



2014-09-01

# Downhill Treadmill Running Does Not Induce Muscle Damage in FVB Mice

Brenda Benson

Brigham Young University - Provo

Follow this and additional works at: <https://scholarsarchive.byu.edu/etd>

 Part of the [Exercise Science Commons](#)

---

## BYU ScholarsArchive Citation

Benson, Brenda, "Downhill Treadmill Running Does Not Induce Muscle Damage in FVB Mice" (2014). *All Theses and Dissertations*. 4259.

<https://scholarsarchive.byu.edu/etd/4259>

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact [scholarsarchive@byu.edu](mailto:scholarsarchive@byu.edu), [ellen\\_amatangelo@byu.edu](mailto:ellen_amatangelo@byu.edu).

Downhill Treadmill Running Does Not Induce Muscle Damage in FVB Mice

Brenda Benson

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Master of Science

Allen C. Parcell, Chair  
Gary W. Mack  
David M. Thomson  
Robert D. Hyldahl

Department of Exercise Sciences

Brigham Young University

September 2014

Copyright © 2014 Brenda Benson

All Rights Reserved

## ABSTRACT

### Downhill Treadmill Running Does Not Induce Muscle Damage in FVB Mice

Brenda Benson  
Department of Exercise Sciences, BYU  
Master of Science

Downhill treadmill running is a commonly used method to cause exercise-induced muscle damage, especially in rodents. Previous studies have evaluated which muscles in rats are more prone to damage. However research using downhill run mice (DHR) has shown some inconsistencies in which muscle is best analyzed for damage. Purpose: The purpose of this study was to quantify the damage in various muscles in a mouse after a single bout of DHR. Methods: Male FVB mice (5 months) were injected with Evans Blue dye (EBD) and then either used as control (CON) or run downhill (-16°) at 20 meters per minute (m/min) for 30 minutes. Twenty-four hours after exercise, the gastrocnemius, soleus, plantaris, tibialis anterior (TA), quadriceps, and triceps brachii muscles were harvested (n = 6 per group per muscle). Cross-sectional slices were obtained, fixed, and mounted to analyze EBD infiltration, dystrophin (Dys), and centralized nuclei. The samples were then imaged using a fluorescent microscope. The entire sample was captured using 20x magnification, and the total number of cells, EBD+, Dys-, and centralized nuclei, were counted. A blood sample was collected to measure plasma creatine kinase (CK) activity. Results: Total number of cells was not different between groups ( $p > 0.05$ ). No significant difference in any of the markers of muscle damage was found in any muscle between CON and DHR ( $p > 0.05$ ). Conclusion: These data suggest that DHR does not induce muscle damage in adult (5 months) male FVB mice.

Keywords: downhill running, mice, muscle damage, Evans Blue dye, dystrophin

## ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Parcell, for his continual patience and help in directing me in this thesis and research. I appreciate all the time and effort that my committee has put into developing the best methods for this research and the help they have given me. I would especially like to thank my fellow graduate students, family, and friends for their support and encouragement during all the changes and problems that occurred. I am grateful for Maggie Shibla's help in making the whole process happen and making sure I had everything in place. I am grateful for Brigham Young University and the College of Life Sciences for the importance they place on learning and the opportunities they allow us to have. I am grateful for Graduate Studies and the research grant they awarded me to help me with my research. I am most grateful for all the divine help and inspiration I received throughout this graduate program, because I would not be here without it.

## Table of Contents

Title.....	i
Abstract.....	ii
Acknowledgements.....	iii
List of Tables.....	v
List of Figures.....	vii
Introduction.....	1
Methods.....	2
<i>Animals</i> .....	2
<i>Downhill Running Protocol</i> .....	3
<i>Tissue Preparation</i> .....	3
<i>Tissue Histochemistry</i> .....	4
<i>Blood Creatine Kinase Analysis</i> .....	5
<i>Statistical Analysis</i> .....	6
Results.....	6
<i>Animals</i> .....	6
<i>Evans Blue Dye Infiltration</i> .....	7
<i>Disrupted Dystrophin</i> .....	7
<i>Centralized Nuclei</i> .....	7
<i>Creatine Kinase</i> .....	8
Discussion.....	8
References.....	14
Appendix A.....	20

## List of Tables

### Table

1. Totals and Percentages of Markers of Muscle Damage..... 16

## List of Figures

### Figure

1. Total Number of EBD Positive Cells..... 17
2. Total Number of Dystrophin Negative Cells ..... 18
3. Total Number of Centralized Nuclei..... 19

## Introduction

Eccentric or lengthening contractions provide a unique stimulus to muscle. They have been shown to result in greater damage to the muscle fiber (29) when compared to isometric or concentric contractions. In order to assess damage to a muscle after a bout of exercise, several direct and indirect markers have been used. Direct markers of muscle damage include histological evidence of degenerating or necrotic fibers (25), z-line streaming (39), disrupted cell membranes (7, 25), and the presence of inflammatory cells (29). Indirect markers include muscle soreness (14, 35), reduced maximal force output (14, 35), increased serum levels of creatine kinase (CK) and myoglobin (14, 35), muscle swelling (14), and decreased relaxed joint angle (14, 35).

Most studies investigating exercise-induced muscle damage typically use eccentric muscle contraction via either resistance exercise (14, 34) or downhill running (10, 38). Resistance exercise utilizing lengthening contractions is typically done in humans and can easily isolate the eccentric motion only. This method also tends to isolate a single muscle or muscle group. However, the insult to the muscle is greater because of the intensity that is generally used, resulting in indices of damage that are difficult to compare with other methods, such as downhill running (36). Downhill running is common in both animal and human models, uses multiple groups of muscles, and produces a more typical response (level of muscle) in damage that might be seen after a bout of exercise (clinically) (36).

The effects of downhill running on muscle damage have been characterized in the rat, looking at the triceps brachii, quadriceps, gastrocnemius, soleus and tibialis anterior (3), but a broad study of the impact of downhill running in the muscle tissue of a mouse has not been conducted. Many muscle damage studies using downhill running in mice base their methods on



the initial rat studies. However the selection of the muscle to be analyzed varies among studies (7, 28, 40, 44). Mice are smaller and their skeletal muscle tends to express a high percentage of type II muscle fibers compared to rats (1). Lower body mass and different muscle fiber composition could influence which specific muscle groups are excessively loaded during downhill running and/or decrease the extent of damage to a given muscle.

In preliminary studies using mice, we did not find the level of damage after a single bout of downhill running as predicted by the existing literature (6, 7). Our downhill running protocol was based on established downhill running protocols, but the literature was unclear as to which muscle would be most damaged during downhill running and therefore the best muscle to be analyzed. The purpose of this study was to identify which skeletal muscles in the mouse were damaged by downhill running and the extent of muscle damage in each muscle group. We used infiltration of Evans Blue dye in the muscle cells and the presence of muscle creatine kinase in the blood to identify muscle damage.

## **Methods**

### *Animals*

Nineteen adult (5 months) male FVB albino strain mice were used (Charles River). Animals were housed in standard cages on a 12-hour light-dark cycle (6am–6pm) in a temperature controlled room. Standard mouse chow and water were provided *ad libitum*. Animals were picked up and handled daily to accustom them to human handling. Mice were randomly assigned to a downhill running (DHR) group or a cage control (CON) group. All procedures were performed with BYU Institutional Animal Care and Use Committee (IACUC) approved procedures.

### *Downhill Running Protocol*

Mice in the DHR group were subjected to a single bout of downhill running. Treadmill familiarization was performed prior to the downhill running bout to ensure animal cooperation during the exercise. Familiarization consisted of placing the animals in the declined treadmill chamber for five minutes with no treadmill speed. The downhill run consisted of a warm-up of gradually increasing speed from 0–20 meters per minute ( $m \cdot \text{min}^{-1}$ ) over 5 mins, and then the mice ran for 30 mins at a speed of  $20 m \cdot \text{min}^{-1}$  with a decline of  $20^\circ$  on a standard mouse treadmill (Exer-3/6 treadmill, Columbus Instruments). This protocol was reported to elicit muscle damage in muscles of the hindlimb in mice (6, 7, 26). We used an active shock grid (2 Hz) located at the end of the treadmill belt, and coarse bristle bottle brushes were inserted inside the treadmill chamber immediately in front of the shock grid to encourage the mice to run. Mice that consistently stayed on the shock grid and would not run were not used in the study. After running, the mice were returned to their cages until tissue harvest.

### *Tissue Preparation*

Evans Blue dye infiltration in the cytoplasm of cells was used to identify damage to the sarcolemma of the muscles. An Evans Blue dye solution (1%) in sterile phosphate-buffered saline (PBS) (0.1 mg/ml) was injected intraperitoneally at a volume of 0.01 ml/10 grams of body weight (6, 17, 26). DHR mice were injected with Evans Blue dye 90 mins prior to the downhill running bout, and tissue harvest took place 24 hours after the bout of exercise. CON mice were sacrificed 24 hours after Evans Blue injection (17). The animals were anesthetized with 2.0–2.5% isoflurane mixed with oxygen at a flow rate of 0.8 liters per min (L/min). Selected muscles of the hindlimb and forelimb were isolated and harvested for analysis: gastrocnemius, soleus, plantaris, tibialis anterior (TA), quadriceps and triceps. Once dissected, the muscle length

was measured (in mm) and the sample was mounted in tragacanth gum with the distal portion of the muscle at the top to ensure that sections from the distal half of the muscle sample were used for analysis (7). The muscle was frozen in isopentane cooled by liquid nitrogen. The contralateral muscle was measured for length, weighed for a wet tissue weight, and flash frozen in liquid nitrogen. These samples were stored at  $-80^{\circ}\text{C}$  for future protein analysis. Finally, a venous blood sample was collected and centrifuged at  $11,180 \times g$  for ten minutes at  $4^{\circ}\text{C}$ . The plasma was transferred to a new tube and frozen at  $-80^{\circ}\text{C}$  until analysis.

### *Tissue Histochemistry*

The frozen, mounted tissue sample was serially sectioned ( $8 \mu\text{m}$ ) using a cryostat microtome (Microm HM 525, Thermo Scientific) and mounted on slides. Section counts were monitored on the microtome to ensure that muscle sections were taken from the distal half of the muscle sample. One slide was analyzed for Evans Blue dye infiltration and the other slide was treated and analyzed for dystrophin and centralized nuclei.

Evans Blue dye is an indicator of membrane permeability in rodents by infiltrating muscle fibers with a damaged sarcolemma and can then be viewed with fluorescent microscopy (17). For determination of Evans Blue dye infiltration in skeletal muscle cells, sections were fixed in cold acetone ( $-20^{\circ}\text{C}$ ) for 1 min and air-dried. The sections were then dipped in xylene, allowed to dry, and mounted with Canada balsam. Sections were imaged using fluorescent microscopy (20x objective, Excitation<sub>560</sub>/Emission<sub>590</sub> ; Olympus IX73) and total number of cells and total Evans Blue-positive cells were counted.

Specific damage to the sarcolemma was determined by decreased or discontinuous staining of the cytoskeletal protein dystrophin. Only the Evans Blue-positive cells were assessed for dystrophin-negative staining as a means to confirm the damage indicated by the dye.

Centralized nuclei were counted because they are an indicator that the cell is undergoing a regenerative process. Muscle sections were fixed in 4% paraformaldehyde for 5 mins and then rinsed in phosphate buffered saline (PBS). Sections were blocked with a 5% FBS, 2% BSA, 0.2% triton X-100, 0.1% sodium azide in PBS solution for 30 mins. Sections were incubated in primary antibody for dystrophin (MF20, Developmental Studies Hybridoma Bank) (1:50) overnight at 4 °C and then rinsed (3 x 5 mins) with PBS. Finally, sections were also incubated with a cocktail containing a secondary Alexa-Fluor-488 fluorescent antibody (sc-2010, Santa Cruz Biotechnology) (1:100) and a DAPI nucleic acid stain for 30 mins at 37 °C. The sections were rinsed (3 x 5 mins) in PBS and mounted with Canada balsam. The samples were then imaged on a fluorescent microscope (Olympus IX73) at 20x magnification (dystrophin: Excitation<sub>488</sub>/Emission<sub>520</sub>; DAPI: Excitation<sub>350</sub>/Emission<sub>460</sub>). Only Evans Blue-positive cells were assessed for discontinuous or disrupted dystrophin staining. The parameters to be counted as a dystrophin-negative fiber required a complete absence or break in membrane staining and a contrast between the dystrophin stain and the rest of the cell (43). The total number of Evans Blue-positive cells that also had discontinuous dystrophin staining were counted. Images of dystrophin and DAPI were merged to identify centralized nuclei, and the total number of centralized nuclei were counted per section.

#### *Blood Creatine Kinase Analysis*

Creatine kinase activity levels were assessed using a microplate kit (MAK116, Sigma). Activity is measured through a coupled reaction that produces a byproduct, which is measured by ultraviolet spectrophotometry at 340 nm and is proportionate to the amount of creatine kinase present. Absorbance values were converted to U/L based upon a standard curve.

### *Statistical Analysis*

Based on the sample sizes of similar, existing studies, a sample size of six animals per group was determined to be adequate in order to find significant differences between groups. All analyses were made using T-Tests to compare CON and DHR groups in respect to body mass, muscle mass, total cell number, Evans Blue-positive cells, dystrophin negative cells, centralized nuclei, and creatine kinase activity. Data are presented as mean  $\pm$  SE, and significance was set at  $p < 0.05$ .

## **Results**

### *Animals*

The animals ranged in age from 17 to 25 weeks old. The mean age for the CON group was  $20.3 \pm 0.7$  (n = 9) and the DHR group was  $21.4 \pm 0.9$  (n = 10), and the groups were not significantly different (Table 1). The mean body weight of the animals was  $29.2 \pm 0.6$ g (n = 9) in the CON animals and  $30.5 \pm 0.7$ g (n = 10) in the DHR animals with no significant difference between groups (Table 1). Initially, only six animals were used per group; however it was necessary to acquire additional samples for the soleus and plantaris because of freeze fracture making the samples unanalyzable in order to maintain a sample size of six. We used animals C1–C6 and DHR1–DHR6 wherever possible, including the gastrocnemius, TA, quadriceps, and triceps. For the soleus, animals C3–C5, C7–C9, DHR1, DHR3, and DHR7–DHR10 were analyzed. For the plantaris, animals C1–C6 and DHR2–DHR7 were analyzed (Table 1). For the creatine kinase activity, plasma samples from animals C1–C6 and DHR1–DHR6 were analyzed. The harvest muscle weight was similar between groups and the size ranged from a mean of  $5.7 \pm 0.1$ mg in the soleus to  $141.7 \pm 5.2$ mg in the quadriceps. The total number of cells ranged from a mean of 287 cells in the soleus to 2105 cells in the gastrocnemius (Table 1).

### *Evans Blue Dye Infiltration*

Total number of Evans Blue-positive fibers in each muscle are reported in Table 1 and Figure 1. In the control group, the mean percentage of Evans Blue-positive cells across all the muscles was 3.9%, and Evans Blue dye infiltration ranged from a low of  $1.7 \pm 0.3\%$  in the soleus to a high of  $6.6 \pm 2.2\%$  in the TA. After a single bout of downhill running, the DHR group had a mean percentage of Evans Blue dye infiltration across all muscles of 4.3%, with a range of  $2.6 \pm 0.8\%$  in the triceps to  $6.6 \pm 1.3\%$  in the TA (Table 2). There was no significant difference between groups.

### *Disrupted Dystrophin*

The respective Evans Blue-positive cells were then assessed for discontinuous or absence of dystrophin staining. The total number of Evans Blue-positive fibers with discontinuous dystrophin staining are shown in Figure 2. There was no difference between groups for the number of fibers showing discontinuous or absent dystrophin, and the mean percentage of Evans Blue-positive fibers showing discontinuous dystrophin staining was 65% in CON and 60% in DHR.

### *Centralized Nuclei*

The total number and relative percentage of centralized nuclei were not significantly different between groups (Figure 3). The percentage of centralized nuclei in the CON group ranged from a low  $1.5 \pm 0.1\%$  in the soleus to  $3.2 \pm 0.4\%$  in the triceps brachii and the combined mean for all muscles in the CON was 2.3%. In the DHR group, percentage of centralized nuclei ranged from a low of  $1.5 \pm 0.3\%$  in the TA to  $3.9 \pm 0.7\%$  in the plantaris, with a mean of 2.7% in all the muscles.

### *Creatine Kinase*

The mean blood creatine kinase activity in CON was  $146 \pm 13.2$  U/L and in DHR it was  $150 \pm 10.8$  U/L (Figure 4). These groups were not found to be significantly different ( $p > 0.05$ ).

### **Discussion**

Early rat studies found significant damage after a bout of downhill running, particularly in the triceps brachii, soleus, and vastus intermedius muscles using a hemotoxylin and eosin (H&E) stain (3). Subsequent studies using downhill running conducted on mice have used these muscles and measured damage primarily using H&E stains (40), Evans Blue dye infiltration (6), plasma creatine kinase levels (40), and maximal isometric force (26). Most studies analyzed the gastrocnemius-soleus complex, possibly because of the small size of the soleus muscle in mice (6, 7, 26). The purpose of this study was to identify the pattern and extent of muscle damage in the mouse after a single bout of downhill running.

We found that downhill treadmill running in the FVB strain of mice did not induce significant damage in any of the six individual muscles when compared to a control group. Our results were unexpected, considering that several studies using similar methods have reported damage after a single bout of downhill running (6, 7, 19, 21, 26, 28, 40). The current study takes a more comprehensive view of muscles in the mouse and suggests that downhill treadmill running may not be a reliable method to induce muscle damage in all conditions.

The primary marker of muscle damage in our study was Evans Blue dye infiltration. Evans Blue vital staining has been shown to be reliable and a simple way of identifying muscle membrane disruption and/or muscle damage (17). Evans Blue dye is used as an indicator of damage in rodents because it will infiltrate muscle fibers with a permeable membrane and can be

seen when viewed with fluorescent microscopy. It has been compared to an H&E stain and has shown positive infiltration for the same fibers that appeared damaged in the H&E stain (33).

In our study, we found some level of Evans Blue-positive fibers in almost every sample, including controls, but there was no significant difference between groups in response to downhill running. Other studies using downhill running in mice and rats have used Evans Blue dye as a marker of damage and found significant amounts of infiltration (6, 7, 23, 26, 40). Their sample sizes per group ranged from two to eight animals, which is similar to our sample size of six. In order to maximize the potential for damage, our methods imitated the greatest decline used in the existing literature, which was 20° (6, 26), with a relatively fast speed of 20 m • min<sup>-1</sup> (30). Running times in the literature ranged from as little as 5 mins (41) to as long as 150 mins (11-13, 16, 18, 21), and the mice in our study ran for a time of 30 mins. While this is a shorter run time, studies have measured significance in markers of muscle damage with only 30 mins (2, 6, 7). These conditions were presumed to be sufficient to induce muscle damage, and the fact that there was not damage suggests that either the methods of analyzing the damage were not sensitive enough or the muscles analyzed were not damaged.

We also analyzed dystrophin staining, centralized nuclei, and plasma CK activity as additional markers of muscle damage. Dystrophin is a structural protein that is crucial to connecting the z-discs to the sarcolemma. Studies have shown that eccentric contractions produce a loss of dystrophin staining (23, 43), and the loss of staining was correlated with Evans Blue dye infiltration (8, 23). Our dystrophin findings were consistent with the Evans Blue data with no difference between groups. Not all of our Evans Blue-positive fibers had discontinuous staining, albeit the majority of fibers did. The parameters to be counted as a dystrophin-negative



fiber required a complete absence or break in membrane staining, and a contrast between the dystrophin stain and the rest of the cell (43).

Centralized nuclei are not a direct marker of muscle damage, but an indicator that a cell is undergoing a regenerative process, which could be a result of damage from a bout of downhill running. A common method used to measure damage with an H&E stain defines centralized nuclei as a parameter to indicate muscle damage (3, 38). We measured centralized nuclei to determine the relationship between Evans Blue-positive fibers and centralized nuclei. We found no difference between DHR and CON in number of centralized nuclei per muscle sample, although, in all but one muscle, the trend was toward a greater percentage of centralized nuclei in the DHR group. The centralized nuclei did not correlate with the Evans Blue-positive fibers, which is similar to the results found in a study conducted by Whitehead et al. (42). We analyzed the muscles at 24 hours postexercise, similar to the study by Whitehead et al., which may be too early for the regenerative processes caused by exercise-induced muscle damage to be apparent (37). In order to get a reliable measure of the response of the nuclei from exercise-induced muscle damage, a later time point may be more effective.

Creatine kinase is an enzyme specific to the cytosol of muscle cells and is found in blood when the cell membrane becomes damaged. Creatine kinase typically has a robust response following a damaging bout of exercise, unless it is a repeated bout of damaging exercise or the subject is a nonresponder (34, 36). Due to its variability in response, it is often used in conjunction with other measures of muscle damage. We found no difference in plasma CK values between groups. This is consistent with all other markers of muscle damage that were analyzed in our study. The mice we used did not undergo any treadmill running prior to the downhill bout, only a familiarization period with no treadmill movement, so it is not likely that

the mice had any adaptations to prevent a robust CK response to the exercise. This leads us to believe that no significant damage was incurred from the downhill treadmill exercise.

Our conclusion that the downhill running in the current mice did not result in muscle damage is inconsistent with much of the existing literature. A few differences in our study could contribute to or explain the lack of damage. These differences include the age and the strain of mice used in our study and, to a lesser extent, our method of counting.

Genetic differences between strains of mice could affect the propensity for muscle damage. Our study used FVB mice because that is the primary strain with which our lab works. This differs from the existing literature, which primarily used C57BL/6 mice (6-9, 11-13, 20-22, 26, 27, 32, 40-42). Strain comparison studies using level treadmill exercise show that C57BL/6 perform poorly on treadmills. One study demonstrated that FVB mice considerably outperformed C57BL/6 mice in treadmill studies by running faster and farther (24). These results are in agreement with Massett et al. (31), showing that FVB had the highest intrinsic exercise capacity while C57BL/6 had the lowest. In discussions with other researchers, they report that mice with the C57BL background have some difficulty running and are awkward while running downhill. While the decline and the speed of the treadmill were similar between our study and the rest of the literature, the speed likely represented a higher percentage of exercise capacity in the studies using C57BL mice. These differences in exercise capacity and the mechanics of running could affect the amount of damage found in the muscle between strains of mice.

When comparing our mice to the rest of the literature using downhill running in mice, our mice were older. The mean age of the current animals was 21 weeks, and other studies used mice as young as 5–6 weeks (6, 7, 18, 26), most were 8–10 weeks (2, 8, 9, 11-13, 16, 21, 22, 42), a few were 12 weeks (19, 27, 28), and only three studies used mice the same age or older than our

mice (20, 32, 41). Studies comparing adaptations in young and old rats after contraction-induced injury found that old rats responded differently in both the injury and adaptation to stretch-shortening contractions (5, 15). This model has been shown to lead to muscle injury, adaptation, and/or maladaptation (4), and when used in old rats, it was reported to cause significant deficits in performance in the unexpected absence of degenerative or damaged fibers (15). Likewise, a similar study reports signs of degenerative myofibers in young rats but not old rats after a bout of injurious exercise (5). The authors suggested that differences in the response to contraction-induced injury was not due to fiber necrosis, but perhaps a lack of response within the muscle due to age factors. While our mice aren't classified as "old," they were at least twice as old as most of the mice used in other studies, and a diminished responsiveness to the DHR exercise due to age could be a factor in the current results (4).

In the current animals, the entire cross-section of the muscle was imaged and all the fibers were counted. This differs from other studies using Evans Blue dye, which used the gastrocnemius-soleus complex together and/or only selected a number of frames to count the damaged fibers (6, 7, 20, 26). By analyzing the entire muscle cross-section of each muscle individually, we were able to better assess the total amount of damage. If only a certain number of frames are analyzed, then the results could be skewed and not accurately reflect actual damage in the muscle.

We conclude that a typical downhill treadmill running protocol does not cause muscle damage in adult male FVB mice. The current data suggest that downhill treadmill running in mice may not be the ideal model for yielding consistent muscle damage in all conditions. We recommend that downhill running protocol parameters (i.e., intensity (speed, grade) and

duration) should be assessed and optimized for the particular characteristics (i.e., strain, age, genotype, etc.) of the mouse to be studied.

## References

1. **Agbulut O, Li Z, Mouly V, and Butler-Browne GS.** Analysis of skeletal and cardiac muscle from desmin knock-out and normal mice by high resolution separation of myosin heavy-chain isoforms. *Biol cell* 88: 131-135, 1996.
2. **Aoi W, Naito Y, Tokuda H, Tanimura Y, Oya-Ito T, and Yoshikawa T.** Exercise-induced muscle damage impairs insulin signaling pathway associated with IRS-1 oxidative modification. *Physiol Res* 61: 81-88, 2012.
3. **Armstrong RB, Ogilvie RW, and Schwane JA.** Eccentric exercise-induced injury to rat skeletal muscle. *J Appl Physiol: Respirat, Environ Exercise Physiol* 54: 80-93, 1983.
4. **Baker BA, and Cutlip RG.** Skeletal muscle injury versus adaptation with aging: novel insights on perplexing paradigms. *Exerc Sport Sci Rev* 38: 10-16, 2010.
5. **Baker BA, Hollander MS, Mercer RR, Kashon ML, and Cutlip RG.** Adaptive stretch-shortening contractions: diminished regenerative capacity with aging. *Appl Physiol Nutr Me* 33: 1181-1191, 2008.
6. **Boppard MD, Burkin DJ, and Kaufman SJ.** Alpha7beta1-integrin regulates mechanotransduction and prevents skeletal muscle injury. *Am J Physiol Cell Physiol* 290: C1660-1665, 2006.
7. **Boppard MD, Volker SE, Alexander N, Burkin DJ, and Kaufman SJ.** Exercise promotes alpha7 integrin gene transcription and protection of skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 295: R1623-1630, 2008.
8. **Brussee V, Merly F, Tardif F, and Tremblay JP.** Normal myoblast implantation in mdx mice prevents muscle damage by exercise. *Biochem Bioph Res Co* 250: 321-327, 1998.
9. **Brussee V, Tardif F, and Tremblay JP.** Muscle fibers of mdx mice are more vulnerable to exercise than those of normal mice. *Neuromuscular Disord* 7: 487-492, 1997.
10. **Byrnes WC, Clarkson PM, White JS, Hsieh SS, Frykman PN, and Maughan RJ.** Delayed onset muscle soreness following repeated bouts of downhill running. *J Appl Physiol* 59: 710-715, 1985.
11. **Carmichael MD, Davis JM, Murphy EA, Brown AS, Carson JA, Eugene MB, and Ghaffar A.** Recovery of running performance following muscle-damaging exercise: relationship to brain IL-1 beta. *Brain Behav Immun* 19: 445-452, 2005.
12. **Carmichael MD, Davis JM, Murphy EA, Brown AS, Carson JA, Mayer EP, and Ghaffar A.** Role of brain IL-1 beta on fatigue after exercise-induced muscle damage. *Am J Physiol-Reg I* 291: R1344-R1348, 2006.
13. **Carmichael MD, Davis JM, Murphy EA, Carson JA, Van Rooijen N, Mayer E, and Ghaffar A.** Role of brain macrophages on IL-1 beta and fatigue following eccentric exercise-induced muscle damage. *Brain Behav Immun* 24: 564-568, 2010.
14. **Clarkson PM, Nosaka K, and Braun B.** Muscle function after exercise-induced muscle damage and rapid adaptation. *Med Sci Sport Exer* 24: 512-520, 1992.
15. **Cutlip RG, Baker BA, Geronilla KB, Mercer RR, Kashon ML, Miller GR, Murlasits Z, and Alway SE.** Chronic exposure to stretch-shortening contractions results in skeletal muscle adaptation in young rats and maladaptation in old rats. *Appl Physiol Nutr Me* 31: 573-587, 2006.

16. **Davis JM, Murphy EA, Carmichael MD, Zielinski MR, Groschwitz CM, Brown AS, Gangemi JD, Ghaffar A, and Mayer EP.** Curcumin effects on inflammation and performance recovery following eccentric exercise-induced muscle damage. *Am J Physiol-Reg I* 292: R2168-R2173, 2007.
17. **Hamer PW, McGeachie JM, Davies MJ, and Grounds MD.** Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability. *J Anat* 200: 69-79, 2002.
18. **Haramizu S, Ota N, Hase T, and Murase T.** Catechins suppress muscle inflammation and hasten performance recovery after exercise. *Med Sci Sport Exer* 45: 1694-1702, 2013.
19. **Haubold KW, Allen DL, Capetanaki Y, and Leinwand LA.** Loss of desmin leads to impaired voluntary wheel running and treadmill exercise performance. *J Appl Physiol* 95: 1617-1622, 2003.
20. **Howard AC, McNeil AK, Xiong F, Xiong WC, and McNeil PL.** A novel cellular defect in diabetes membrane repair failure. *Diabetes* 60: 3034-3043, 2011.
21. **Kawanishi N, Kato K, Takahashi M, Mizokami T, Otsuka Y, Imaizumi A, Shiva D, Yano H, and Suzuki K.** Curcumin attenuates oxidative stress following downhill running-induced muscle damage. *Biochem Bioph Res Co* 441: 573-578, 2013.
22. **Knoblauch MA, O'Connor DP, and Clarke MSF.** Obese mice incur greater myofiber membrane disruption in response to mechanical load compared with lean mice. *Obesity* 21: 135-143, 2013.
23. **Lehti TM, Kalliokoski R, and Komulainen J.** Repeated bout effect on the cytoskeletal proteins titin, desmin, and dystrophin in rat skeletal muscle. *J Muscle Res Cell M* 28: 39-47, 2007.
24. **Lerman I, Harrison BC, Freeman K, Hewett TE, Allen DL, Robbins J, and Leinwand LA.** Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. *J Appl Physiol (1985)* 92: 2245-2255, 2002.
25. **Lieber RL, Thornell LE, and Friden J.** Muscle cytoskeletal disruption occurs within the first 15 min of cyclic eccentric contraction. *J Appl Physiol* 80: 278-284, 1996.
26. **Lueders TN, Zou K, Huntsman HD, Meador B, Mahmassani Z, Abel M, Valero MC, Huey KA, and Boppart MD.** The alpha7beta1-integrin accelerates fiber hypertrophy and myogenesis following a single bout of eccentric exercise. *Am J Physiol Cell Physiol* 301: C938-946, 2011.
27. **Lynch GS, Fary CJ, and Williams DA.** Quantitative measurement of resting skeletal muscle [Ca<sup>2+</sup>]<sub>i</sub> following acute and long-term downhill running exercise in mice. *Cell Calcium* 22: 373-383, 1997.
28. **Magalhaes J, Fraga M, Lumini-Oliveira J, Goncalves I, Costa M, Ferreira R, Oliveira PJ, and Ascensao A.** Eccentric exercise transiently affects mice skeletal muscle mitochondrial function. *Appl Physiol Nutr Metab* 38: 401-409, 2013.
29. **Malm C, Sjodin B, Sjoberg B, Lenkei R, Renstrom P, Lundberg IE, and Ekblom B.** Leukocytes, cytokines, growth factors and hormones in human skeletal muscle and blood after uphill or downhill running. *J Physiol-London* 556: 983-1000, 2004.
30. **Maruhashi Y, Kitaokai K, Yoshiki Y, Nakamura R, Okano A, Nakamura K, Tsuyama T, Shima Y, and Tomita K.** ROS scavenging activity and muscle damage prevention in eccentric exercise in rats. *J Physiol Sci* 57: 211-216, 2007.

31. **Masset MP, Fan R, and Berk BC.** Quantitative trait loci for exercise training responses in FVB/NJ and C57BL/6J mice. *Physiol Genomics* 40: 15-22, 2009.
32. **Mathur S, Vohra RS, Germain SA, Forbes S, Bryant ND, Vandeborne K, and Walter GA.** Changes in muscle T2 and tissue damage after downhill running in mdx mice. *Muscle Nerve* 43: 878-886, 2011.
33. **Matsuda R, Nishikawa A, and Tanaka H.** Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. *J Biochem* 118: 959-964, 1995.
34. **Nosaka K, and Clarkson PM.** Muscle damage following repeated bouts of high force eccentric exercise. *Med Sci Sport Exer* 27: 1263-1269, 1995.
35. **Nosaka K, Clarkson PM, Mcguiggin ME, and Byrne JM.** Time course of muscle adaptation after high force eccentric exercise. *Eur J Appl Physiol O* 63: 70-76, 1991.
36. **Paulsen G, Mikkelsen UR, Raastad T, and Peake JM.** Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? *Exerc Immunol Rev* 18: 42-97, 2012.
37. **Podhorska-Okolow M, Sandri M, Zampieri S, Brun B, Rossini K, and Carraro U.** Apoptosis of myofibres and satellite cells: exercise-induced damage in skeletal muscle of the mouse. *Neuropath Appl Neuro* 24: 518-531, 1998.
38. **Smith HK, Maxwell L, Rodgers CD, McKee NH, and Plyley MJ.** Exercise-enhanced satellite cell proliferation and new myonuclear accretion in rat skeletal muscle. *J Appl Physiol* 90: 1407-1414, 2001.
39. **Staron RS, Hikida RS, Murray TF, Nelson MM, Johnson P, and Hagerman F.** Assessment of skeletal-muscle damage in successive biopsies from strength-trained and untrained men and women. *Eur J Appl Physiol O* 65: 258-264, 1992.
40. **Tsivitse SK, Peters MG, Stoy AL, Mundy JA, and Bowen RS.** The effect of downhill running on Notch signaling in regenerating skeletal muscle. *Eur J Appl Physiol* 106: 759-767, 2009.
41. **Vilquin JT, Brussee V, Asselin I, Kinoshita I, Gingras M, and Tremblay JP.** Evidence of mdx mouse skeletal muscle fragility in vivo by eccentric running exercise. *Muscle Nerve* 21: 567-576, 1998.
42. **Whitehead NP, Streamer M, Lusambili LI, Sachs F, and Allen DG.** Streptomycin reduces stretch-induced membrane permeability in muscles from mdx mice. *Neuromuscul Disord* 16: 845-854, 2006.
43. **Zhang BT, Yeung SS, Allen DG, Qin L, and Yeung EW.** Role of the calcium-calpain pathway in cytoskeletal damage after eccentric contractions. *J Appl Physiol* 105: 352-357, 2008.
44. **Zou K, Meador BM, Johnson B, Huntsman HD, Mahmassani Z, Valero MC, Huey KA, and Boppart MD.** The  $\alpha_7\beta_1$  integrin increases muscle hypertrophy following multiple bouts of eccentric exercise. *J Appl Physiol* 2011.

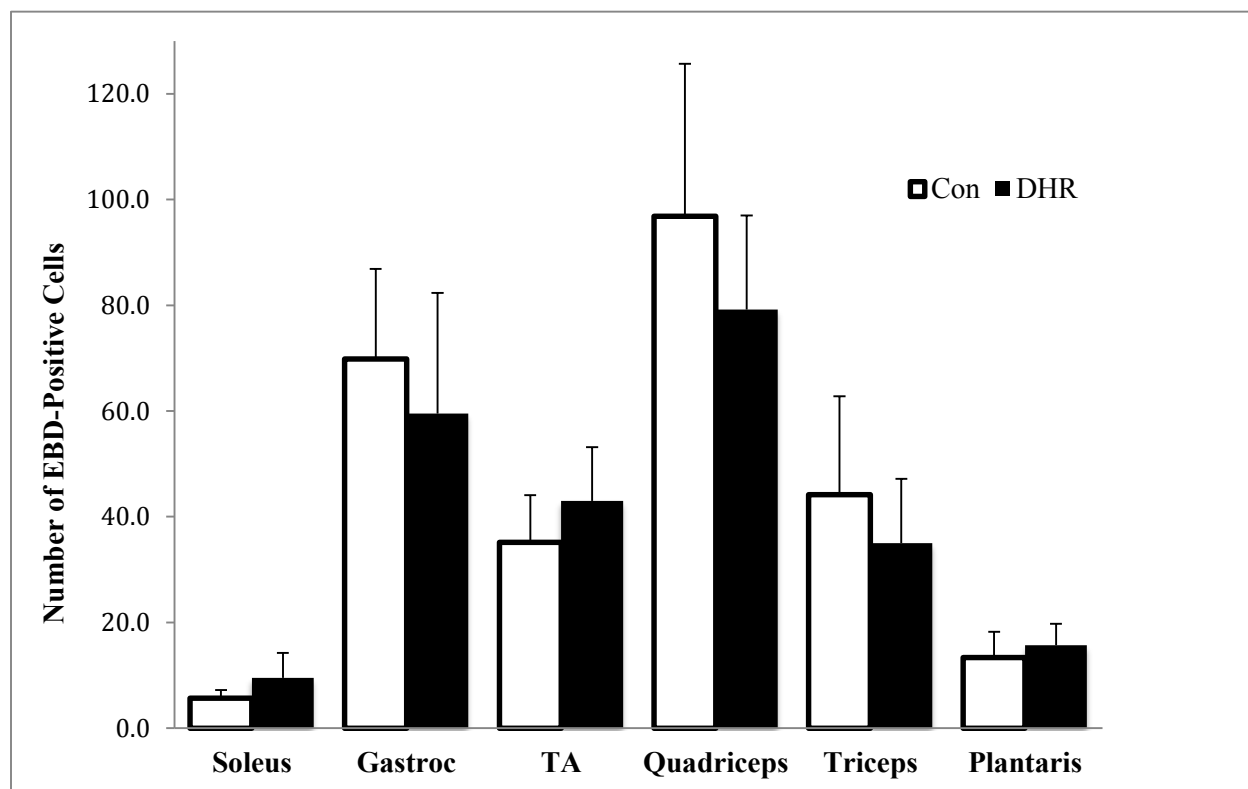
**Table 1. Totals and Percentages of Markers of Muscle Damage**

Muscle	EBD Positive		Centralized Nuclei		Dystrophin Negative	
	CON	DHR	CON	DHR	CON	DHR
<b>Soleus</b>	5.7 ± 1.5	9.5 ± 4.7	4.8 ± 0.9	6.2 ± 1.6	4.2 ± 1.9	6.3 ± 3.2
	1.7% ± 0.3	3.4% ± 1.3	1.5% ± 0.1	2.3% ± 0.5	1.1% ± 0.4	2.2% ± 0.9
<b>(total cell #)</b>	(308 ± 48)	(267 ± 27)				
<b>Gastroc</b>	69.8 ± 17.1	59.5 ± 22.8	33.5 ± 7.1	51.7 ± 14.7	52.5 ± 15.7	33.0 ± 9.7
	3.3% ± 0.7	3.0% ± 1.3	1.6% ± 0.4	2.6% ± 0.7	2.4% ± 0.7	1.6% ± 0.5
<b>(total cell #)</b>	(2148 ± 312)	(2062 ± 235)				
<b>Plantaris</b>	13.3 ± 4.9	15.7 ± 4.1	9.5 ± 3.1	11.5 ± 2.9	4.7 ± 1.3	5.2 ± 1.2
	4.0% ± 1.4	5.9% ± 1.7	2.9% ± 1.1	3.9% ± 0.7	1.4% ± 0.4	1.9% ± 0.5
<b>(total cell #)</b>	(370 ± 47)	(290 ± 35)				
<b>TA</b>	35.2 ± 8.9	43.0 ± 10.1	13.7 ± 2.4	10.5 ± 2.4	28.3 ± 8.1	16.3 ± 4.6
	6.6% ± 2.2	6.6% ± 1.3	2.5% ± 0.7	1.5% ± 0.3	5.0% ± 1.4	2.8% ± 0.9
<b>(total cell #)</b>	(603 ± 101)	(658 ± 74)				
<b>Quadriceps</b>	96.8 ± 28.8	79.2 ± 17.8	42.5 ± 6.0	42.0 ± 6.0	78.3 ± 25.2	54.3 ± 11.3
	5.0% ± 1.6	4.1% ± 1.0	2.1% ± 0.3	2.2% ± 0.4	4.0% ± 1.4	2.9% ± 0.7
<b>(total cell #)</b>	(2013 ± 59)	(2020 ± 120)				
<b>Triceps</b>	44.2 ± 18.6	35.0 ± 12.2	53.8 ± 11.1	46.7 ± 8.4	32.8 ± 17.0	23.5 ± 9.7
	2.8% ± 1.1	2.6% ± 0.8	3.2% ± 0.4	3.6% ± 0.6	2.1% ± 1.0	1.7% ± 0.7
<b>(total cell #)</b>	(1648 ± 159)	(1311 ± 67)				

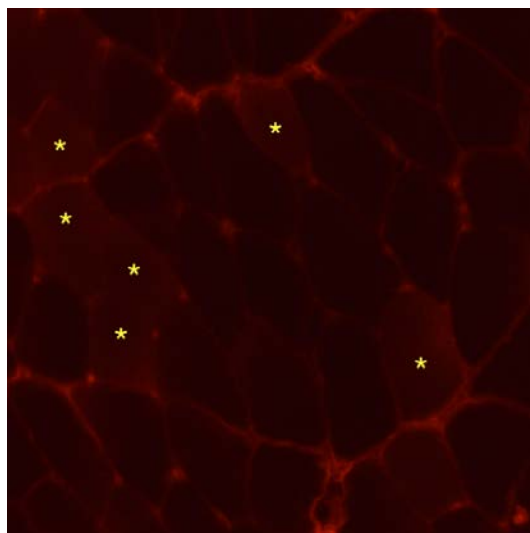
Values represent mean ± SE for the absolute number and percentage of Evans Blue-positive fibers, centralized nuclei and dystrophin-negative cells. Total cell # represents the mean ± SE for the total number of cells for that muscle. Percentages are calculated as absolute number of positive cells divided by the total number of fibers examined for each respective muscle.



A.



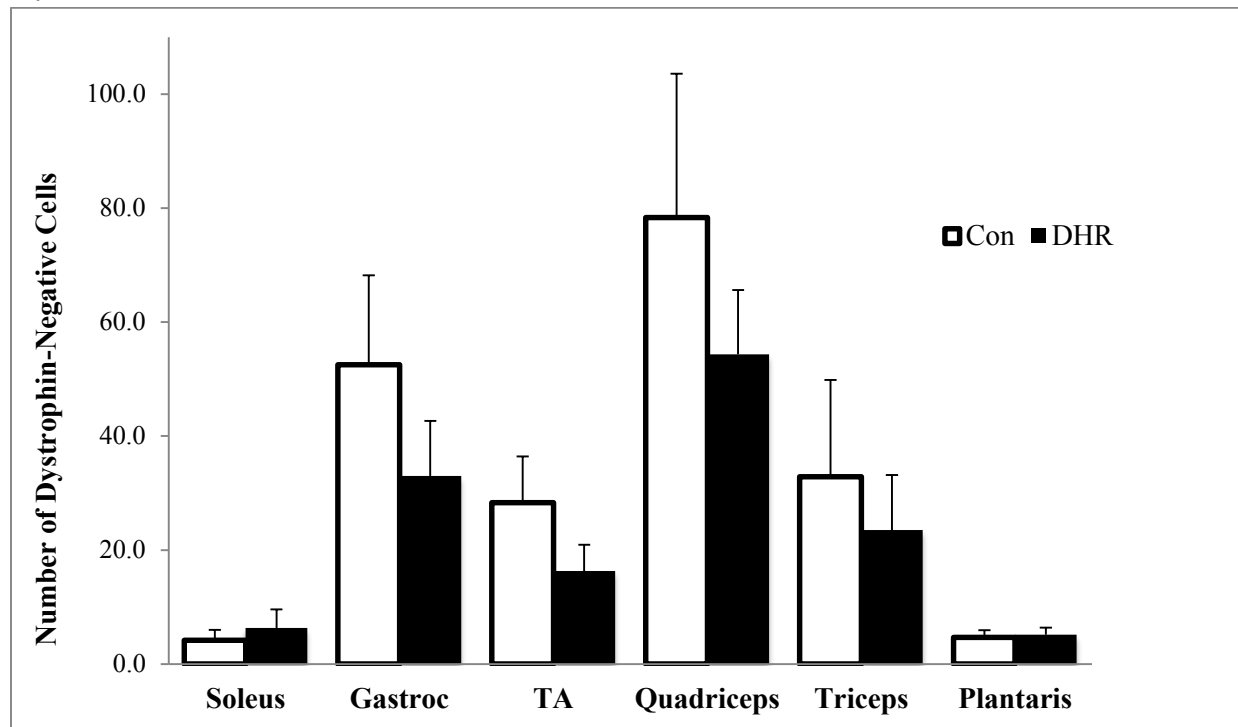
B.

**Figure 1**

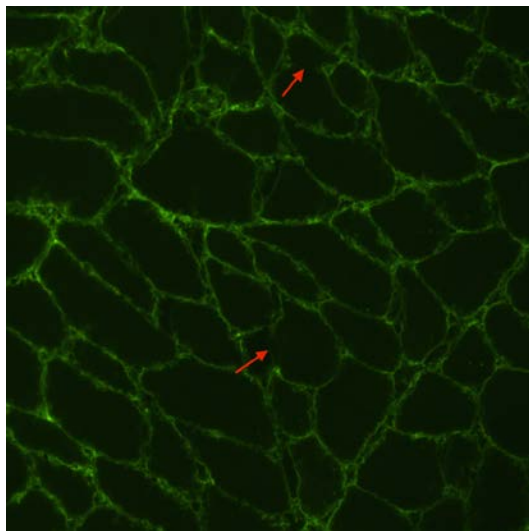
A. Total number of Evans Blue-positive cells counted in both Control (Con) and Downhill running (DHR) mice muscles. Values are presented as means  $\pm$  SE ( $n = 6$ ). EBD, Evans Blue dye; Gastroc, gastrocnemius; TA, tibialis anterior.

B. IHC image of a muscle cross-section demonstrating Evans Blue infiltration (marked with a star).

A.



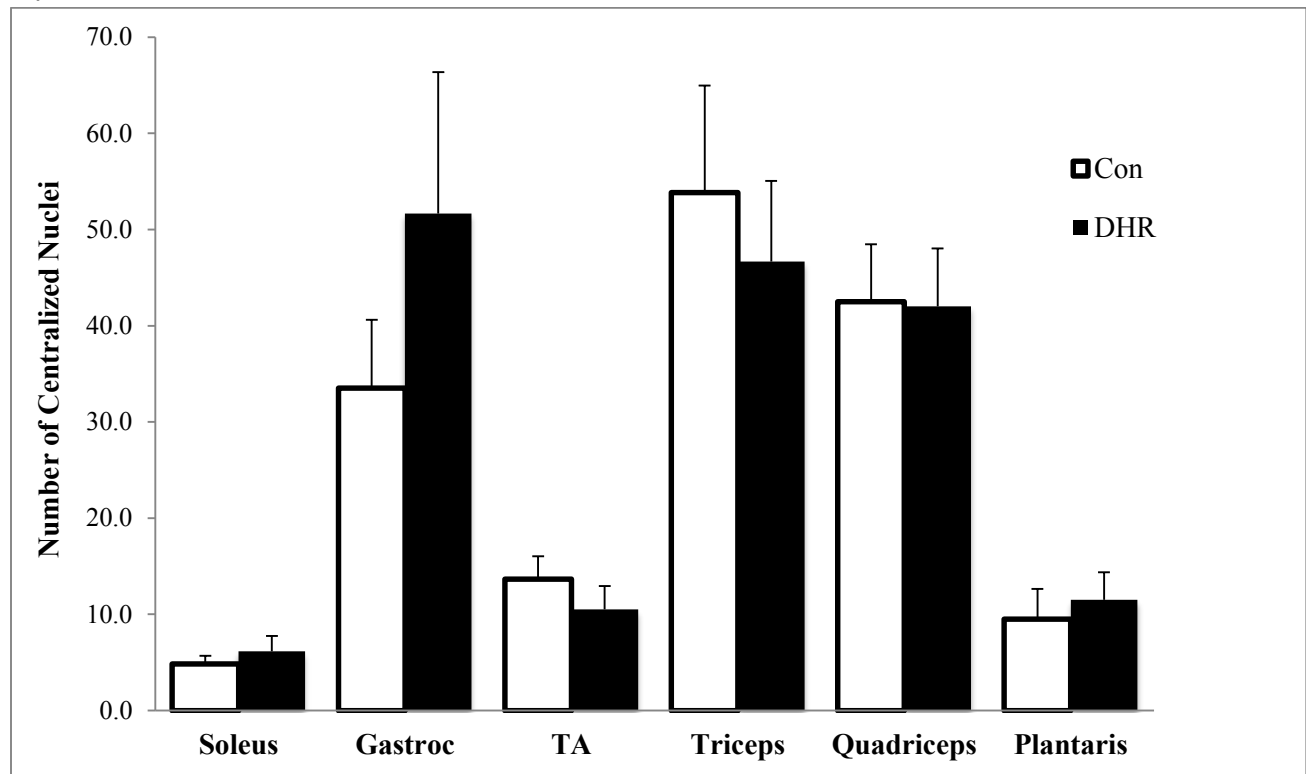
B.

**Figure 2**

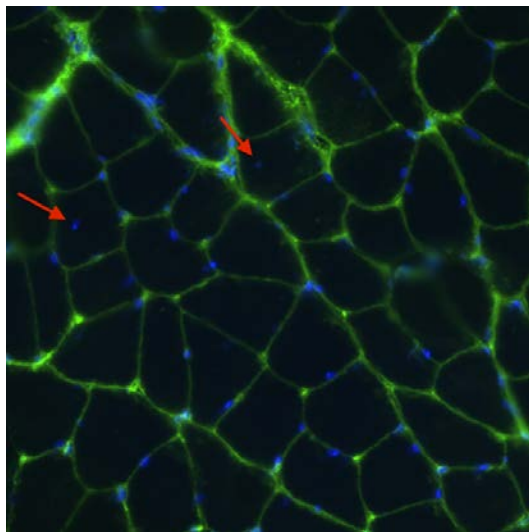
A. Total number of dystrophin-negative cells. Evans Blue-positive cells were assessed for dystrophin-negative staining in Control (Con) and Downhill running (DHR) muscles. Values represent means  $\pm$  SE of the total number of dystrophin-negative cells for each muscle ( $n = 6$ );  $p > 0.05$  for all groups.

B. IHC image of a muscle cross-section demonstrating dystrophin-negative staining, indicated by arrows, of an Evans Blue-positive cell.

A.



B.

**Figure 3**

A. Total number of centralized nuclei was counted per muscle. Means  $\pm$  SE (n = 6)  $p > 0.05$  for all groups.

B. A muscle cross-section with a merged image of both dystrophin and DAPI stains to show centralized nuclei, indicated by arrows.

**Appendix A. List of articles using downhill treadmill running in mice**

Author	Year	Strain	S	Age	Grade	Speed m/min	Time min	Bouts	Muscles	Time of Sacrifice	Markers of Muscle Damage*							
											EBD	H&E	CK	DYS	Whl	Td-E	F	Oth
Aoi	2012	ICR		10 wk	-5	30	30	1	soleus	24h			+					
Armand	2003	Swiss	F	adult	-14	30/40	90/30	1	soleus	24-168h								+
Boppart	2006	sj6/c57bl6	F	5 wk	-20	17	30	1	gastroc-soleus	24h	+							
Boppart	2008	c57bl6-a7bgal	F	5 wk	-20	17	30	1 or 2	gastroc-soleus	0,3h, 24h, 1wk	+							
Brussee	1997	c57bl10j	M	2 mo	-15	10	10	3	ta, EDL, soleus, gastroc, BB, TB, diaphragm	0	+							
Brussee	1998	c57bl/10j mdx	M	2mo	-15	10	10	3	BB	24h	+			+				
Carmichael	2005	c57bl/6	M	8wk	-14	22	150	1		24h			+		+	+		
Carmichael	2006	c57bl6	M	8wk	-14	22	150	1		8d					+	+		
Carmichael	2010	c57bl/6	M	8wk	-14	22	150	1		24h							+	
Davis	2007	ICR	M	8wk	-14	22	150	1	soleus	24h, 48h			+		+	+		
Haramizu	2013	ICR	M	6wk	-14	22	150	1	gastroc, soleus, plantaris	0, 24h			+		+	+	+	
Haubold	2003	WT129, desmin null	M	3 mo	-16	10	5	1		24h			+		+	+		
Howard	2011	c57bl/jinsr, c57bl/6,		6-7 mo	-15	10	60	1	quad, gastroc	24h	+							
Kawanishi	2013	c57bl6	M	9wk	-15	22	150	1	gastroc	24h			+					
Knoblauch	2013	B6.V Lepob/J, C57bl6/j		10wk	-12+	7	90	1	gastroc	0								+

Author	Year	Strain	S	Age	Grade	Speed m/min	Time min	Bouts	Muscles	Time of Sacrifice	Markers of Muscle Damage*						
											EBD	H&E	CK	DYS	Whl	Td-E	F
Lueders	2011	SJL/c57bl6, mck-a7bx2	F	5-8wk	-20	17	60	1	plantorflexors (force), gastroc-soleus	1, 2, 4, 7d	+	+					+
Lynch	1997	c57bl/10	M	80 d	-16	13	60	1	Soleus, EDL	24h, 48h		+	+				+
Magalhaes	2013	CD-1	M	12wk	-16	25-30	120	1	Soleus, gastroc, quad, spinotrapezius	0, 48h			+				
Mathur	2011	c57bl/10scsn	M	5-15 mo	-14	8-10	45	1	TA, EDL, FDL, soleus, gastroc	48h	+						
Tsivitse	2009	c57bl/j6	M	5 wk	-15	22	40	1	gastroc, soleus,	24h, 48h, 72h, 96h, 120h	+	+					
Vilquin	1998	c57bl10 scsn CD1TnILacZ1/29	B	9-14mo	-16	10	5	1	TA	3d			+	+			
Whitehead	2006	c57bl10 scsn	M	7-10 wk	-17	10	45	1	EDL	24h	+						+

\*A plus sign indicates which markers of muscle damage were assessed for that study, abbreviations are as follows: EBD, Evans Blue dye; H&E, Hematoxylin and eosin stain; CK, creatine kinase; DYS, dystrophin-negative staining; Whl, wheel running; Td-E, treadmill endurance test; F, maximal contractile force; Oth, other.