).
61 However, myostatin dysfunction has also been associated with low specific force of skeletal
62 muscles (Amthor et al. 2007; Matsakas et al. 2010). Interestingly, endurance training can lead
63 to normalization of specific muscle force in myostatin null mice (Matsakas et al. 2012). Food
64 restriction was also associated with an increase in specific muscle force of these mice
65 (Matsakas et al. 2013). Both endurance training and food restriction caused a reduction in

66 muscle mass, which might improve intramuscular force transmission. Furthermore, myostatin
67 dysfunction is also associated with a shift in muscle fiber composition towards faster
68 contracting fiber types (Amthor et al. 2007). Type 2 muscle fibers characterized by a faster
69 contraction time and are more sensitive to exercise-induced muscle damage than slow
70 contracting type 1 fibers (Macaluso et al. 2012; Chapman et al. 2013). Thus myostatin
71 inhibition may increase susceptibility of skeletal muscles to damage (Mendias et al. 2006).

72

73 It appears that myostatin effects are complex, vary between the skeletal muscles and can be
74 further complicated by excessive muscle hypertrophy. The aim of our study was to examine
75 effects of myostatin dysfunction on contractile properties and CK efflux in skeletal muscles
76 of young mice before they reached adult body and muscle size. We have studied *extensor*
77 *digitorum longus* (EDL) and *soleus* (SOL) muscles from Berlin high (BEH) mice with mutant
78 myostatin, known as *compact* allele, and the wild type myostatin allele (BEH^{+/+}) (Amthor et
79 al. 2007; Lionikas et al. 2013). The BEH and BEH^{+/+} mice were matched by muscle mass to
80 minimize the influence of excessive muscle hypertrophy as a possible confounding factor.

81

82 MATERIALS AND METHODS

83 Animals and experiments

84 All procedures of this experiment were approved by the Lithuanian State Food and
85 Veterinary Service (Nr. 0223). BEH^{+/+} females were generated by crossing animals from
86 BEH and Berlin Low (BEL) strains and then repeatedly backcrossing the offspring to BEH
87 using marker assisted selection for the wild type myostatin (Amthor et al. 2007; Lionikas et
88 al. 2013). The data on age, body mass and muscle mass of these animals are presented in
89 Table 1. BEH mice were younger than BEH^{+/+} mice when matched by the muscle mass of
90 SOL or EDL. The age difference between the strains was particularly significant in case of

4

91 EDL. Thus additional measurements were carried out on EDL of BEH mice of a similar age
92 as BEH+/+ mice. Prior to the *in vitro* experiments, animals were kept in standard cages (cage
93 dimensions: 267 x 207 x 140 mm) at 20-22° C temperature and 55±10% humidity with 12/12-
94 h light/dark cycle. As in our previous studies (Kilikevicius et al. 2013, Lionikas et al. 2013),
95 mice were housed one to three mice per cage, fed standard rodent diet (58.0 % kcal from
96 carbohydrates, 28.5 % kcal from protein, 13.5 % kcal from fat; LabDiet 5001, LabDiet, St.
97 Louis, USA) and received tap water *ad libitum*.

98 **Muscle properties and CK efflux**

99 All experiments were performed at room temperature (~25 °C). Mice were euthanized by the
100 cervical dislocation. Afterwards, SOL or EDL muscle of the right leg was dissected, freed
101 from tendons, blotted and weighed (Kern, ABS 80-4, Germany). Muscles of the left leg were
102 used for assessment of contractile properties and total muscle CK efflux as described
103 previously (Baltusnikas et al. 2014). The muscles were dissected and placed immediately in
104 the organ bath containing Tyrode solution (121 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 1.8
105 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.1 mM NaEDTA, 24 mM NaHCO₃, 5.5 mM glucose) which
106 was bubbled with 95 % O₂ and 5 % CO₂ to attain a pH of ~7.4. Muscles were fixed between
107 two platinum plate electrodes of the muscle test system (1200A-LR Muscle Test System,
108 Aurora Scientific Inc., Aurora, Canada). Then the muscle length was increased progressively
109 in steps until peak force was reached in 150-Hz tetani of 0.5-s or 2-s duration which were
110 induced every 2 min in EDL or SOL, respectively. Single stimulus was then delivered to
111 assess twitch contraction time and 90% twitch relaxation time, and this was followed by a
112 measurement of peak tetanic force as well as 90% contraction and relaxation times. Then
113 muscles were subjected to 100 eccentric contractions at a frequency of 0.1 Hz. During the
114 exercise, muscles were stimulated at 150 Hz stimulation for 700 ms. During the last 200 ms
115 of this stimulation a ramp stretch was performed followed by 200 ms gradual return of the

116 muscle to the initial length without any stimulation. The amplitude of the stretch was
117 equivalent to 2.5 fiber lengths per second in case of both SOL and EDL muscles (Brooks and
118 Faulkner 1988). After the eccentric exercise muscles were photographed with the length scale
119 in the background for assessment of optimal muscle length (L_0). Then these muscle as well as
120 muscles from the control experiment without any exercise, were incubated in 2 ml of Tyrode
121 solution for 2 h at room temperature. 250 μ l of Tyrode solution was sampled for assessment
122 of CK activity using biochemical analyser (SpotchemTM EZ SP-4430, Menarini Diagnostics,
123 UK) with the reagent strips (Arkray Factory, Inc., Shiga, Japan).

124 The physiological cross-sectional areas (pCSA) of SOL and EDL were estimated by dividing
125 muscle weight by the product of fiber length and 1.06 kg l^{-1} , the density of mammalian
126 skeletal muscle (Brooks and Faulkner 1988). Muscle fiber length was assumed to be equal to
127 45% and 70% of muscle length for EDL and SOL, respectively. Muscles tended to show a
128 slight increase in weight after the experimental protocol involving repetitive exercise. Thus
129 weights of the contralateral muscles were used in these assessments. In a large set of samples
130 ($n=101$) we dissected both left and right solei of adult mice to immediately measure wet
131 muscle mass; there was no difference (paired t-test $p=0.953$) found in weights of the
132 contralateral muscles.

133 **Statistical analysis**

134 All data analysis was performed using Prism 5.0 software. Data for SOL and EDL were
135 analyzed separately. The two factor analysis of variance (ANOVA) was used to assess effects
136 of experimental intervention (exercise or rest) and mouse strain (BEH^{+/+} or BEH) on muscle
137 CK efflux. Repeated measures ANOVA was used for the analysis of peak isometric force
138 during eccentric exercise. The post hoc testing was carried out using t-tests with a Bonferroni
139 correction for multiple comparisons. Non parametric Mann–Whitney U test was used in all
140 other cases. All the tests were two-tailed with significance level was set at $P < 0.05$.

141

142 **RESULTS**

143 Data on muscle properties of BEH^{+/+} and BEH mice are presented in Table 2. There were no
144 strain differences for SOL muscle. However, strain effects were found in EDL. BEH^{+/+} mice
145 had a longer L_0 ($P < 0.01$) and a smaller ($P < 0.01$) pCSA of EDL compared to BEH mice.
146 The older BEH mice showed the greatest pCSA ($P < 0.01$) of this muscle. In spite of greater
147 pCSA, EDL of young BEH generated less force ($P < 0.05$) and showed a lower ($P < 0.01$)
148 specific force compared to BEH^{+/+}. The older BEH mice had the highest ($P < 0.01$) peak
149 force for EDL, but their specific force was similar as in young BEH mice and lower ($P <$
150 0.01) compared to BEH^{+/+} mice.

151

152 The contraction speed of a single twitch and tetanus of the muscles from BEH^{+/+} and BEH
153 mice are presented in Table 2. For SOL, BEH mice had shorter contraction times in single
154 twitch ($P < 0.01$) and 150-Hz tetani ($P < 0.01$) than BEH^{+/+} mice. Data for the EDL were
155 less consistent than for SOL. BEH mice had longer contraction times ($P < 0.01$), but shorter
156 ($p < 0.05$) relaxation times in single twitches compared to BEH^{+/+} mice. The opposite was
157 true for tetani. Relaxation times were longer ($P < 0.01$) for BEH mice compared to BEH^{+/+}.
158 Only older, but not younger BEH mice showed shorter ($p < 0.05$) contraction times of tetanus
159 than BEH^{+/+} mice.

160

161 Data on peak isometric force for SOL and EDL during repeated isometric-eccentric exercise
162 are shown in Fig. 1. BEH^{+/+} and young BEH mice showed similar loss ($P < 0.001$) of peak
163 isometric force for both muscles during the exercise. For EDL, older BEH mice showed a
164 greater ($P < 0.05-0.01$) decline in isometric force compared to both young BEH and BEH^{+/+}

165 mice after initial ten and twenty contractions, respectively. Afterwards, however, the relative
166 decline of peak isometric force was similar in all mice.

167

168 Data on the total CK efflux from the muscles of mice are presented in Fig. 2. There were no
169 differences between the strains in muscle CK efflux when measurements were performed at
170 rest, i.e. without prior exercise. After the exercise muscle CK efflux increased ($P < 0.05-0.01$)
171 and younger BEH mice showed a greater ($P < 0.05$) CK efflux from SOL compared to
172 BEH^{+/+} mice. There were no differences between these mice for the EDL. However, older
173 BEH mice showed a greater ($P < 0.05$) CK efflux from EDL compared to the age-matched
174 BEH^{+/+} and younger BEH.

175

176 **DISCUSSION**

177 The aim of the study was to examine the effects of myostatin dysfunction on the contractile
178 properties and total CK efflux from SOL and EDL muscles at rest and after exercise. The
179 results of the study show that BEH mice with myostatin dysfunction had lower specific force
180 than BEH^{+/+} mice with the wild type myostatin in the faster contracting EDL, but not in the
181 slower contracting SOL. Furthermore, BEH mice demonstrated greater exercise-induced
182 muscle CK efflux compared to BEH^{+/+} when mice of similar age were compared, but not at
183 young age. These results show that effects of myostatin dysfunction vary between skeletal
184 muscles and depend on the age of mice.

185

186 Myostatin dysfunction is associated with excessive muscle hypertrophy (McPherron et al.
187 1997) and reduction in specific muscle force of the fast contracting EDL (Amthor et al. 2007;
188 Mendias et al. 2006). It has been hypothesized that enlargement of muscle fibers might
189 impair force transmission within the skeletal muscles due to an increase in muscle fiber

190 pennation angles (Amthor et al. 2007). However, muscle fibers of myostatin null mice might
191 also show an intrinsic reduction in force output due to a low content of contractile proteins
192 (Qaisar et al. 2012). We studied skeletal muscles of young mice before they developed
193 excessive muscularity. This approach minimized confounding effects of muscle hypertrophy.
194 Nevertheless, EDL of BEH mice showed lower specific force compared to BEH+/+ mice in
195 both cases, i.e. when muscles were matched by weight or age. Thus impairment in force
196 generating capacity of EDL muscle in myostatin deficient mice was independent of muscle
197 size, and appears to be due to reduced force output at the level of single muscle fibers (Qaisar
198 et al. 2012). Interestingly, BEH+/+ and BEH mice did not differ in the specific force of SOL
199 muscle. Similar findings on the differences between EDL and SOL muscles have been
200 reported for adult mice (Mendias et al. 2006). Endurance training can improve specific force
201 of skeletal muscles in myostatin null mice (Matsakas et al. 2012). It might be that motor
202 activity helps to maintain specific force of SOL in BEH mice in spite of myostatin deficiency.
203 SOL muscle shows markedly greater involvement in locomotion than other leg muscles
204 which prevail in daily activity of mice including EDL (Roy et al. 1991).

205

206 BEH mice showed shorter contraction times in both single twitches and tetani of SOL
207 compared to BEH+/+ mice. This might be associated with a shift in muscle fiber composition
208 towards faster contracting fiber types in SOL muscle of mice with myostatin deficiency
209 compared to the wild type mice (Girgenrath et al. 2005; Amthor et al. 2007; Matsakas et al.
210 2010). Fast twitch muscle fibers of mice and humans are more susceptible to damage after
211 eccentric exercise than slow twitch muscle fibers (Mendias et al. 2006; Chapman et al. 2013).
212 Indeed, SOL muscle of BEH mice showed greater CK efflux after exercise compared to
213 BEH+/+ mice.

214

215 Effects of myostatin dysfunction were less consistent for EDL than SOL. This might be
216 associated with differences in myostatin effects on fiber type composition of EDL and SOL.
217 Myostatin dysfunction causes a marked increase in content of 2X and 2B fibers at the
218 expense of type 1 fibers in SOL, but induces only a small increase in content of type 2B
219 fibers at the expense of type 2X fibers in EDL (Girgenrath et al. 2005). Age of the studied
220 mice might also be of importance here. BEH mice of similar age as BEH+/+ mice, but not
221 young BEH mice showed elevated CK efflux from EDL after eccentric exercise compared to
222 BEH+/+ mice. A study by Gokhin et al. 2008 demonstrated that contractile force, fiber cross-
223 sectional area, area of the fibers occupied by the contractile proteins, and percentage of type
224 2B fibers increase rather drastically in mouse tibialis anterior muscle between day 1 and day
225 21 after birth. Then the changes between day 21 and day 28 are much more subtle. For
226 instance, area of the fibers occupied by the contractile proteins – the most relevant index in
227 relation to the specific force, does not change between these time points; and proportion of
228 type 2B fibres is comparable to that of the adult animals (Bloemberg & Quadriatello 2012)
229 already at the age of 21 days. Because young BEH mice were at 26 days of age and BEH+/+
230 at 37 days, both have already passed the phase where developmental differences might had
231 played a sizable role. However, muscle resistance to exercise-induced protein efflux is
232 dependent on other factors than specific force. Collagen content of extracellular matrix might
233 be of particular importance here. Procollagen processing increases after eccentric exercise in
234 both rats and humans (Han et al. 1999; Crameri et al. 2004). Myostatin belongs to
235 transforming growth factor (TGF- β) family of cytokines that signal through Smad2/3, TAK1-
236 p38 MAPK pathways (Lee 2004; Tsukada et al. 2005). Inhibition of TGF- β signaling
237 suppresses collagen expression in EDL of mice after injury (Gumucio et al. 2013). It could be
238 that concentration and/or properties of structural proteins become insufficient to sustain high
239 mechanical loads during the phase of rapid muscle growth between 26 and 40 days and

240 susceptibility to exercise-induced muscle damage increases in mice with myostatin
241 dysfunction.

242

243 We did not observe any significant difference in loss of peak isometric force between BEH
244 and BEH+/+ muscles during eccentric exercise. Muscle contractions were separated by 10 s
245 periods of rest that should minimize metabolic inhibition during the exercise (Allen et al.
246 1995). Our exercise protocol included stretches of similar amplitude, but at half of the
247 velocity compared to the previous study of myostatin effects on muscles of adult (10-12
248 month old) mice (Mendias et al. 2006). In general, exercise-induced CK efflux from skeletal
249 muscles is not always associated with changes in muscle force. Eccentric contractions often
250 induce an impairment in excitation-contraction coupling of muscle fibers without any clear
251 sign of muscle damage (Warren et al., 1993, Allen, 2001). Such impairment will lead to
252 inactivation of muscles fibers and will protect them from damaging effects of exercise. It
253 appears that changes in force generating capacity can be dissociated from alterations in
254 permeability of sarcolemma and muscle CK efflux during and after exercise. Indeed, the
255 regenerated SOL muscle showed a particularly low exercise-induced CK efflux in spite of
256 relatively modest improvement in fatigue resistance compared to the control muscles
257 (Baltusnikas et al. 2014).

258

259 In summary, myostatin dysfunction leads to impairment in muscle force generating capacity
260 of faster contracting EDL and increased susceptibility of both SOL and EDL to protein efflux
261 after eccentric exercise.

262

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267

268 **CONFLICTS OF INTEREST**

269 We declare that we have no conflict of interests.

270

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- 381

382 **Table 1.** Age, body mass and muscle mass of *soleus* (SOL) and *extensor digitorum longus*
 383 (EDL) muscles in BEH+/+ and BEH mice with the wild type and dysfunctional myostatin,
 384 respectively. Values are means and S.D.

	SOL		EDL		
	BEH+/+ (n=25)	BEH (n=22)	BEH+/+ (n=15)	BEH (n=15)	BEH (Older) (n=11)
Age (days)	36.5 ± 5.5	31.7 ± 0.4	36.9 ± 2.7	26.2 ± 1.2	38.5 ± 1.7
Body mass (g)	26.8 ± 2.6	22.9 ± 3.0	27.1 ± 1.8	16.3 ± 1.3	31.4 ± 1.5
Muscle mass (mg)	5.7 ± 0.5	6.0 ± 0.6	7.8 ± 0.4	7.8 ± 0.9	13.8 ± 0.9

385

386

387 **Table 2.** Muscle properties of BEH+/+ and BEH mice with the wild type and dysfunctional
 388 myostatin, respectively. SOL is *soleus* muscle; EDL is *extensor digitorum longus* (EDL). L₀
 389 is optimal muscle length. pCSA is physiological cross-sectional area. Values are means and
 390 S.D. ** P < 0.01 BEH+/+ vs BEH, ## P < 0.01 BEH vs BEH (Older).

	SOL		EDL		
	BEH+/+	BEH	BEH+/+	BEH	BEH (Older)
L ₀ (mm)	12.4 ± 0.9	12.5 ± 0.5	14.3 ± 0.6	12.0 ± 0.5 **	13.0 ± 0.6 **
pCSA (mm ²)	0.84 ± 0.06	0.92 ± 0.08	1.18 ± 0.11	1.40 ± 0.11 **	2.15 ± 0.15 **, ##
Peak isometric force (mN)	173.8 ± 17.6	180.5 ± 13.7	160.3 ± 23.6	145.1 ± 10.5 **	219.9 ± 25.4 **, ##
Specific force (mN/mm ²)	273.8 ± 33.3	271.0 ± 35.2	137.1 ± 23.2	104.3 ± 12.1 *	102.3 ± 9.8, **

391

392

393 **Table 3.** Twitch and tetanus contraction and relaxation times in skeletal muscles of BEH+/+
 394 and BEH mice, respectively. SOL is *soleus* muscle; EDL is *extensor digitorum longus* (EDL).

395 Values are means and S.D.; * P < 0.05, ** P < 0.01 BEH vs BEH+/+, # P < 0.05, ## P < 0.01

396 BEH (Older) vs BEH.

	SOL		EDL		
	BEH+/+	BEH	BEH+/+	BEH	BEH (Older)
Twitch contraction time (ms)	69.5 ± 8.1	56.9 ± 9.1 **	21.6 ± 0.7	28.3 ± 1.3 **	25.2 ± 1.6 **, #
Twitch relaxation time (ms)	313.9 ± 144.2	304.4 ± 91.5	120.6 ± 23.5	96.2 ± 6.4 *	86.2 ± 8.4 *
Tetanus contraction time (ms)	573.8 ± 54.6	473.0 ± 72.8 **	132.6 ± 9.8	143.0 ± 6.0	125.6 ± 5.7 **, ##
Tetanus relaxation time (ms)	200.7 ± 18.2	163.2 ± 30.7 **	58.7 ± 2.4	68.6 ± 2.4 **	68.4 ± 3.9 **

397

398

399 **FIGURE CAPTIONS**

400 **Figure 1.** Peak isometric force for *soleus* (A) and *extensor digitorum longus* (B) muscles of
401 BEH+/+ and BEH mice with the wild type and mutant myostatin, respectively, during 100
402 contractions repeated every 10 s. The data for older BEH mice with the mutant myostatin,
403 BEH (Older), is also shown. * $P < 0.05$ for BEH+/+ vs BEH (Older); # $P < 0.05$, ## $P < 0.01$
404 for BEH vs BEH (Older), respectively. Values are means with S.D.

405

406 **Figure 2.** The total CK efflux at rest and after eccentric exercise from *soleus* (SOL, A) and
407 *extensor digitorum longus* (EDL, B) muscles of BEH and BEH+/+ mice with the mutant and
408 wild type myostatin, respectively. The data for older BEH mice with mutant myostatin, BEH
409 (Older), is also shown (B). * $P < 0.05$, *** $P < 0.001$ for BEH+/+ vs BEH; # $P < 0.001$ for
410 BEH vs BEH (Older) mice. Values are means with S.D.

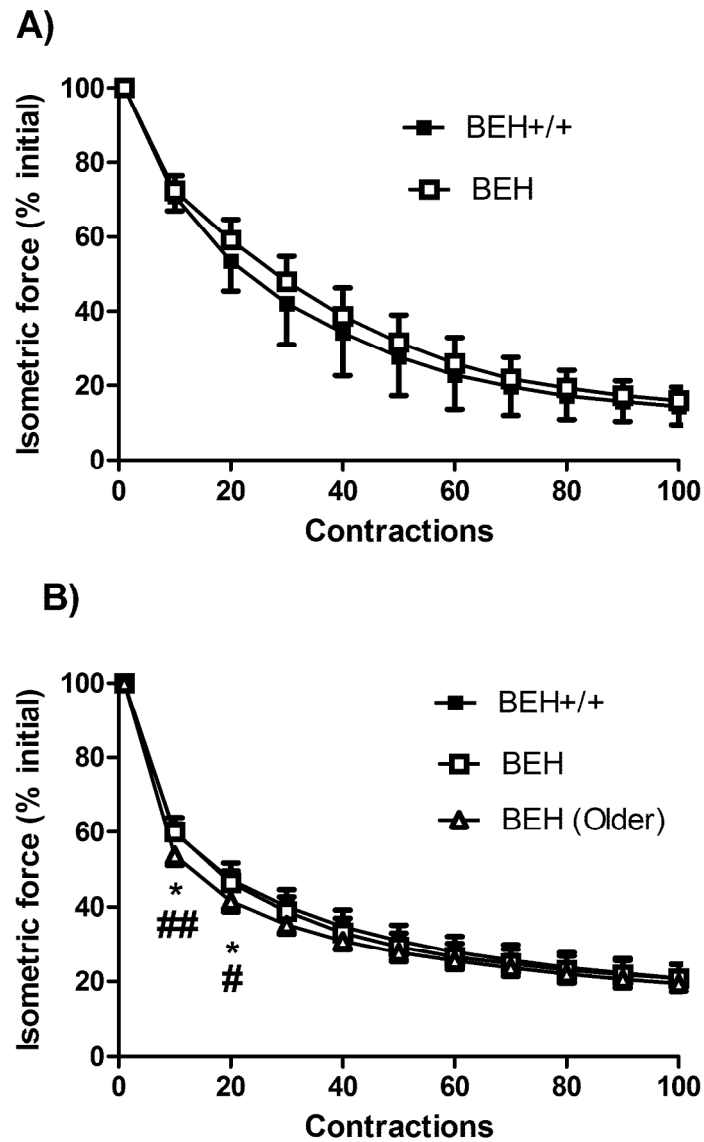


Figure 1. Peak isometric force for soleus (A) and extensor digitorum longus (B) muscles of BEH+/+ and BEH mice with the wild type and mutant myostatin, respectively, during 100 contractions repeated every 10 s. The data for older BEH mice with the mutant myostatin, BEH (Older), is also shown. * $P < 0.05$ for BEH+/+ vs BEH (Older); # $P < 0.05$, ## $P < 0.01$ for BEH vs BEH (Older), respectively. Values are means with S.D. 181x267mm (300 x 300 DPI)

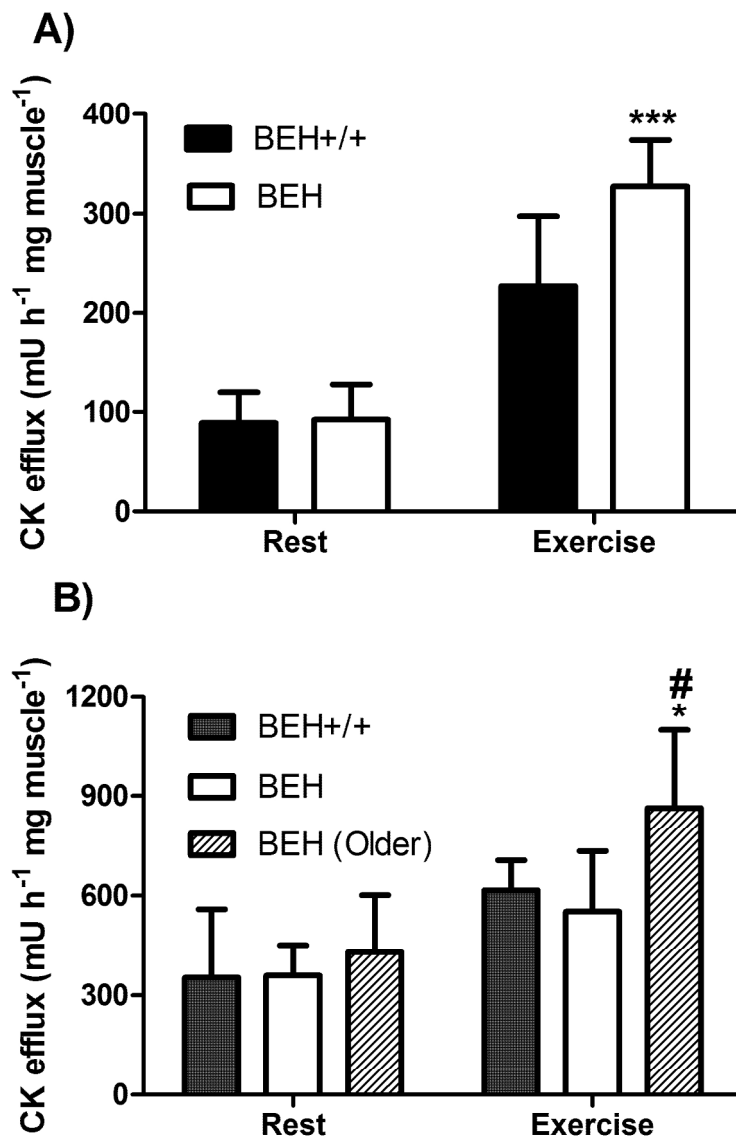


Figure 2. The total CK efflux at rest and after eccentric exercise from soleus (SOL, A) and extensor digitorum longus (EDL, B) muscles of BEH and BEH+/+ mice with the mutant and wild type myostatin, respectively. The data for older BEH mice with mutant myostatin, BEH (Older), is also shown (B). * $P < 0.05$, *** $P < 0.001$ for BEH+/+ vs BEH; # $P < 0.001$ for BEH vs BEH (Older) mice. Values are means with S.D.

180x242mm (300 x 300 DPI)