

## Original Article

# Myostatin dysfunction does not protect from fasting-induced loss of muscle mass in mice

Andrej Fokin<sup>1</sup>, Petras Minderis<sup>1</sup>, Tomas Vencunas<sup>1,2</sup>, Arimantas Lionikas<sup>3</sup>, Mindaugas Kvedaras<sup>1</sup>, Aivaras Ratkevicius<sup>2</sup>

<sup>1</sup>Institute of Sport Science and Innovations, Lithuanian Sports University, Kaunas, Lithuania; <sup>2</sup>Department of Applied Biology and Rehabilitation, Lithuanian Sports University, Kaunas, Lithuania; <sup>3</sup>School of Medicine, Medical Sciences and Nutrition, College of Life Sciences and Medicine, University of Aberdeen, Aberdeen, Scotland, UK

## Abstract

**Objectives:** The aim of the study was to investigate if myostatin dysfunction can ameliorate fasting-induced muscle wasting. **Methods:** 18-week old males from Berlin high (BEH) strain with myostatin dysfunction and wild type myostatin (BEH+/+) strain were subjected to 48-h food deprivation (FD). Changes in body composition as well as contractile properties of soleus (SOL) and extensor digitorum longus (EDL) muscles were studied. **Results:** BEH mice were heavier than BEH+/+ mice (56.0±2.5 vs. 49.9±2.8 g,  $P<0.001$ , respectively). FD induced similar loss of body mass in BEH and BEH+/+ mice (16.6±2.4 vs. 17.4±2.2%,  $P>0.05$ ), but only BEH mice experienced wasting of the gastrocnemius, tibialis anterior and plantaris muscles. FD induced a marked decrease in specific muscle force of SOL. EDL of BEH mice tended to be protected from this decline. **Conclusion:** Myostatin dysfunction does not protect from loss of muscle mass during fasting.

**Keywords:** Myostatin, Caloric Restriction, Fasting, Muscle Atrophy, Muscle Specific Force

## Introduction

Skeletal muscles play a key role in the overall health and well-being<sup>1</sup>. Many chronic diseases and conditions, such as cardiovascular heart diseases, diabetes, rheumatoid arthritis, cancer and various forms of muscular dystrophies are associated with muscle wasting<sup>2,3</sup>. Fasting and caloric restriction improve metabolic health, but also lead to decrease in skeletal muscle mass<sup>4,5</sup>. It would be beneficial to develop interventions that preserve muscle mass during fasting and other catabolic conditions that are associated with inadequate nutrition and/or low levels of physical activity<sup>1</sup>.

Myostatin belongs to transforming the growth factor  $\beta$ -superfamily and is a negative regulator of skeletal

muscle growth and development<sup>6,7</sup>. C57BL/6J mice with dysfunctional myostatin have enlarged skeletal muscles due to muscle fiber hypertrophy and/or hyperplasia<sup>6,8</sup>. Consistently with an inhibitory effect, overexpression of myostatin causes a decrease in skeletal muscle mass<sup>9,10</sup>. Myostatin signaling inhibits protein synthesis and promotes proteolysis<sup>11,12</sup>. Myostatin inhibition helps to increase muscle mass and improve muscle function in *mdx* mouse, which is a popular model of Duchenne muscle dystrophy<sup>13</sup>. These findings speak for potential of myostatin inhibition therapies. Some strategies including treatment with myostatin binding follistatin<sup>14-16</sup>, myostatin antibodies<sup>13,17</sup> and *Mstn* gene silencing with systemic siRNAs delivery<sup>16</sup> have already shown promising results.

There are also undesirable consequences of myostatin inhibition. Myostatin dysfunction is associated with reduction in specific muscle force which is assessed as peak tetanic force normalized to muscle mass or cross-sectional area<sup>8,18-20</sup>. In some cases myostatin inhibition might lead to muscle hypertrophy without a corresponding improvement in muscle function<sup>21</sup>. However, C57BL/6J mice lacking myostatin showed an increase in specific force of EDL after a period of caloric restriction<sup>22</sup> as well as after endurance training<sup>22,23</sup>. It was reported that myostatin dysfunction

The authors have no conflict of interest.

Corresponding author: Dr. Aivaras Ratkevicius, Department of Applied Biology and Rehabilitation, Lithuanian Sports University, Sporto g. 6, LT-44221 Kaunas, Lithuania  
E-mail: Aivaras.Ratkevicius@lsu.lt

Edited by: G. Lyrītis  
Accepted 15 March 2019



prevents loss of mass of tibialis anterior muscle after 48-h, but not after 24-h fasting<sup>24</sup>. On the other hand, higher rates of skeletal muscle loss have been found in C57BL/6J mice lacking myostatin compared to the wild type controls after 24 h of fasting<sup>25</sup> and after 5-week 40% caloric restriction<sup>22,25</sup>. It appears that other models of myostatin dysfunction might be useful in resolving this controversy about effects of myostatin dysfunction on skeletal muscles during fasting.

A great majority of studies of myostatin dysfunction were carried out using C57BL/6J mouse strain. Studies of the other strains may be helpful for a better understanding of physiological effects of myostatin dysfunction. Berlin high mouse strain (BEH) presents such a model. BEH strain, with its counterpart Berlin low strain, BEL, were derived from heterogeneous population of mice in 1970s by selection for protein content and body weight at the age of 60 days<sup>26</sup>. It was later discovered that BEH hypermuscularity is associated with a mutation in the myostatin gene, *Mstn*, in particular a 12bp deletion known as the compact allele<sup>27</sup>. The BEH+/+ strain differs from BEH by 0.01% of genome, ~0.4 Mb region on chromosome 1 engulfing the *Mstn* gene<sup>28</sup>. This region in BEH+/+ contains wild type *Mstn* which was introgressed from the BEL strain by marker-assisted selection for many generations<sup>7</sup>. It is highly unlikely that any other genes than *Mstn* are involved in causing the hypermuscular phenotype of BEH strain compared to BEH+/+ strain. Interestingly, on the C57BL/6J background myostatin dysfunction induces both fiber hypertrophy and hyperplasia<sup>8</sup>, whereas on the BEH background it promotes fiber hypertrophy only<sup>28</sup>. Thus, effects of myostatin dysfunction appear to be influenced by the genetic background.

The overall aim of our study was to test the hypothesis that myostatin dysfunction prevents loss of muscle mass and function during fasting. We compared physiological responses to 48-h food deprivation (FD) in Berlin high mouse strain with dysfunctional myostatin (BEH) and wild type myostatin (BEH+/+).

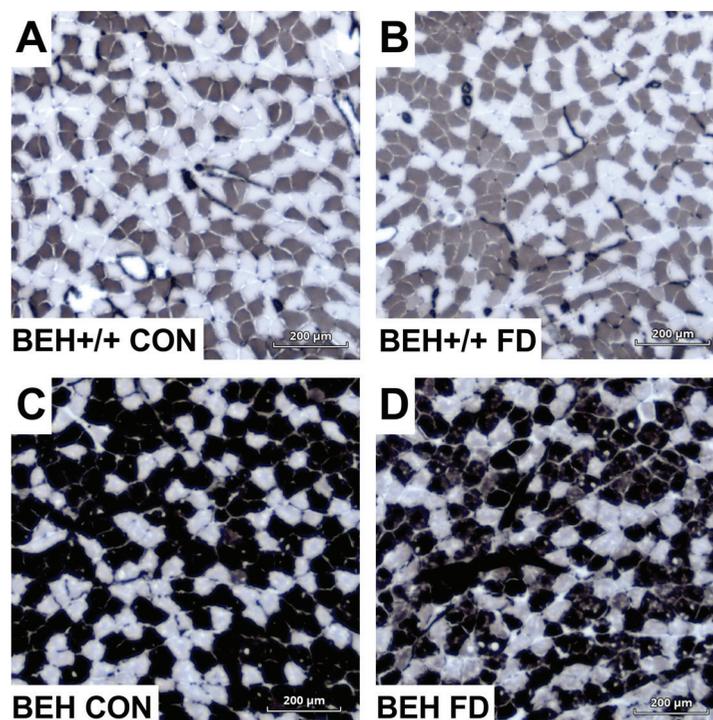
## Methods

**Animals and experiments.** This study was carried out in accordance with the recommendations of the Lithuanian State Food and Veterinary Service which approved all the procedures and interventions concerning mice (Ref. # O223 for 2012 and Ref. # 10 for 2014). 18-week old males of BEH+/+ (n=45) and BEH (n=37) strains of mice were studied. BEH mice are homozygous for *Mstn*Cmpt-dl1Abc (Compact; Cmpt) mutation which is associated with myostatin dysfunction<sup>7,27-29</sup>. BEH+/+ mice with the wild type myostatin were generated by crossing BEH mice with the Berlin Low (BEL) strain and then repeatedly backcrossing the offspring to BEH using marker assisted selection for the functional myostatin<sup>7,28</sup>. The breeding pairs of the BEH and BEH+/+ were a generous gift of prof. Lutz Bünger (Scotland's Rural College, UK). Before experiments mice were bred and housed in the animal facilities of Lithuanian Sports University. They were

kept in standard cages (cage dimensions: 267 x 207 x 140 mm) at a temperature of 20-21°C and 40-60% humidity with the normal 12/12-h light/dark cycle reversed. Animals were fed standard chow diet (56.7 kcal% carbohydrate, 29.8 kcal% protein, 13.4 kcal% fat; LabDiet 5001, USA) and received tap water *ad libitum*.

**Fasting.** Mice were subdivided into the control (CON) groups for BEH+/+ (n=18) and BEH (n=14) strains and food deprivation (FD) groups for BEH+/+ (n=27) and BEH (n=23) strains, respectively. The CON mice were provided with *ad libitum* access to food and water. The FD mouse had *ad libitum* access to water, but did not receive any food. Mice were weighed at 0 h, 24 h and 48 h of the intervention (Kern 440-45N, Germany). Food intake for the CON mice was determined individually over 48 h period by subtracting the weight of all remaining food in the cage from the weight of food provided initially. At the end of the intervention mice were sacrificed by the exposure to CO<sub>2</sub>. Immediately afterwards, as in our previous study<sup>30</sup>, the heart and the skeletal muscles of the randomly selected hindlimb including gastrocnemius (GAS), plantaris (PL), soleus (SOL), tibialis anterior (TA) and extensor digitorum longus (EDL) were dissected and weighed with a precision of 0.1 mg (Kern, ABS 80-4, Germany). Before weighing the muscles were freed from all visible tendons and blotted dry rapidly on filter paper. The combined mass of all five muscles was used as index of muscle mass in comparison of mouse strains and assessment of effects of food deprivation in the studied mice. These muscles were not subjected to any exercise. The muscles of the contralateral leg were used for assessment of muscle force generating capacity. Liver and fat from four different sites were also removed and weighed. The combined fat mass was assessed and included the posterior subcutaneous fat (SF), gonadal fat (GF), visceral fat (VF) including mesenteric and perirenal fat, and interscapular fat (IF).

**Muscle force generating capacity.** Contractility of soleus (SOL) and extensor digitorum longus (EDL) muscles (n=10 in each group) was examined as in our previous studies<sup>21,30</sup>. After sacrifice SOL and EDL muscles were excised with 5-0 silk suture tied securely to the proximal and distal tendons and placed in 100 ml Radnoti tissue bath filled with the Tyrode solution (121 mM NaCl, 5 mM KCl, 0.5 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM NaEDTA, 24 mM NaHCO<sub>3</sub>, 5.5 mM glucose) that was bubbled with a gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> at pH 7.4. The bath was maintained at room temperature of ~21-23°C during all experiments. The contractile properties of the muscles were assessed one after another, while SOL was examined, EDL was kept intact in Tyrode solution. The pilot experiments showed that this additional time in queuing does not affect muscle force generating capacity. For these measurements the muscle was suspended vertically between two platinum plate electrodes in the bath with the proximal tendon attached securely to the lever arm of muscle test system (1200A-LR Muscle Test System, Aurora Scientific Inc., Canada) and distal tendon to a stable iron hook. Optimal length of the muscle (L<sub>0</sub>) was assessed by twitch contractions. This procedure was



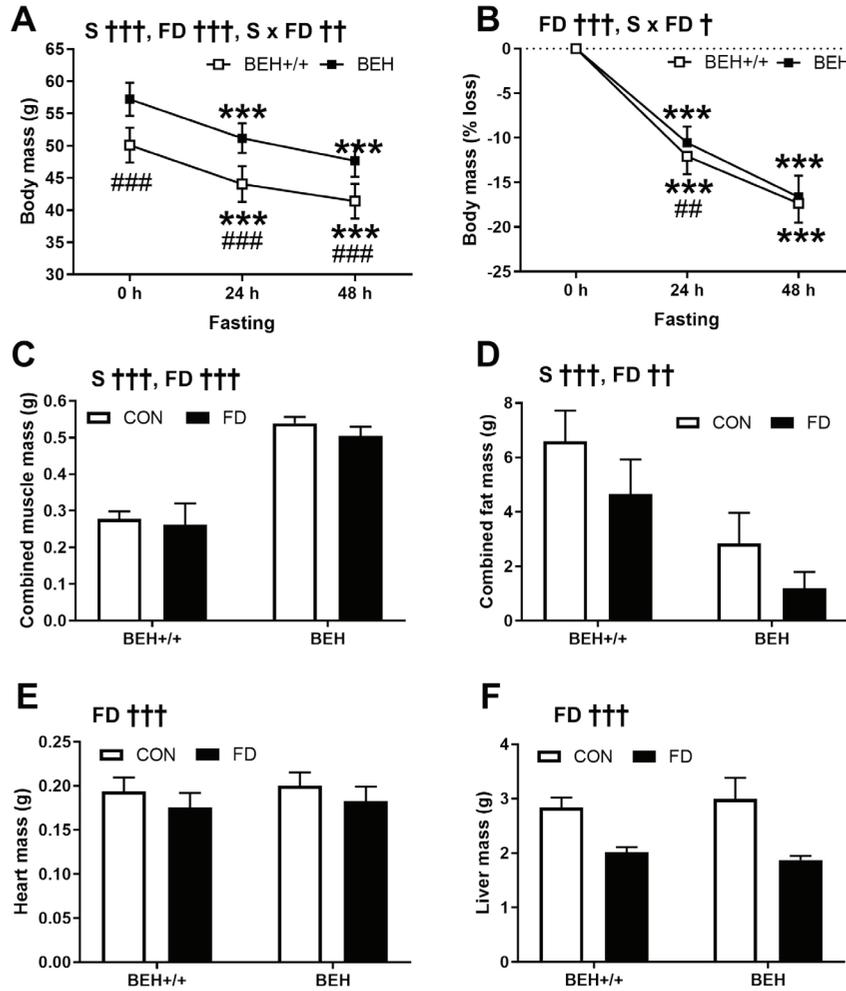
**Figure 1.** Typical images of mouse soleus muscle (SOL) cross section stained for ATPase activity. Images were obtained from the muscles of BEH+/+ (A, B) and BEH (C, D) strains in the control (CON) and food deprivation (FD) groups for analysis of muscle fiber type composition. Type 1 fibers are darkly stained.

continued until twitch force did not increase with the increase in muscle length. The muscle was then photographed with the length scale in the background to assess muscle length with a precision of 0.1 mm. The muscle was kept at this optimal length during the assessment of contractile properties. The muscle was subjected to 300-ms (EDL) and 900-ms (SOL) trains of stimuli at 20, 50, 80, 100, 150 and 200 Hz for assessment of peak tetanic force from a force-frequency curve. Specific tetanic force was calculated as peak tetanic force divided by muscle physiological cross-sectional area (PCSA) which estimated by dividing muscle wet mass by the product of fiber length ( $L_f$ ) and the skeletal muscle density ( $1.06 \text{ g/cm}^3$ ) assuming  $L_f$  to  $L_0$  ratios of 0.45 and 0.70 for EDL and SOL, respectively<sup>31</sup>.

**Energy expenditure and physical activity.** BEH+/+ (n=9) and BEH (n=9) mice were studied using Panlab metabolism system (Physiocage 00, Panlab Harvard Apparatus, Spain). Assessment of energy expenditure (EE) and respiration exchange ratio (RER) of freely moving mice was performed using the metabolic cage of standard size. The cage was connected to the gas analyzer (LE405, Panlab Harvard Apparatus, Spain) and the switching device (LE400, Panlab Harvard Apparatus, Spain) for the control of the air flow. Air flow rate was set to  $250 \text{ ml min}^{-1}$  and the switching time of 3 min, which refers to a transition time between measurements  $\text{O}_2$  and  $\text{CO}_2$  concentrations in

the metabolic cage and the external environment. Physical activity of mice was assessed using strain gauges mounted on the supporting constructions of the metabolic cage. Any movement of mice will start with the application of ground reaction force which will be recorded and provide an indirect measure of physical activity of mice. The gas analyzer LE405 was calibrated at the high point (50%  $\text{O}_2$ , 1.5%  $\text{CO}_2$ ) and at the low point (20%  $\text{O}_2$ , 0%  $\text{CO}_2$ ) prior to the experiment. All the metabolic measurements on mice were performed at light cycle (from 9:00 a.m. to 18:00 p.m.). Each mouse was weighed (Kern, ABS 80-4, Germany) and transferred into metabolic cage for 3-h assessment of EE under the free moving condition. Afterwards the mouse was weighed again and transferred into a separate cage for 48 h with *ad libitum* access to water without food. Then the mouse was weighed once more and 3-h assessment of EE was repeated. At the end of experiment, the mouse was weighed for the last time and sacrificed as explained previously. The EE ( $\text{kcal day}^{-1}$ ) was calculated as the average values of the last 2 of 3 h metabolic measurements<sup>32</sup>.

**CS enzyme activity.** Citrate synthase (CS) enzyme activity was assessed as previously described<sup>30,33</sup>. From 40 to 70 mg samples of the gastrocnemius muscle (n=8 in each group) were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 100 mM  $\text{KHPO}_4$ , 2 mM ethylenediaminetetraacetic acid

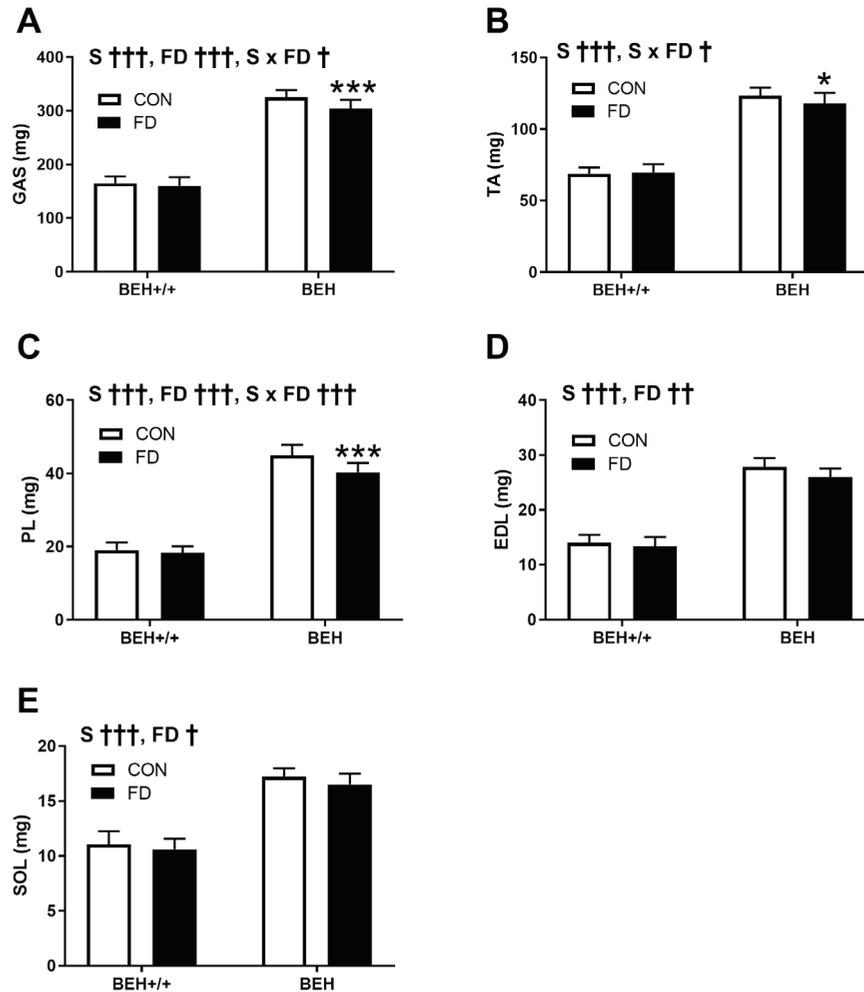


**Figure 2.** The morphometric data for BEH+/+ and BEH strains including body mass (A, B) during 48-h food deprivation as well as the combined hindlimb muscle mass (C), the combined fat mass (D), heart mass (E) and liver mass (F) in the control (CON) and food deprivation (FD) groups. The combined muscle mass is the sum of masses of gastrocnemius (GAS), tibialis anterior (TA) plantaris (PL), extensor digitorum longus (EDL) and soleus (SOL) muscles. The combined fat mass includes the subcutaneous fat (SF), gonadal fat (GF), visceral fat (VF) and interscapular fat (IF). Data are shown as mean  $\pm$  SD; A and B, n=27 for BEH+/+, n=23 for BEH; C, n=18 in CON, n=27 in FD for BEH+/+; n=14 in CON, n=23 in FD for BEH; D and F, n=8 in each group for BEH+/+, n=4 in each group for BEH; E, n=10 in all the groups.  $\dagger\dagger P < 0.01$ ,  $\dagger\dagger\dagger P < 0.001$ , two-way ANOVA for effects of strain (S), food deprivation (FD) and S x FD interaction, respectively;  $*** P < 0.001$ , post-hoc testing for differences from the initial value (0 h);  $\#\# P < 0.01$ ,  $\#\#\# P < 0.001$  differences between the strains, respectively.

(EDTA), 0.2% wt/vol bovine serum albumin, pH was adjusted to 7.0) in frozen liquid nitrogen. Then, the homogenates were defrosted by shaking for 60 min and centrifuged at 13,000 g for 10 min at 4°C for extraction of soluble proteins. The Bradford reagent (B6916, Merck, Germany) was used for assessment of protein concentration of supernatants. Measurements of CS activity were carried out using CS reaction reagent (100 mM triethanolamine-HCl, 100  $\mu$ M dithionitrobenzoic acid (DTNB), 0.5 mM Triton-X (0.25% vol/vol), 0.5 mM oxaloacetate, 0.31 mM acetyl CoA, pH 8.0) and spectrophotometer (T60 UV, PG Instruments Limited, UK) at room temperature of 21°C. 10  $\mu$ L of supernatant was added to start the reaction in 1000  $\mu$ L. The CS from a porcine

heart was used as a standard (C3260, Merck, Germany) for assay calibration. The wavelength of 412 nm and molar extinction coefficient of 13,600 M<sup>-1</sup> cm<sup>-1</sup> were used to assess the maximum CS activity ( $V_{max}$ ) during the first 2 min of the reaction.

**Muscle fiber type composition.** Fiber type composition and fiber size of soleus muscle (SOL) was determined using similar methods as in our previous study<sup>34</sup>. The transverse sections from the belly of the muscle were cut at a thickness of 10  $\mu$ m with a cryotome (Leica CM1850UV) at -20°C. The staining for myosin ATPase was performed after pre-incubation at pH of 4.34<sup>35</sup>. ImageJ software (NIH - version 1.43) was used in analysis of microscopic images of the cross sections which



**Figure 3.** Mass of specific hindlimb muscles of BEH+/+ and BEH strains in the control group (CON) and after 48-h food deprivation (FD). Abbreviations: GAS, gastrocnemius muscle; TA, tibialis anterior muscle; PL, plantaris muscle; EDL, extensor digitorum longus muscle; SOL, soleus muscle. Data are presented as mean  $\pm$  SD; n=18 in CON, n=27 in FD for BEH+/+ strain and n=14 in CON, n=23 in FD for BEH strain, respectively. †  $P<0.05$ , ††  $P<0.01$ , †††  $P<0.001$ , two-way ANOVA for effects of strain (S), food deprivation (FD) and S x FD interaction, respectively; \*  $P<0.05$ , \*\*\*  $P<0.001$  CON versus FD, respectively.

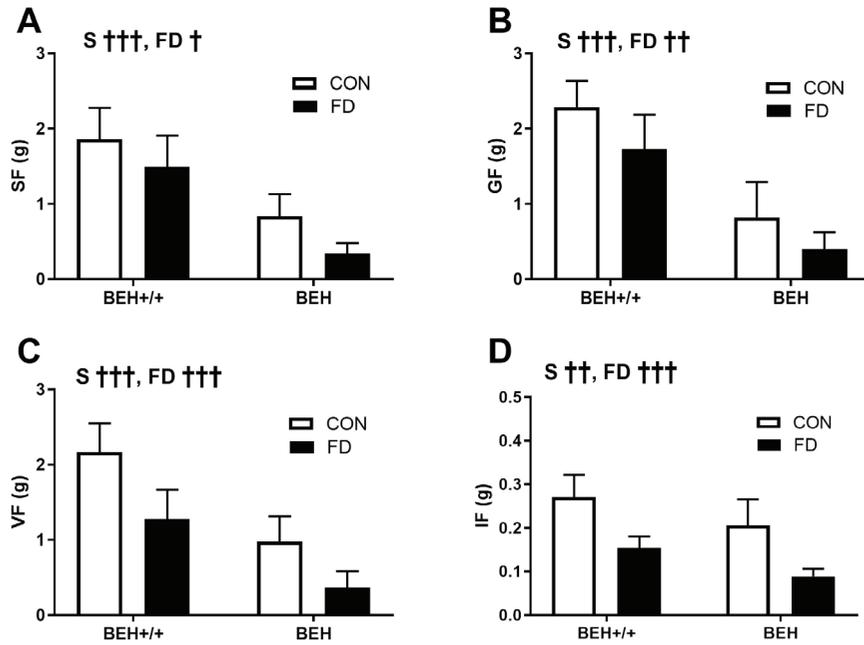
were taken with four times magnification. The typical images for BEH+/+ and BEH mice in CON and FD groups are shown in Figure 1. Total fiber number for type 1 and 2 fibers was counted. Afterwards, the average fiber cross sectional area was assessed using data from 25% of randomly selected fibers.

**Statistical analysis.** Statistical analysis was performed using Prism 7.0 and IBM SPSS Statistics (v20) software. Two-way and/or three-way ANOVA with Bonferroni's post hoc test was used to assess differences between the strains (BEH and BEH+/+) and between conditions (CON and after FD). Covariance analysis with body mass as a covariate was used to compare energy expenditure (EE) between mouse strains. Student's t-test was used when assessing differences between BEH and BEH+/+ strains in

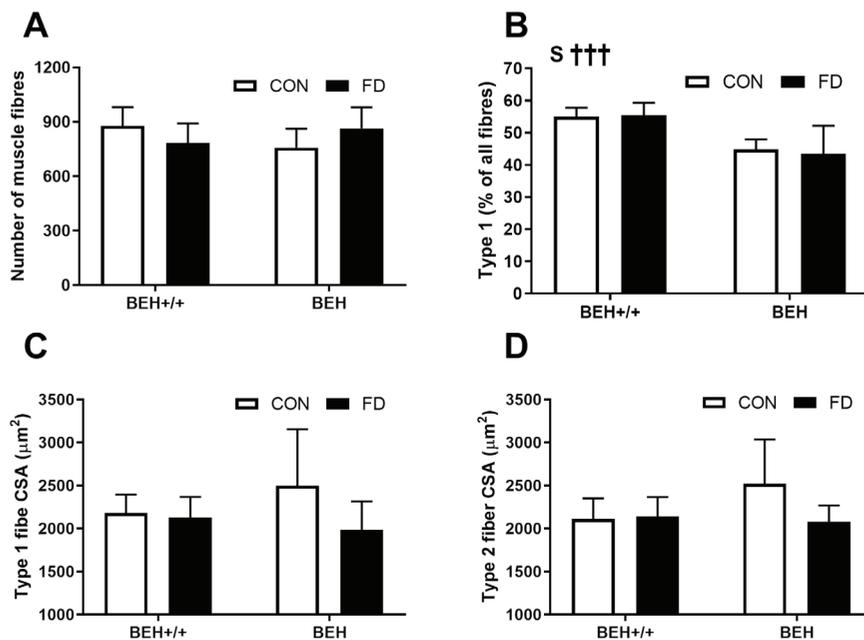
food intake. The level of significance was set at  $P<0.05$ . All data are presented as means  $\pm$  SD.

## Results

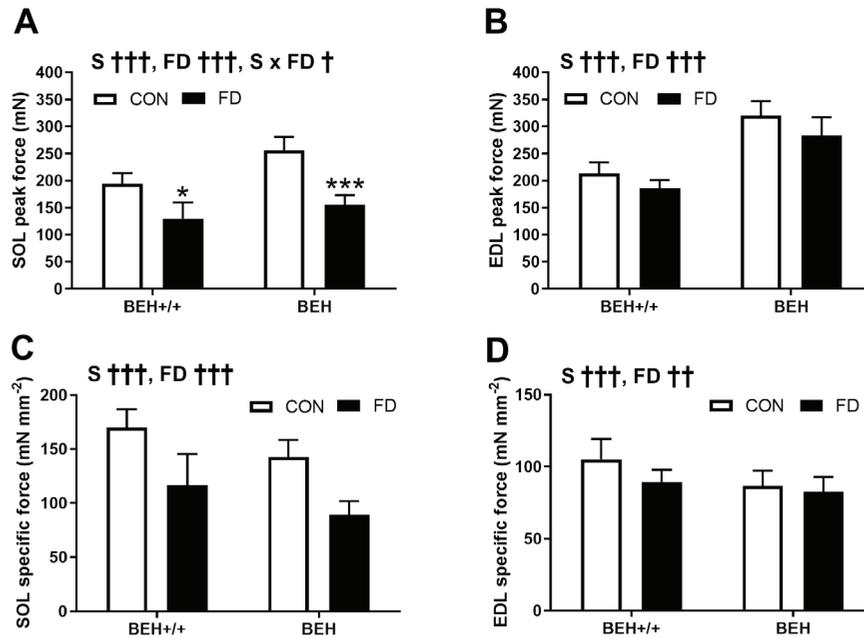
**Body composition.** The Figure 2 shows the morphometric data for BEH+/+ and BEH strains in the control group (CON) and after 48-h food deprivation (FD). BEH mice were heavier, had greater hindlimb muscle mass, but less fat than BEH+/+ mice when fed *ad libitum* (Figure 2 A, C and D). Neither heart nor liver mass differed significantly between these two mouse strains under these conditions (Figure 2 E and F). FD lead to a gradual decrease in body mass (Figure 2A and B). This decrease was faster in BEH+/+ than BEH strain during the first 24 h, but there was no difference between



**Figure 4.** Fat mass at four different sampling sites is shown for BEH+/+ and BEH strain in the control group (CON) and after 48-h food deprivation (FD). Abbreviations: SF, subcutaneous fat; GF, gonadal fat; VF, visceral fat; IF, interscapular fat. Data are presented as mean  $\pm$  SD; n=8 for BEH+/+ strain and n=4 for BEH strain, respectively. †  $P < 0.05$ , ††  $P < 0.01$ , †††  $P < 0.001$ , two-way ANOVA for effects of strain (S), food deprivation (FD) and S x FD interaction, respectively.



**Figure 5.** Total number of muscle fibres (A), muscle fiber type composition (B) and cross sectional area for type 1 (C) and type 2 (D) fibers in control (CON) mice as well as in mice after 48-h of food deprivation (FD) for BEH+/+ and BEH strains, respectively. Numbers of samples analyzed; 8 for BEH+/+ and 4 for BEH. Data are shown as mean  $\pm$  SD. †††  $P < 0.001$ , two-way ANOVA for strain (S) effects.



**Figure 6.** Force generating capacity of soleus (SOL) and extensor digitorum longus (EDL) muscles in BEH+/+ and BEH mice under the control conditions (CON) of *ad libitum* feeding and after 48 h food deprivation (FD). Peak force (A and B) and specific force (C and D) are shown as mean  $\pm$  SD; n=10 in each group, except for SOL of BEH in CON group n=9. †  $P < 0.05$ , ††  $P < 0.01$ , †††  $P < 0.001$ , two-way ANOVA for effects of strain (S), food deprivation (FD) and S x FD interaction, respectively.

the strains in the relative weight loss after 48 h of FD. Both strains experienced a relatively small (~6%) decrease in the combined hindlimb muscle mass (Figure 2C) and a large (>30%) depletion of fat reserves (Figure 2D). BEH+/+ and BEH strains also experienced a modest (~9%) heart mass loss and a substantial (~30%) liver wasting (Figure 2E and F).

**Isolated skeletal muscle mass.** Data on isolated skeletal muscle mass is presented in Figure 3. Three-way ANOVA showed effects of muscle ( $P < 0.001$ ), strain ( $P < 0.001$ ) and FD ( $P < 0.01$ ) on muscle mass. There were also interactions of muscle x strain ( $P < 0.001$ ), muscle x FD ( $P < 0.001$ ), FD x strain ( $P < 0.05$ ), muscle x FD x strain ( $P < 0.05$ ). Muscle mass was greater in BEH compared to BEH+/+ strain, as BEH to BEH+/+ muscle mass ratio was 1.6 for SOL, 1.8 for TA, 2.0 for EDL, 2.0 for GAS and 2.4 for PL. The two-way ANOVA showed significant interactions between effects of FD and strain on GAS, TA and PL as these muscles showed decline in BEH, but not in BEH+/+ mice (Figure 3A, B and C). EDL and SOL wasting did not differ significantly between the strains (Figure 3D and E).

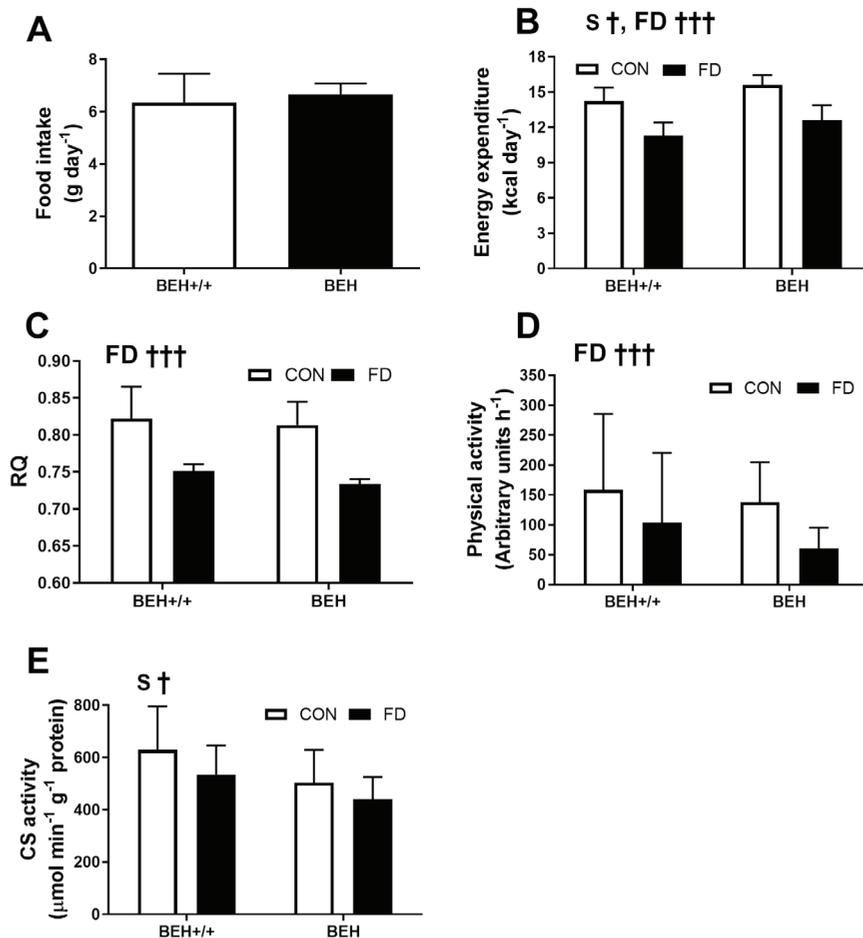
**Fat distribution.** Data on fat from four different sampling sites is presented in Figure 4. Three-way ANOVA showed significant effect of sampling site ( $P < 0.001$ ), strain ( $P < 0.001$ ) and FD ( $P < 0.01$ ) with interactions of sampling site x strain ( $P < 0.001$ ), sampling site x FD ( $P < 0.001$ ), FD x strain ( $P < 0.05$ ). Fat mass was larger in BEH+/+ mice compared to BEH mice and FD induced loss of fat at all sampling sites. This

FD induced fat depletion tended to be greater for VF and IF compared to SF and GF.

**Fiber type composition.** Data on muscle fiber properties of SOL muscle is presented in Figure 5. BEH+/+ and BEH mice did not differ in the total fiber number which was not affected by FD. BEH+/+ mice had greater percentage of type 1 fibers than BEH mice. FD did not affect muscle fiber type composition. Three-way ANOVA did not show any significant effect of fiber type, strain or FD on muscle fiber cross-sectional area. The FD effect was just below the significance level ( $P = 0.065$ ) in this analysis. For CON group, however, two-way ANOVA showed a significant strain effect ( $P = 0.036$ ) but no fiber type effect as cross-sectional area of type 1 and 2 fibers was larger by 15% and 19% in BEH mice compared to BEH+/+ mice, respectively.

**Muscle force output.** Force generating capacity of SOL and EDL is shown in Figure 6. Three-way ANOVA showed significant effects of muscle ( $P < 0.001$ ), strain ( $P < 0.001$ ) and FD ( $P < 0.001$ ) on peak force. There was also a significant interaction of muscle x strain ( $P < 0.001$ ), muscle x FD ( $P < 0.001$ ) and strain x FD ( $P < 0.05$ ). SOL generated more force than EDL, and both muscles were stronger in BEH strain than BEH+/+ mice. FD induced a decrease in the peak force and this decline was greater in SOL than EDL. For SOL, the decrease in peak force was greater for BEH than BEH+/+ strain.

Three-way ANOVA also showed significant effects of muscle ( $P < 0.001$ ), strain ( $P < 0.001$ ) and FD ( $P < 0.001$ ) on



**Figure 7.** Food intake (A), energy expenditure (B), respiratory quotient (RQ) (C), physical activity (D) and citrate synthase (CS) activity (E) in BEH<sup>+/+</sup> and BEH mice under the control conditions (CON) of *ad libitum* feeding and after 48 h food deprivation (FD). Data are shown as mean  $\pm$  SD,  $n=9$  in each group for A, B, C, D and  $n=8$  in E. †  $P<0.05$ , †††  $P<0.001$ , two-way ANOVA for effects of strain (S), food deprivation (FD) and S  $\times$  FD interaction, respectively.

specific muscle force with significant interactions of muscle  $\times$  strain ( $P<0.05$ ), muscle  $\times$  FD ( $P<0.001$ ). Specific muscle force was greater in EDL than SOL and muscles of BEH<sup>+/+</sup> mice performed better compared to BEH mice. SOL showed larger FD induced decline than EDL. BEH mice tended to show smaller changes in specific force of EDL than BEH<sup>+/+</sup> though this difference was not significant.

**Metabolism and physical activity.** Metabolic characteristics and physical activity of mice are presented in Figure 7. Food intake did not differ between BEH<sup>+/+</sup> and BEH strains. Energy expenditure (EE) was greater in BEH compared to BEH<sup>+/+</sup> mice, but the covariance analysis using body mass as a covariate did not show any significant difference between the strains. FD induced a decrease in EE of both strains. RQ as well as physical activity did not differ between the strains and decreased after FD. CS enzyme activity of GAS muscle was lower in BEH mice compared to BEH<sup>+/+</sup> mice and the negative effect of FD was below the significance level ( $P=0.086$ ).

## Discussion

The main aim of the study was to test the hypothesis that myostatin dysfunction protects against muscle atrophy during fasting. Our results do not support this hypothesis. On the contrary, after 48-h food deprivation BEH mice with myostatin dysfunction were affected by greater decrease in GAS, TA and PL mass compared to BEH<sup>+/+</sup> mice which carry the wild type myostatin. In general, skeletal muscles and heart showed relatively small mass loss compared to liver and fat reserves. On the other hand, 48-h food deprivation induced a large decrease in specific SOL force in both mouse strains. EDL also showed a decrease in specific force, but this decline tended to be somewhat smaller in BEH compared to BEH<sup>+/+</sup> strain. Our results show that changes in muscle mass cannot be used in assessment of functional impairments of skeletal muscles during fasting.

Comparison of muscle mass between BEH and BEH<sup>+/+</sup>

mice suggests that myostatin dysfunction lead to greater hypertrophy of EDL, PL and GAS than SOL. Similar findings were reported for C57BL/6 mice null for the *Mstn* gene<sup>35</sup>. It is believed that these differences between skeletal muscles are due to fiber type composition as density of myostatin receptors is greater in type 2 fibers than type 1 fibers in C57BL/6 mice<sup>8</sup>. Myostatin promotes proteolysis in slow-twitch muscle fibers of SOL while it interferes with both protein synthesis and proteolysis in fast-twitch fibers of EDL<sup>36</sup>. EDL, PL and GAS have greater proportions of type 2 fibers compared to SOL<sup>8,19,37</sup>. However, type 1 and type 2 fiber showed similar enlargement in BEH compared to BEH+/+ mice and differences in fiber type composition could not account for differences in SOL mass between these two strains. It is likely that other factors modulate effects of myostatin dysfunction on muscle fibers. This is supported by findings on the heart muscle. There was no difference in heart mass between BEH and BEH+/+ mice though myostatin suppresses protein synthesis and promotes proteolysis in cardiomyocytes<sup>36</sup>.

Mice were fasted as in other studies of myostatin dysfunction<sup>24,25</sup>. After 48-h food deprivation there was ~17% decrease in body weight of BEH+/+ and BEH mice. Allen et al.<sup>24</sup> reported 25-28% decrease in body mass of C57BL/6J mice which have two fold smaller body mass than BEH mice. Body mass normalized metabolic rate decreases with increase in body mass of mammals<sup>38</sup>. Obese women weighing approximately 80 kg lost only 2.5% of body mass after 48-h fasting<sup>39</sup>. Our measurements of energy expenditure suggest that BEH mice expend at least 6 times more energy per unit of body mass than humans<sup>40</sup>. Differences in weight loss between mice and humans suggest that metabolic rate is a key factor in fasting-induced weight loss.

Skeletal muscle and heart mass decreased by less than 10% while there was 30-40% liver wasting and considerable loss of body fat after 48-h food deprivation. Liver wasting is probably due to depletion of glycogen<sup>41</sup> and protein loss which is associated with reduced metabolic requirements in fasted animals<sup>42</sup>. RQ fell from 0.82 to 0.72 over the 48-h period fasting in both mouse strains suggesting that fat was the major source of energy during fasting. As in C57BL/6 mice, BEH mice with myostatin dysfunction had less body fat than BEH+/+ mice when fed *ad libitum*<sup>7,43,44</sup>. Low body fat is probably of major importance for greater muscle wasting in BEH compared to BEH+/+ mice. In contrast to this line of reasoning, Allen et al.<sup>24</sup> reported greater resistance to atrophy in TA of C57BL/6 mice null for myostatin compared to the wild type controls after 48-h fasting. Only 4 or 5 animals per group were used by Allen et al.<sup>24</sup> who presented data for SOL and TA only. Small sample size can lead to aberrant results as mice of the same strain can vary in body and muscle mass. We analyzed at least 14 muscles in each experimental group in order to minimize unpredictable effects of variations in muscle mass between mice. This helped to provide robust evidence about differences in muscle atrophy between BEH and BEH+/+ strains.

Differences in metabolism between C57BL/6 and BEH

mice might also affect degree of muscle atrophy. BEH and BEH+/+ strains did not differ in RQ while C57BL/6 mice null for myostatin show greater RQ compared to the wild type controls<sup>43,45</sup>. Thus myostatin dysfunction is associated with a significant shift from fat to carbohydrate oxidation in C57BL/6 strain, but not in BEH mice. This shift is expected to promote gluconeogenesis from amino acids and increase loss of muscle mass in C57BL/6 mice null for myostatin compared to the wild type controls. This would provide another line of evidence contradicting findings of Allen et al.<sup>24</sup>. Unfortunately, no RQ data is available for C57BL/6 mice null for myostatin during food deprivation to support our contention. Nevertheless, our data provides another line of evidence that genetic background is of importance in mediating physiological effects of myostatin dysfunction on the whole body metabolism in addition to skeletal muscle properties<sup>28</sup>.

48-h fasting led to a decrease in energy expenditure in both BEH and BEH+/+ strains. Decreases in physical activity, diet induced thermogenesis as well as alteration in hormone profile could be of significance under these conditions<sup>46-48</sup>. The observed decrease in physical activity is in contrast to feeding anticipatory behavior which increases physical activity in mice subjected to 30% caloric restriction<sup>49</sup>. We have also noted a significant reduction in interscapular fat which is dominated by a brown fat<sup>50</sup>. This reduction might also play a role in lower heat production and reduction in energy expenditure of the fasted mice<sup>50</sup>.

Fasting induced a large decrease in specific SOL force in both strains. We could not find any other study reporting such a decrease in specific SOL force after fasting as studies of C57BL/6 mice focused primarily on EDL<sup>25,34</sup>. SOL shows high involvement in postural activities and locomotion and its impairment could have significant functional implications<sup>51</sup>. Hypothetically, changes in muscle fiber type composition might affect specific force of skeletal muscles as type 1 fibers have lower specific force than type 2 fibers<sup>52</sup>. Caloric restriction leads to a greater decrease in the cross sectional area of type 2 fibers compared to type 1 fibers<sup>53</sup>. However, areas of both type 1 and type 2 fibers tended to decrease in BEH mice while BEH+/+ did not show a significant decline in fiber sizes after 48-h food deprivation. Thus, relative muscle cross-sectional area occupied by type 1 did not increase and could not be responsible for a decline in specific SOL force. Fasting did not have a significant effect on CS activity which was lower in the gastrocnemius of BEH mice compared to BEH+/+ mice. CS is a popular marker of mitochondrial content in mice and humans<sup>54,55</sup>. Thus, it is unlikely that the decline in specific muscle force was due to impaired capacity for aerobic ATP resynthesis. Glycogen depletion could potentially interfere with excitation contraction coupling of SOL and cause force loss<sup>56</sup>. Detailed studies of excitation contraction coupling and force generation by contractile filaments are needed to explore mechanisms for a decline in specific SOL force after fasting. This was beyond the scope of the current investigation.

The comparison of changes in specific EDL force between

BEH and BEH+/+ mice suggests that myostatin dysfunction tended to reduce the fall in specific EDL force in BEH mice. This is largely in agreement with findings of Collins-Hooper et al.<sup>25</sup> who reported improvement in specific force of EDL from C57BL/6 mice after 24-h fasting which was associated with a decrease in accumulation of non-contractile proteins in fast twitch muscle fibers of these mice null for the *Mstn* gene. Increases in specific EDL force in C57BL/6 mice null for the *Mstn* gene were also reported after 40% CR and endurance training<sup>22,23</sup>. We observed significantly less pronounced effects of 48-h food deprivation on EDL of BEH mice compared to the findings on C57BL/6 mice null for the *Mstn* gene<sup>22,23</sup>. Thus, effects of myostatin dysfunction on muscle specific force also depend on the strain background.

There were no differences between strains in the heart mass loss after 48 h food deprivation. Fasting-induced impairment in heart function is a serious health risk during prolonged fasting<sup>57</sup>. This decline could be associated with the decrease in physical activity and it would be important to ascertain what levels of physical activity are required to prevent a decline in heart function during fasting, but this is beyond the scope of the current study.

Fasting is associated with upregulation of proteolytic and autophagy programs in skeletal muscles<sup>25</sup>. Lack of data on molecular mechanisms contributing to muscle atrophy might be considered as a limitation of the current study. On the other hand, extent of muscle wasting and differences between BEH and BEH+/+ strains were rather small and varied significantly between the muscles. It appears that muscle cell cultures with strict control of experimental conditions are better suited for studies of molecular mechanisms of myostatin effects<sup>36</sup>.

In summary, our results show that myostatin dysfunction does not protect from skeletal muscle wasting during fasting. The 48-h food deprivation is associated with a significant decrease in physical activity and energy expenditure of mice. It is also associated with a decrease in fat reserves and pronounced liver wasting. Food deprivation leads to a marked decline in specific SOL force. The decline of specific EDL force was relatively small especially in BEH mice with myostatin dysfunction. It appears that myostatin dysfunction is associated with accumulation of excessive amounts of protein in skeletal muscles and FD induces less severe functional impairments in the fast-twitch muscles of BEH mice compared to BEH mice with functional myostatin.

#### Acknowledgements

We would like to thank prof. Lutz Bünger for providing BEH and BEH+/+ mice for this study and Mrs Indrė Libnickienė for her excellent technical assistance provided during the project.

#### Author Contributions

A.F., P.M. and A.R. conceived and designed research; A.F. and P.M. performed experiments; A.F., P.M. M.K. and A.R. analyzed data; A.F., P.M., T.V., A.L., M.K. and A.R. interpreted results of experiments; A.F., P.M. and A.R. prepared figures; A.R. drafted manuscript; A.F., P.M., T.V., A.L., M.K. and A.R. edited and revised manuscript; A.F., P.M., T.V., A.L., M.K. and A.R. approved final version of manuscript.

## References

1. Wolfe RR. The underappreciated role of muscle in health and disease. *The American journal of clinical nutrition* 2006;84(3):475-82.
2. Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 2005; 37(10):1974-84.
3. Sakuma K, Aoi W, Yamaguchi A. The intriguing regulators of muscle mass in sarcopenia and muscular dystrophy. *Front Aging Neurosci* 2014;6:230.
4. Donnelly JE, Jakicic J, Gunderson S. Diet and body composition. Effect of very low calorie diets and exercise. *Sports Med* 1991;12(4):237-49.
5. Saris WH. Very-low-calorie diets and sustained weight loss. *Obes Res* 2001;9 Suppl 4:295s-301s.
6. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 1997;387(6628):83-90.
7. Amthor H, Macharia R, Navarrete R, et al. Lack of myostatin results in excessive muscle growth but impaired force generation. *Proc Natl Acad Sci U S A* 2007;104(6):1835-40.
8. Mendias CL, Marciniak JE, Calderon DR, Faulkner JA. Contractile properties of EDL and soleus muscles of myostatin-deficient mice. *Journal of applied physiology* (Bethesda, Md: 1985) 2006;101(3):898-905.
9. Zimmers TA, Davies MV, Koniaris LG, et al. Induction of cachexia in mice by systemically administered myostatin. *Science (New York, NY)* 2002;296(5572):1486-8.
10. Reisz-Porszasz S, Bhasin S, Artaza JN, et al. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. *Am J Physiol Endocrinol Metab* 2003;285(4):E876-88.
11. Lipina C, Kendall H, McPherron AC, Taylor PM, Hundal HS. Mechanisms involved in the enhancement of mammalian target of rapamycin signalling and hypertrophy in skeletal muscle of myostatin-deficient mice. *FEBS Lett* 2010;584(11):2403-8.
12. Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J* 2013;280(17):4294-314.
13. Bogdanovich S, Krag TO, Barton ER, et al. Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 2002;420(6914):418-21.
14. Tsuchida K. Myostatin inhibition by a follistatin-derived peptide ameliorates the pathophysiology of muscular dystrophy model mice. *Acta Myol* 2008;27:14-8.
15. Smith RC, Lin BK. Myostatin inhibitors as therapies for muscle wasting associated with cancer and other disorders. *Curr Opin Support Palliat Care* 2013; 7(4):352-60.
16. Khan T, Weber H, DiMuzio J, et al. Silencing Myostatin Using Cholesterol-conjugated siRNAs Induces Muscle Growth. *Mol Ther Nucleic Acids* 2016;5(8):e342.
17. St Andre M, Johnson M, Bansal PN, et al. A mouse anti-myostatin antibody increases muscle mass and

- improves muscle strength and contractility in the mdx mouse model of Duchenne muscular dystrophy and its humanized equivalent, domagrozumab (PF-06252616), increases muscle volume in cynomolgus monkeys. *Skelet Muscle* 2017;7(1):25.
18. Gentry BA, Ferreira JA, Phillips CL, Brown M. Hindlimb skeletal muscle function in myostatin-deficient mice. *Muscle Nerve* 2011;43(1):49-57.
  19. Ploquin C, Chabi B, Fouret G, et al. Lack of myostatin alters intermyofibrillar mitochondria activity, unbalances redox status, and impairs tolerance to chronic repetitive contractions in muscle. *Am J Physiol Endocrinol Metab* 2012;302(8):E1000-8.
  20. Qaisar R, Renaud G, Morine K, Barton ER, Sweeney HL, Larsson L. Is functional hypertrophy and specific force coupled with the addition of myonuclei at the single muscle fiber level? *FASEB J* 2012;26(3):1077-85.
  21. Minderis P, Kilikevicius A, Baltusnikas J, et al. Myostatin dysfunction is associated with reduction in overload induced hypertrophy of soleus muscle in mice. *Scandinavian Journal of Medicine & Science in Sports* 2016;26(8):894-901.
  22. Matsakas A, Romanello V, Sartori R, et al. Food restriction reverses the hyper-muscular phenotype and force generation capacity deficit of the myostatin null mouse. *Int J Sports Med* 2013;34(3):223-31.
  23. Matsakas A, Macharia R, Otto A, et al. Exercise training attenuates the hypermuscular phenotype and restores skeletal muscle function in the myostatin null mouse. *Exp Physiol* 2012;97(1):125-40.
  24. Allen DL, Cleary AS, Lindsay SF, Loh AS, Reed JM. Myostatin expression is increased by food deprivation in a muscle-specific manner and contributes to muscle atrophy during prolonged food deprivation in mice. *Journal of applied physiology (Bethesda, Md: 1985)* 2010;109(3):692-701.
  25. Collins-Hooper H, Sartori R, Giallourou N, et al. Symmorphosis through dietary regulation: a combinatorial role for proteolysis, autophagy and protein synthesis in normalising muscle metabolism and function of hypertrophic mice after acute starvation. *PLoS One* 2015;10(3):e0120524.
  26. Bunger L, Laidlaw A, Bulfield G, et al. Inbred lines of mice derived from long-term growth selected lines: unique resources for mapping growth genes. *Mamm Genome* 2001;12(9):678-86.
  27. Varga L, Szabo G, Darvasi A, Muller G, Sass M, Soller M. Inheritance and mapping of Compact (Cmpt), a new mutation causing hypermuscularity in mice. *Genetics* 1997;147(2):755-64.
  28. Lionikas A, Kilikevicius A, Bunger L, et al. Genetic and genomic analyses of musculoskeletal differences between BEH and BEL strains. *Physiol Genomics* 2013; 45(20):940-7.
  29. Lionikas A, Smith CJ, Smith TL, Bunger L, Banks RW, Bewick GS. Analyses of muscle spindles in the soleus of six inbred mouse strains. *J Anat* 2013;223(3):289-96.
  30. Kvedaras M, Minderis P, Fokin A, Ratkevicius A, Venckunas T, Lionikas A. Forced Running Endurance Is Influenced by Gene(s) on Mouse Chromosome 10. *Front Physiol* 2017;8:9.
  31. Brooks SV, Faulkner JA. Contractile properties of skeletal muscles from young, adult and aged mice. *J Physiol* 1988;404:71-82.
  32. Speakman JR. Body size, energy metabolism and lifespan. *J Exp Biol* 2005;208(Pt 9):1717-30.
  33. Ratkevicius A, Carroll AM, Kilikevicius A, et al. H55N polymorphism as a likely cause of variation in citrate synthase activity of mouse skeletal muscle. *Physiol Genomics* 2010;42a(2):96-102.
  34. Kilikevicius A, Venckunas T, Zelniene R, et al. Divergent physiological characteristics and responses to endurance training among inbred mouse strains. *Scandinavian Journal of Medicine & Science in Sports* 2013;23(5):657-68.
  35. Brooke MH, Kaiser KK. Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. *The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society* 1970;18(9):670-2.
  36. Manfredi LH, Paula-Gomes S, Zanon NM, Kettelhut IC. Myostatin promotes distinct responses on protein metabolism of skeletal and cardiac muscle fibers of rodents. *Braz J Med Biol Res* 2017;50(12):e6733.
  37. Bloemberg D, Quadrilatero J. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One* 2012;7(4):e35273.
  38. Hochachka PW, Darveau CA, Andrews RD, Suarez RK. Allometric cascade: a model for resolving body mass effects on metabolism. *Comp Biochem Physiol A Mol Integr Physiol* 2003;134(4):675-91.
  39. Solianik R, Sujeta A. Two-day fasting evokes stress, but does not affect mood, brain activity, cognitive, psychomotor, and motor performance in overweight women. *Behav Brain Res* 2018;338:166-72.
  40. Pontzer H, Durazo-Arvizu R, Dugas LR, et al. Constrained Total Energy Expenditure and Metabolic Adaptation to Physical Activity in Adult Humans. *Curr Biol* 2016;26(3):410-7.
  41. Baldwin KM, Fitts RH, Booth FW, Winder WW, Holloszy JO. Depletion of muscle and liver glycogen during exercise. Protective effect of training. *Pflugers Archiv: European Journal of Physiology* 1975;354(3):203-12.
  42. Salin K, Villasevil EM, Anderson GJ, et al. Decreased mitochondrial metabolic requirements in fasting animals carry an oxidative cost. *Functional Ecology* 2018;32(9):2149-57.
  43. McPherron AC, Lee SJ. Suppression of body fat accumulation in myostatin-deficient mice. *The Journal of Clinical Investigation* 2002;109(5):595-601.
  44. Zhao B, Wall RJ, Yang J. Transgenic expression of myostatin propeptide prevents diet-induced obesity and insulin resistance. *Biochem Biophys Res Commun*

- 2005;337(1):248-55.
45. Guo T, Jou W, Chanturiya T, Portas J, Gavrilova O, McPherron AC. Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. *PLoS One* 2009;4(3):e4937.
  46. Hambly C, Speakman JR. Contribution of different mechanisms to compensation for energy restriction in the mouse. *Obes Res* 2005;13(9):1548-57.
  47. Mitchell SE, Tang Z, Kerbois C, et al. The effects of graded levels of calorie restriction: I. impact of short term calorie and protein restriction on body composition in the C57BL/6 mouse. *Oncotarget* 2015;6(18):15902-30.
  48. Mitchell SE, Tang Z, Kerbois C, et al. The effects of graded levels of calorie restriction: VIII. Impact of short term calorie and protein restriction on basal metabolic rate in the C57BL/6 mouse. *Oncotarget* 2017(11);8:17453-74.
  49. van Norren K, Rusli F, van Dijk M, et al. Behavioural changes are a major contributing factor in the reduction of sarcopenia in caloric-restricted ageing mice. *Journal of Cachexia, Sarcopenia and Muscle* 2015;6(3):253-68.
  50. Connolly E, Morrisey RD, Carnie JA. The effect of interscapular brown adipose tissue removal on body-weight and cold response in the mouse. *The British Journal of Nutrition* 1982;47(3):653-8.
  51. Roy RR, Hutchison DL, Pierotti DJ, Hodgson JA, Edgerton VR. EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming. *Journal of applied physiology (Bethesda, Md: 1985)* 1991;70(6):2522-9.
  52. Bottinelli R, Canepari M, Pellegrino MA, Reggiani C. Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. *J Physiol* 1996;495(Pt 2):573-86.
  53. Goldspink G, Ward PS. Changes in rodent muscle fibre types during post-natal growth, undernutrition and exercise. *J Physiol* 1979;296:453-69.
  54. Jacobs RA, Diaz V, Meinild AK, Gassmann M, Lundby C. The C57Bl/6 mouse serves as a suitable model of human skeletal muscle mitochondrial function. *Exp Physiol* 2013;98(4):908-21.
  55. Vigelso A, Andersen NB, Dela F. The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training. *Int J Physiol Pathophysiol Pharmacol* 2014;6(2):84-101.
  56. Westerblad H, Place N, Yamada T. Mechanisms of skeletal muscle weakness. *Adv Exp Med Biol* 2010;682:279-96.
  57. Spencer IO. Death during therapeutic starvation for obesity. *Lancet* 1968;1(7555):1288-90.