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Published in:
Applied Physiology, Nutrition and Metabolism

DOI:
10.1139/apnm-2014-0513

First published: 06/04/2015

Document Version
Peer reviewed version

Canadian Science Publishing
NRC Research Press journals | Publishing Services

Applied Physiology, Nutrition, and Metabolism
Physiologie appliquée, nutrition et métabolisme

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Journal:	*Applied Physiology, Nutrition, and Metabolism*
Manuscript ID:	apnm-2014-0513.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Baltusnikas, Juozas; Lithuanian Sports University, Kilikevicius, Audrius; Lithuanian Sports University, Venckunas, Tomas; Lithuanian Sports University, Fokin, Andrej; Lithuanian Sports University, Bünger, Lutz; Scotland's Rural College (SRUC), Lionikas, Arimantas; University of Aberdeen, Ratkevicius, Aivaras; University of Aberdeen, School of Medical Sciences; Lithuanian Sports University,
Keyword:	muscle damage < muscle

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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Animals and experiments

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

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

Muscle properties and CK efflux

All experiments were performed at room temperature (~25 °C). Mice were euthanized by the cervical dislocation. Afterwards, SOL or EDL muscle of the right leg was dissected, freed from tendons, blotted and weighed (Kern, ABS 80-4, Germany). Muscles of the left leg were used for assessment of contractile properties and total muscle CK efflux as described previously (Baltusnikas et al. 2014). The muscles were dissected and placed immediately in the organ bath containing Tyrode solution (121 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.1 mM NaEDTA, 24 mM $NaHCO_3$, 5.5 mM glucose) which was bubbled with 95 % O_2 and 5 % CO_2 to attain a pH of ~7.4. Muscles were fixed between two platinum plate electrodes of the muscle test system (1200A-LR Muscle Test System, Aurora Scientific Inc., Aurora, Canada). Then the muscle length was increased progressively in steps until peak force was reached in 150-Hz tetani of 0.5-s or 2-s duration which were induced every 2 min in EDL or SOL, respectively. Single stimulus was then delivered to assess twitch contraction time and 90% twitch relaxation time, and this was followed by a measurement of peak tetanic force as well as 90% contraction and relaxation times. Then muscles were subjected to 100 eccentric contractions at a frequency of 0.1 Hz. During the exercise, muscles were stimulated at 150 Hz stimulation for 700 ms. During the last 200 ms of this stimulation a ramp stretch was performed followed by 200 ms gradual return of the

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

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

Statistical analysis

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

RESULTS

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

ACKNOWLEDGEMENTS

This study was funded by a grant (No. MIP-067/2012) from the Research Council of Lithuania. We are thankful to Petras Jeneckas, Audrius Capskas, and Indre Libnickiene for their technical assistance.

CONFLICTS OF INTEREST

We declare that we have no conflict of interests.

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Table 1. Age, body mass and muscle mass of *soleus* (SOL) and *extensor digitorum longus* (EDL) muscles in BEH+/+ and BEH mice with the wild type and dysfuctional myostatin, 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

Table 2. Muscle properties of BEH+/+ and BEH mice with the wild type and dysfuctional myostatin, respectively. SOL is *soleus* muscle; EDL is *extensor digitorum longus* (EDL). L_0 is optimal muscle length. pCSA is physiological cross-sectional area. Values are means and 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, **

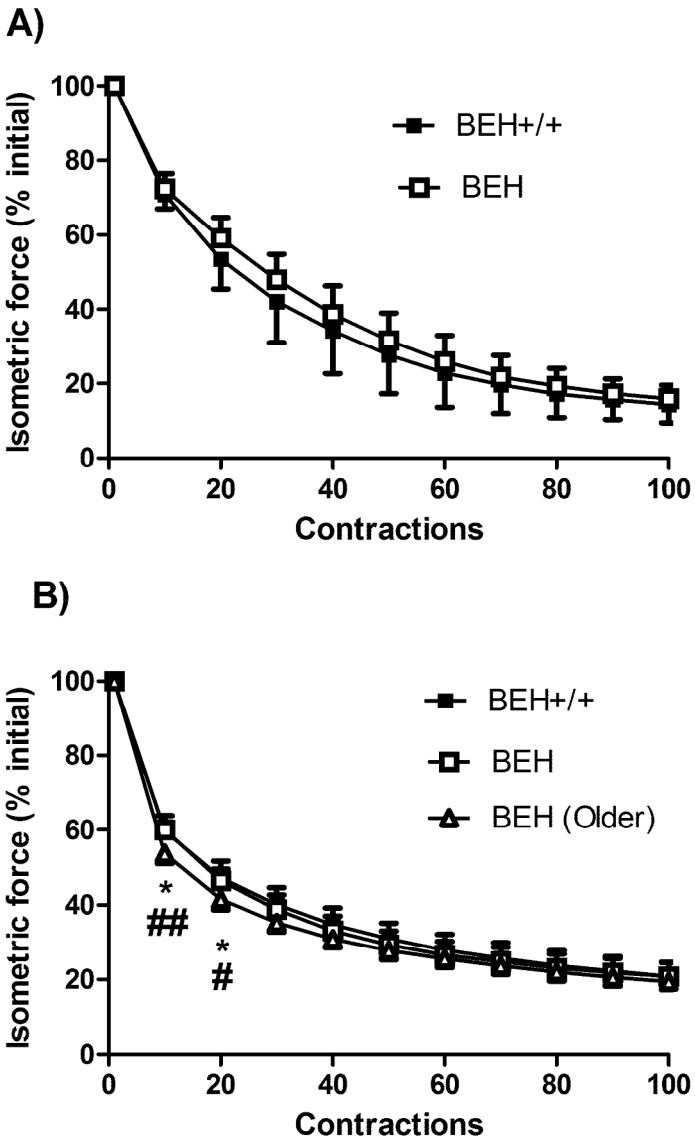
Table 3. Twitch and tetanus contraction and relaxation times in skeletal muscles of BEH+/+ and BEH mice, respectively. SOL is *soleus* muscle; EDL is *extensor digitorum longus* (EDL). Values are means and S.D.; * P < 0.05, ** P < 0.01 BEH vs BEH+/+, # P < 0.05, ## P < 0.01 BEH (Older) vs BEH.

	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 **

FIGURE CAPTIONS

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.

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.



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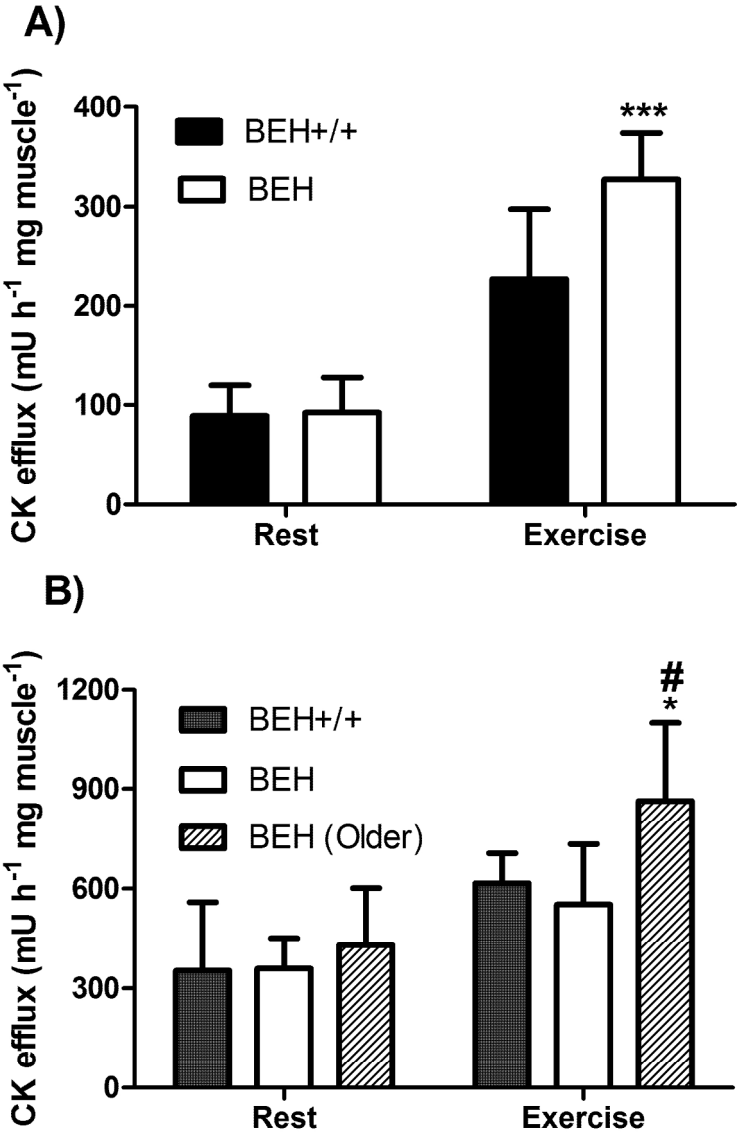
181x267mm (300 x 300 DPI)



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180x242mm (300 x 300 DPI)