

## Original Article

# Genetic variability affects the response of skeletal muscle to disuse

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## Abstract

**Objective:** To examine whether genetic variability plays a role in skeletal muscle response to disuse. **Methods:** We examined skeletal muscle response to disuse in five different strains of mice: CAST/EiJ, NOD/ShiLtJ, NZO/HILtJ, 129S1/SvlmJ and A/J. Mice had one limb immobilized by a cast for three weeks. **Results:** Response to immobilization was dependent on the strain of mice. Skeletal muscle mass/body weight was decreased by immobilization in all strains except 129S1/SvlmJ. Immobilization decreased absolute skeletal muscle mass in quadriceps and gastrocnemius in NOD/ShiLtJ and NZO/HILtJ mice. Three weeks of immobilization resulted in an increase in quadriceps levels of atrogens in CAST/EiJ. Immobilization resulted in an increase in quadriceps and gastrocnemius levels of *Myh4* in CAST/EiJ. A similar trend was observed for *Myh7* in gastrocnemius muscle. Immobilization resulted in a decrease of the p-p70S6K1/total p70S6K1 ratio in quadriceps of NOD/ShiLtJ mice and the gastrocnemius of A/J mice. Immobilization did not affect the p-4EBP1/total 4EBP1 ratio in quadriceps of any of the strains examined. However, the p-4EBP1/total 4EBP1 ratio in gastrocnemius was greater in immobilized, relative to control, limbs in CAST/EiJ mice. **Conclusion:** Genetic variability affects the response of skeletal muscle to disuse.

**Keywords:** Immobilization, Skeletal Muscle Atrophy

## Introduction

The maintenance of skeletal muscle mass is critical for optimal quality of life, and mechanical loading plays an important role in maintaining skeletal muscle mass<sup>1</sup>. Different types of mechanical unloading, such as that resulting from bed rest, immobilization, spaceflight and decreased physical activity can result in disuse atrophy with serious consequences including increased propensity for falls, severe functional decline and eventually death<sup>2</sup>.

Unfortunately, the mechanism underlying disuse atrophy is only partially understood.

A better understanding of the mechanism underlying unloading-induced skeletal muscle loss, or disuse atrophy, could lead to the development of novel and more effective countermeasures and therapeutics for disuse atrophy. In humans, heritability is approximately 66% for skeletal muscle mass and 80% for lean body mass. This suggests that genetic variability may contribute to skeletal muscle mass and therefore may contribute to skeletal muscle adaptation to unloading. The role of genetics has also been studied in mouse models. For instance, Judex et al.<sup>3</sup> identified one Quantitative Trait Loci (QTL) on chromosome 5 for unloading-induced loss of skeletal muscle cross-sectional area in the F2 offspring of a double cross between BALB/cByJ22 and C3H/HeJ23 mice. This QTL accounted for 5% ( $p < 0.001$ ) of the variability in skeletal muscle loss in response to unloading in the F2 mice. We are not aware of other studies that have examined the genetics of unloading-induced skeletal muscle loss. Exploring how genetic variability contributes

The authors have no conflict of interest.

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Edited by: G. Lyritis

Accepted 16 March 2021



to unloading-induced skeletal muscle loss could provide mechanistic insights into disuse atrophy.

To examine the effect of genetic variability on the response of skeletal muscle to unloading, we exposed five different strains of mice to hindlimb immobilization. The strains examined were A/J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HILtJ, and CAST/EiJ. We chose to examine these strains because they are founder strains of Diversity Outbred (DO) mice. DO mice are a recently developed high-resolution mapping population that enables gene discovery for traits such as the response of skeletal muscle to unloading<sup>4</sup>. DO mice can be used to investigate the genetics of a wide range of complex diseases. Examining these founder strains will not only enable us to examine the role of genetic variability in the response of skeletal muscle to unloading but will also provide insight into the usefulness of DO mice in future studies.

To assess the role of genetic variability in disuse atrophy we quantified unloading-induced changes in skeletal muscle mass as well as proteins and genes critical to skeletal muscle turnover. Skeletal muscle atrophy results from an imbalance of muscle protein breakdown (MPB) and muscle protein synthesis (MPS). MPB and MPS balance is at least partially regulated by Akt which activates the protein kinase mTOR to phosphorylate p70S6K1 and 4EBP1 resulting in increased MPS. Inhibition of Akt activates the transcription factor FOXO. FOXO then activates transcription of the genes encoding two atrogenes, Atrogin-1 (encoded by *FbxO32*) and MuRF-1 (encoded by *Trim63*)<sup>5</sup>, leading to MPB<sup>2,6,7</sup>. Thus, increasing levels of p70S6K1 and 4EBP1 reflect increases in MPS while increasing level of Atrogin-1 and Murf-1 reflect increases in MPB.

Atrophy leads to changes in fiber type composition, and how those fibers change is dependent on the type of atrophy that occurs. Unlike age-induced atrophy which results in reduced fast-twitch fibers<sup>8</sup>, disuse atrophy can lead to increased fast-twitch myosin heavy chains. In rats, hindlimb suspension decreased *Myh7* (encodes myosin heavy chain  $\beta$  and is expressed in skeletal muscle with slow-twitch fibers)<sup>9,10</sup> gene expression and caused a gradual transformation to fast-twitch fibers<sup>11</sup>. Shen et al.<sup>12</sup> found different expression of *Myh4*, (encodes myosin heavy chain 4 and is expressed in skeletal muscle with fast-twitch fibers), and *Myh7* in the deltoid and supraspinatus muscle. The deltoid showed an increase in *Myh7*, in response to the microgravity of space, whereas the supraspinatus displayed an increase in *Myh4*. The same increase in fast-twitch fiber type has been shown in various muscles after spaceflight<sup>13-16</sup>. These data suggest that unloading may induce a shift from slow- to fast-twitch fibers.

In this study skeletal muscle atrophy was induced by immobilization of the left hind limb through casting. We hypothesized that there would be differential, strain-dependent effects of unloading on skeletal muscle mass and expression of genes and proteins associated with skeletal muscle turnover in five strains of mice – CAST/EiJ, NOD/ShiLtJ, NZO/HILtJ, 129S1/SvImJ and A/J – after three weeks of unloading using single limb immobilization.

## Materials and Methods

### Animal model

All the animal procedures were performed with the approval of the Virginia Commonwealth University Institutional Animal Care and Use Committee. We used five Diversity Outbred (DO) founder mouse strains: CAST/EiJ, NOD/ShiLtJ, NZO/HILtJ, 129S1/SvImJ and A/J. Six male mice of each strain (they were not littermates) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and arrived at 4-14 weeks of age. The mice were given 2 to 12 weeks to acclimate. At 16 weeks of age mice of each strain had their left hind limb placed in a cast. The mice were sacrificed after three weeks of casting.

### Casting protocol

The left hind limb of the mice was casted as previously described<sup>17</sup>. Mice were placed under anesthesia with isoflurane (2.5%), had the left hind limb shaved using clippers, and the skin swabbed with 70% isopropanol. Surgical tape was tightly wrapped around the left hind limb from the hip to the knee. A microcentrifuge tube was then glued on top of the tape. The tube had the bottom end removed to allow air to flow into it. Right limbs were used as contralateral controls. Mice were single housed and given access to food and water *ad libitum*. Casted mice were able to freely move around the cages, dragging the immobilized limb.

### Physical Activity and Food Consumption

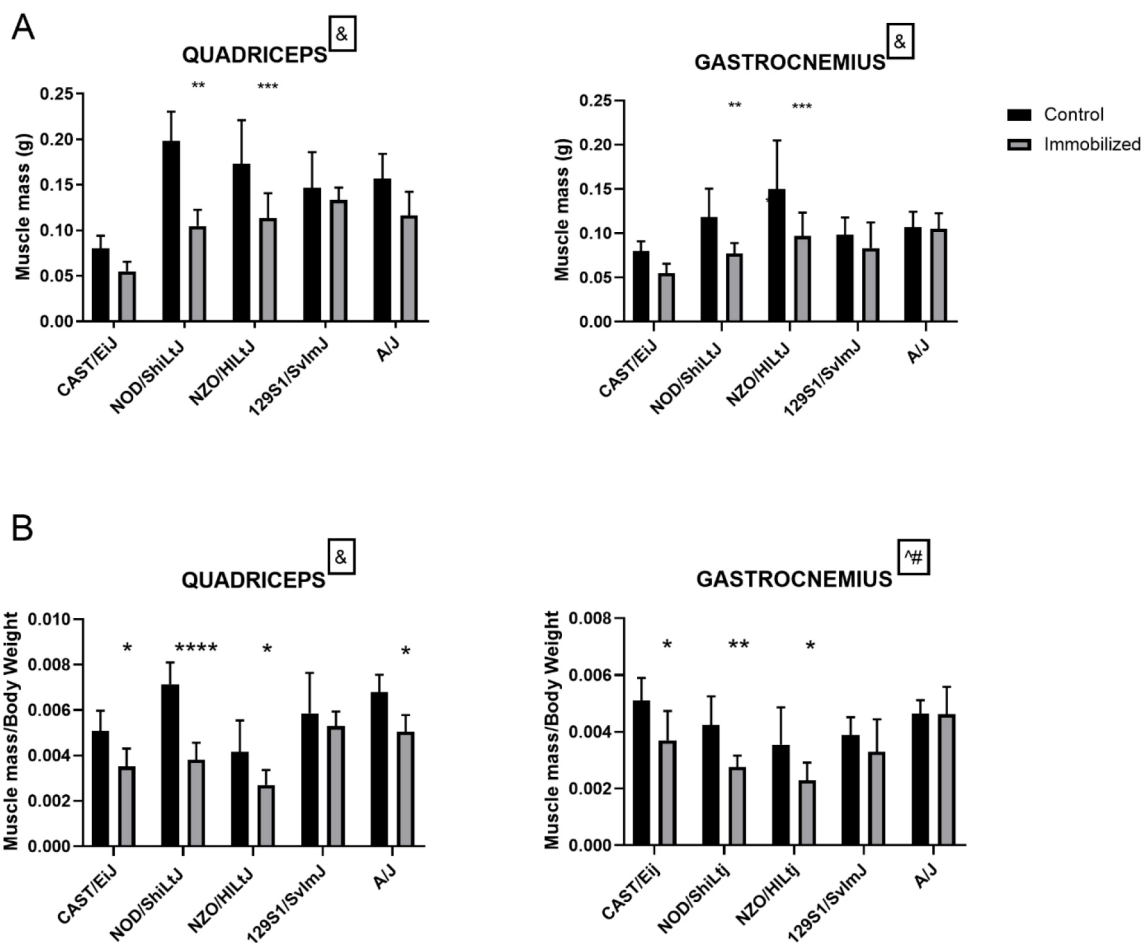
Physical activity and food consumption were recorded over a 24-hour period, before and after casting. Observations were made one week prior to casting surgery and two weeks after casting. To assess individual mouse physical activity, video recordings captured for 24 hours were analyzed using the OpenField Matlab function developed by Patel et al.<sup>18</sup>. Food consumption was measured by weighing food in the cages before and after the 24-hour observation period.

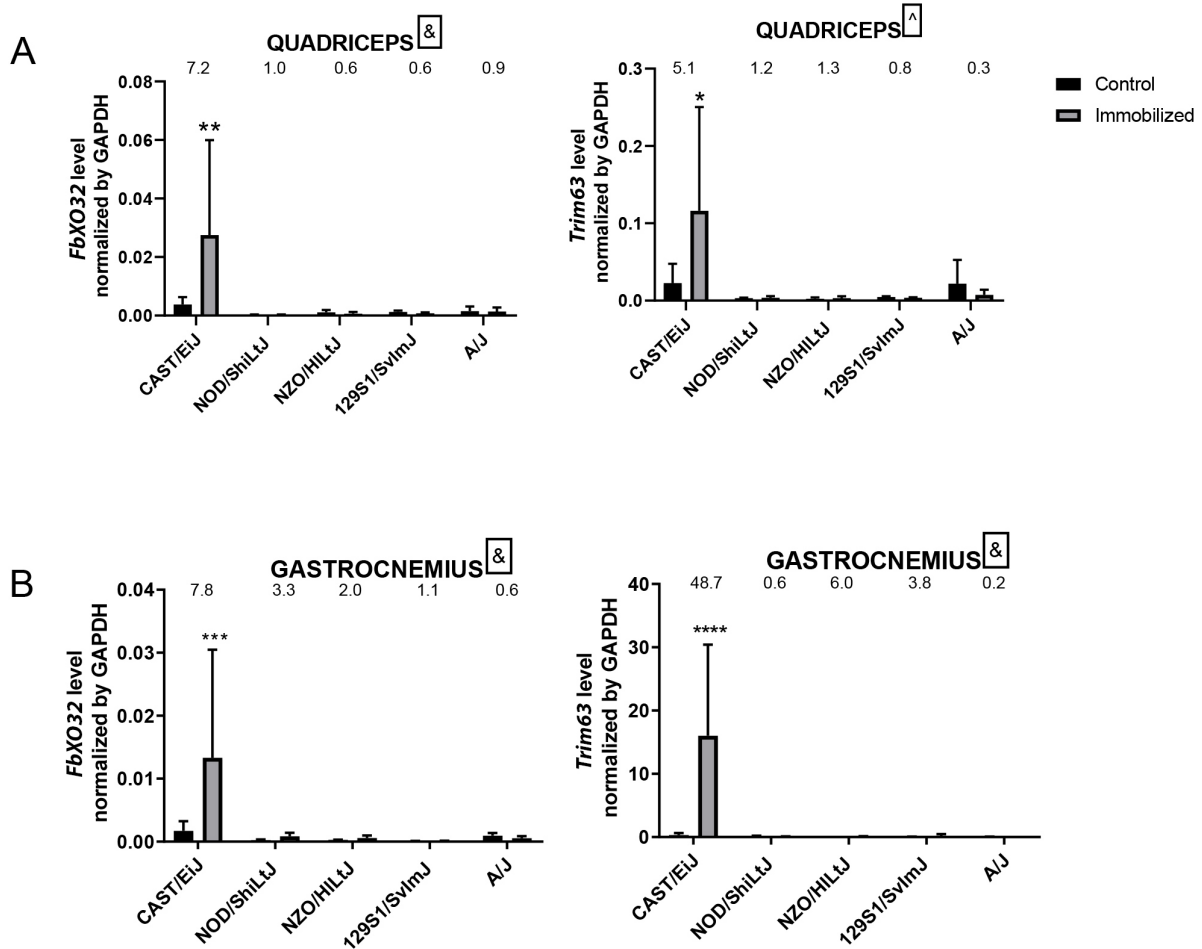
### RNA isolation and quantitative RT – PCR

Total RNA from muscle samples was extracted using RNeasy Fibrous Tissue kit (Qiagen, Valencia, CA, USA). The kit used a mixture of oligo (dT) and random primers to provide an unbiased representation of the 5' and 3' regions of the target genes for freedom in qPCR primer design. RNA was eluted from the column with RNase-free water, and 1  $\mu$ l was used for quantification using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA was reverse transcribed using iScript gDNA Clear cDNA Synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. RT-qPCR was performed using 500 ng of cDNA for genes encoding the atrogenes Atrogin-1 (encoded by *FbxO32*) and MuRF 1 (encoded by *Trim63*), *Myh4*, (fast-twitch fiber), and *Myh7* (slow-twitch fiber). All primer sequences are shown in Supplemental Table 1.

**Table 1.** Mouse body weight, activity and food consumption. \* Significantly different than CAST/EiJ and NOD/ShiLtJ,  $p < 0.05$ . Values are mean  $\pm$  SD. Percent change is the percent change in mean values.

Mouse Strain	CAST/EiJ	NOD/ShiLtJ	NZO/HILtJ	129S1/SvImJ	A/J
Day 1 Weight (g)	17.0 $\pm$ 0.9	30.5 $\pm$ 2.9	48.9 $\pm$ 4.0	28.4 $\pm$ 1.8	27.5 $\pm$ 2.0
Day 21 Weight (g)	15.8 $\pm$ 0.8	27.7 $\pm$ 1.1	42.3 $\pm$ 3.4	25.3 $\pm$ 1.5	22.9 $\pm$ 1.8
% Change	-7.3%	-8.7%	-13%	-11%	-17% *
p-value	0.012	0.015	0.0002	0.001	0.0002
Baseline activity (km/day)	3.01 $\pm$ 1.09	1.65 $\pm$ 0.88	1.49 $\pm$ 0.59	1.91 $\pm$ 0.67	2.34 $\pm$ 0.52
Immobilized activity (km/day)	2.69 $\pm$ 0.70	2.11 $\pm$ 0.66	1.70 $\pm$ 0.79	2.10 $\pm$ 0.72	0.90 $\pm$ 0.74
% Change	-6%	+94%	+63%	+39%	-57%
p-value	0.827	0.486	0.416	0.530	0.024
Baseline food consumption (g/day)	3.7 $\pm$ 2.0	4.0 $\pm$ 0.4	5.0 $\pm$ 0.5	4.5 $\pm$ 1.1	3.3 $\pm$ 0.7
Immobilized food consumption (g/day)	4.4 $\pm$ 2.2	4.9 $\pm$ 0.6	5.0 $\pm$ 1.5	5.7 $\pm$ 1.6	4.4 $\pm$ 1.3
% Change	+22%	+24%	-1.6%	+33%	+43%
p-value	0.149	0.004	0.886	0.176	0.175

**Figure 1.** Effect of three weeks of casting on quadriceps and gastrocnemius muscle mass (A) and muscle mass normalized to body weight (B). Results for grey bars are data from control mice and black bars are data from mice exposed to immobilization by casting. Values are means  $\pm$  SD;  $n = 6$  for immobilized and control samples for five strains of mice. Numbers above bars are the fold changes (immobilized/control) for each strain. & Significant immobilization and mouse strain interaction ( $p < 0.05$ ); ^ Significant main effect of mouse strain ( $p < 0.05$ ); # Significant main effect of immobilization; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .



**Figure 2.** Effect of three weeks of casting on Atrogenes (*FbXO32* and *Trim63*) expression levels in (A) quadriceps and (B) gastrocnemius muscles. Values are means $\pm$ SD; n=6 for immobilized and control samples for five strains of mice. Grey bars are data from control limbs, and black bars are data from limbs exposed to immobilization by casting. Numbers above bars are the fold changes (immobilized/control) for each strain. &Significant immobilization and mouse strain interaction ( $p < 0.05$ ); ^Significant main effect of mouse strain ( $p < 0.05$ ); \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; immobilized vs control within the same strain.

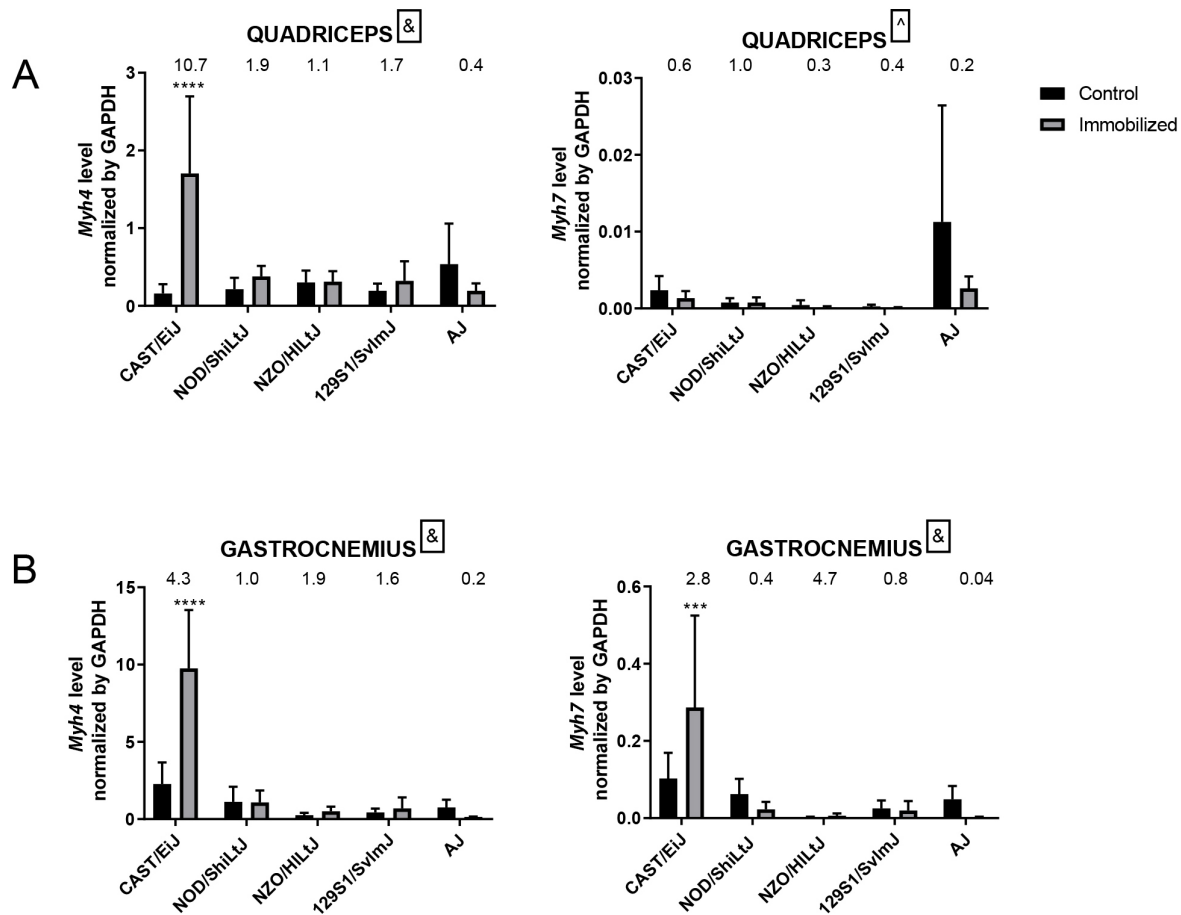
### Western Blot Analysis

Approximately 20 mg muscle was used for protein isolation and before homogenization all fat was removed. Muscle samples were homogenized in ice-cold buffer consisting of (mmol/L): 20 HEPES (pH 7.4), 2 EGTA, 50 sodium fluoride, 100 potassium chloride, 0.2 EDTA, 50  $\beta$ -glycerophosphate, 1 DTT, 0.1 phenylmethane-sulphonylfluride, 1 benzamidine, and 0.5 sodium vanadate. Protein concentration was quantified using a BCA kit (Thermo Fisher scientific), and an equal amount of total protein (30  $\mu$ g) per sample was subjected to standard SDS-PAGE using Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> 4-20% 12-well gels (Bio-Rad, Hercules, CA). Gels were electroblotted onto PVDF membranes (Bio-Rad, Hercules, CA). Western Blot analysis was performed for total and phosphorylated 4EBP1 (T37/46, Cell Signaling Technology; Boston, MA) and

total and phosphorylated p70S6K1 (T389, Cell Signaling Technology; Boston, MA) with both antibodies diluted 1:1000. The secondary antibodies were diluted 1:3000. The bound immune complexes were detected using Clarity Max<sup>™</sup> Western ECL Substrate (Bio-Rad, Hercules, CA).

### Statistical analyses

Repeated measures two-way ANOVAs and post-hoc tests (Tukey's or Sidak's multiple comparisons tests) were used to test for significant differences among the strains after three weeks of casting. GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Heritability of the skeletal muscle phenotypic characteristics was also calculated as previously described<sup>19</sup>.



**Figure 3.** Effect of three weeks of casting on fiber type composition (*Myh4* – fast twitch fiber and *Myh7*- slow twitch fiber) gene levels in (A) quadriceps and (B) gastrocnemius muscles in five strain of mice. Grey bars are data from control mice and black bars are data from mice exposed to immobilization by casting. Values are means $\pm$ SD; n=6 for immobilized and control samples for five strains of mice. Numbers above bars are the fold changes (immobilized/control) for each strain. &Significant immobilization and mouse strain interaction ( $p<0.05$ ); ^Significant main effect of mouse strain ( $p<0.05$ ); \*\*\*\* $p<0.0001$ ; immobilize vs control within same strain.

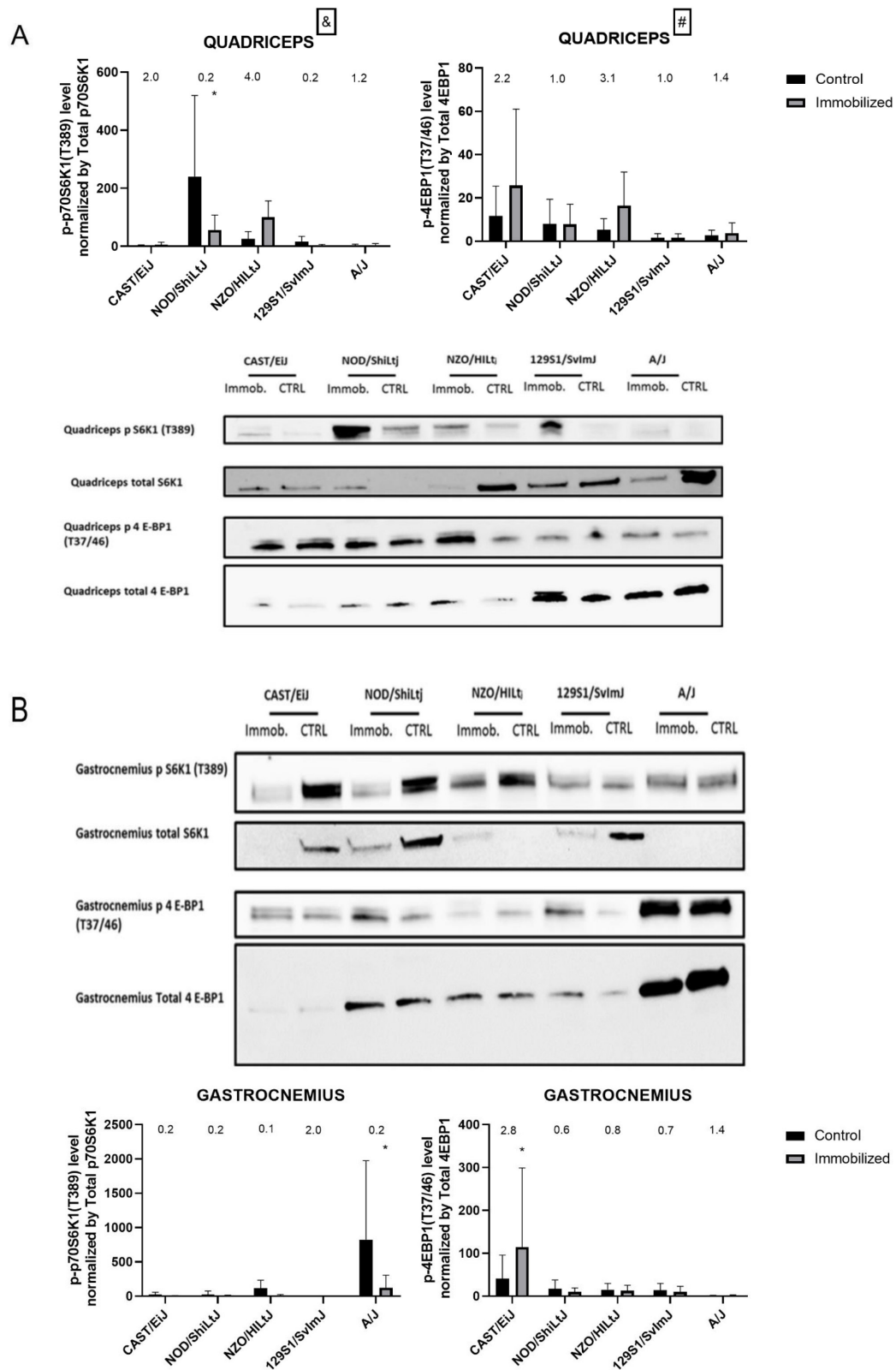
## Results

### Body weight, mouse activity levels and food consumption

Body weights were recorded before and after three weeks of casting (Table 1). All strains displayed significant weight loss. A/J mice had the highest percentage of body weight loss compared with the other strains, and this loss was significantly more than in CAST/EiJ, and NOD/ShiLtJ mice. To analyze mouse activity, we video recorded movement around the cage for a period of 24 hours before and after casting. Immobilization significantly decreased activity only in A/J mice. All other strains had non-significant, slight decreases or large increases in distance travelled. Food consumption was significantly increased in NOD/ShiLtJ mice. All other strains had non-significant increases in food consumption except NZO/HILtJ, which had a non-significant decrease in food consumption.

### Skeletal muscle mass

We assessed skeletal muscle mass both in absolute terms and as corrected for body weight. Assessing absolute skeletal muscle mass is appropriate when evaluating the effects of unloading in one strain. This is because in each mouse the contralateral limb serves as a control for the immobilized limb in the same mouse. However, when evaluating skeletal muscle mass across strains it is necessary to normalize to body weight. There was a significant immobilization and mouse strain interaction for absolute gastrocnemius mass, absolute quadriceps mass, and quadriceps mass/body weight. There were significant main effects of mouse strain and immobilization on gastrocnemius mass/body weight. Interestingly, immobilization significantly decreased quadriceps and gastrocnemius absolute muscle mass in only NOD/ShiLtJ and NZO/HILtJ mice (Figure 1A). CAST/



**Figure 4.** Effect of three weeks of casting on phosphorylation of p70S6K1 and 4E – BP1 in (A) quadriceps and (B) gastrocnemius muscles. All western blots were quantified and bar graphs represent mean  $\pm$  SD; n=6 for immobilized and control samples for five strains of mice. Grey bars are data from control limbs, and black bars are data from limbs exposed to immobilization by casting. Numbers above bars are the fold changes (immobilized/control) for each strain & Significant immobilization and mouse strain interaction (p<0.05); #Significant main effect of immobilization (p<0.05); \* p<0.05 immobilized vs control within the same strain.

EiJ, 129S1/SvlmJ and A/J quadriceps and gastrocnemius absolute muscle mass were not affected by casting. Quadriceps muscle mass/body weight was significantly decreased by immobilization, to varying degrees, in all strains except 129S1/SvlmJ. Immobilization resulted in the most significant decrease in quadriceps muscle mass/body weight in NOD/ShiLtJ mice (Figure 1B). Gastrocnemius muscle mass/body weight was significantly decreased, to varying degrees, in all strains except 129S1/SvlmJ and A/J, and again the most significant loss was in NOD/ShiLtJ mice. Finally, control muscle mass/body weight varied across strains (Figure 1B). Quadriceps mass/body weight was significantly different between 5 strain pairs: CAST/EiJ vs. NOD/ShiLtJ ( $p=0.007$ ); CAST/EiJ vs. A/J ( $0.036$ ); NOD/ShiLtJ vs. NZO/HILtJ ( $p<0.0001$ ); NZO/HILtJ vs. 129S1/SvlmJ ( $p=0.037$ ) and NZO/HILtJ vs. A/J ( $p=0.0003$ ). However, gastrocnemius mass/body weight was significantly different in only one pair, CAST/EiJ vs. NZO/HILtJ ( $p=0.031$ ).

#### Atrogene levels

To determine the level of skeletal muscle atrophy induced by casting, levels of the atrogens, *FbxO32* (encodes Atrogin-1) and *Trim63* (encodes MuRF1), were quantified by RT-qPCR (Figure 2). There was a significant immobilization and mouse strain interaction for quadriceps and gastrocnemius levels of *FbxO32* and gastrocnemius levels of *Trim63*. There was a significant main effect of mouse strain on quadriceps levels of *Trim63*. Three weeks of immobilization resulted in a significant increase in quadriceps and gastrocnemius levels of *FbxO32* and *Trim63* in CAST/EiJ mice but not in any other strain. Control levels *FbxO32* and *Trim63* in both quadriceps and gastrocnemius were similar across all strains.

#### Skeletal muscle fiber type gene levels

As previously mentioned, unloading also affects skeletal muscle fiber type composition. Levels of mRNA for genes associated with fast-twitch fibers, *Myh4*, and slow-twitch fibers, *Myh7*, were quantified (Figure 3). There was a significant immobilization and mouse strain interaction for quadriceps and gastrocnemius levels of *Myh4* and gastrocnemius levels of *Myh7*. There was a significant main effect of mouse strain on quadriceps levels of *Myh7*. Three weeks of immobilization resulted in a significant increase in quadriceps and gastrocnemius levels of *Myh4* in CAST/EiJ mice but not in any other strain. A similar trend was observed for *Myh7* in gastrocnemius muscle. However, *Myh7* levels were not affected by immobilization in quadriceps of any strain. Control levels of *Myh4* and *Myh7* in both quadriceps and gastrocnemius were similar across all strains.

#### Protein synthesis markers

The mTOR pathway is related to the maintenance of MPS. A central regulator of protein synthesis in skeletal muscle is mTORC1. Previous human and animal studies demonstrated that the inhibition of mTORC1 by rapamycin associated with

**Table 2.** Heritability of different properties.

Property	Quadriceps	Gastrocnemius
Body weight	0.4497	0.4497
Muscle mass	0.2299	0.3133
Muscle mass/BW	0.6945	0.5074
FBXO32	0.2560	0.4749
Trim63	0.3898	0.2704
Myh4	0.2400	0.3697
Myh7	0.2807	0.3562
p-p7OS6K1	0.1708	0.1490
p-4EBP1	0.07387	0.02344

exercise did not significantly reduce the protein synthesis rate<sup>20-22</sup>. However, by quantifying phosphorylation of p7OS6K1 and 4EBP1, we indirectly assessed mTOR activity in quadriceps and gastrocnemius muscles.

There was a significant immobilization and mouse strain interaction for quadriceps p-p7OS6K1/total p7O6SK1 ratio. There was a significant main effect of immobilization on quadriceps p-4EBP1/total 4EBP1 ratio. No other main effects were significant for p-p7OS6K1/total p7O6SK1 and p-4EBP1/total 4EBP1. Three weeks of immobilization resulted in a significant decreases of the p-p7OS6K1/total p7O6SK1 ratio in quadriceps of NOD/ShiLtJ mice (Figure 4A) and the gastrocnemius of A/J mice (Figure 4A). immobilization did not affect the p-p7OS6K1/total p7O6SK1 ratio in quadriceps or gastrocnemius of any other strain. Immobilization did not affect the p-4EBP1/total 4EBP1 ratio in quadriceps of any of the strains examined (Figure 4A). However, the p-4EBP1/total 4EBP1 ratio in gastrocnemius was significantly greater in immobilized, relative to control, limbs in CAST/EiJ mice (Figure 4B). The p-p7OS6K1/total p7O6SK1 ratio in control quadriceps was similar in all strains, except NOD/ShiLtJ in which levels were significantly greater than all other strains (NOD/ShiLtJ vs. CAST/EiJ,  $p=0.0015$ ; NOD/ShiLtJ vs. NZO/HILtJ,  $p=0.0028$ ; NOD/ShiLtJ vs. 129S1/SvlmJ,  $p=0.0018$ ; NOD/ShiLtJ vs. A/J,  $p=0.0009$ ). The p-p7OS6K1/total p7O6SK1 ratio in control gastrocnemius was significantly greater in A/J mice relative to other strains (CAST/EiJ vs. A/J,  $p=0.0174$ ; NOD/ShiLtJ vs. A/J,  $p=0.0175$ ; NZO/HILtJ vs. A/J,  $p=0.0442$ ; 129S1/SvlmJ vs. A/J,  $p=0.0133$ ). The p-4EBP1/total 4EBP1 ratio in control quadriceps was similar in all strains examined. However, the p-4EBP1/total 4EBP1 ratio in control gastrocnemius was significantly reduced in A/J mice relative to the other strains examined.

#### Heritability

Considering that genetics may play an important role in the response to unloading, heritability was calculated for each property analyzed. The analysis was done for each muscle (Table 2). Genetics had a greater impact on skeletal

muscle mass/body weight than other traits. Heritability of skeletal muscle mass, *FbxO32*, *Myh4* and *Myh7* was greatest in the gastrocnemius. On the contrary, heritability calculated for skeletal muscle mass/body weight, *Trim63*, p-p70S6K1/total p70S6K1 and 4EBP1/total 4EBP1 was higher in the quadriceps than the gastrocnemius. Phosphorylated p-p70S6K1/total p70S6K1 and 4EBP1/total 4EBP1 were the traits least affected by genetics.

## Discussion

Our results reveal variability in responsiveness to immobilization across the five different genetic strains of mice. Four of the five strains experienced either loss of muscle mass or loss of muscle mass/body weight in response to immobilization. Despite this consistent muscle atrophy among the strains, there was no consistent pattern in changes of atrogenes, fiber type gene expression, or phosphorylation of p70S6K1 or 4EBP1. CAST/EiJ mice had significant changes in atrogenes and fiber type gene expression, A/J and NOD/ShiLtJ mice had significant decreases in phosphorylation of p70S6K1, while NZO/HiLtJ had no significant effects on any of these markers. This suggests the timing of when these commonly affected indicators of muscle atrophy change depend on mouse strain and is influenced by genetic variability. These indicators are typically shown to be affected by immobilization within 14 days and may no longer have been significantly affected by immobilization when we analyzed them on day 21.

NOD/ShiLtJ and NZO/HiLtJ were the only strains that displayed significant skeletal muscle mass loss in response to unloading. These two strains displayed similar changes in activity level, lost similar amounts of body weight following immobilization and neither of these two strains displayed an increase in atrogenes or changes in skeletal muscle fiber type following immobilization. NOD/ShiLtJ mice displayed a decrease in the p-p70S6K1/total p70S6K1 ratio in the quadriceps of the immobilized limbs. Interestingly, the p-4EBP1/total 4EBP1 ratio levels were not affected by immobilization in either NOD/ShiLtJ or NZO/HiLtJ mice. These results are consistent with previous data from our group showing p70S6K1 changes precede 4EBP1 changes following unloading induced by hind limb suspension of C57BL/6J mice<sup>23</sup>. The decrease in p-p70S6K1/total p70S6K1 ratio, which is the downstream target of mTOR kinase, in NOD/ShiLtJ mice suggests that changes mTOR kinase activity and decreased protein synthesis contribute to disuse atrophy in this strain.

Our finding that only NOD/ShiLtJ and NZO/HiLtJ mice displayed skeletal muscle atrophy in the immobilized limb is especially interesting in light of the fact that males of both of these strains spontaneously develop diabetes<sup>24</sup>, while this has not been reported in the other strains examined in this study. Disuse skeletal muscle atrophy is exacerbated in diabetes<sup>25</sup> suggesting that susceptibility of NOD/ShiLtJ and NZO/HiLtJ mice to disuse atrophy may be related to their diabetic phenotype.

CAST/EiJ mice displayed the least body weight loss during the experiment, were the most physically active and displayed an intermediate skeletal muscle mass to body weight ratio in control limbs. Interestingly, CAST/EiJ mice were the only mice that displayed changes in levels of atrogenes, *Myh4* or *Myh7*. Despite being resistant to disuse-induced skeletal muscle atrophy, CAST/EiJ mice displayed an increase in both atrogenes in both quadriceps and gastrocnemius muscles in the immobilized limb. This was accompanied by increases in p-4EBP1, a marker of protein synthesis, in gastrocnemius muscles exposed to immobilization. These results suggest that, in response to immobilization, CAST/EiJ muscles display increased muscle protein breakdown that is compensated for by an increase in protein synthesis, resulting in no net skeletal muscle loss. *Myh4* levels were increased in both the gastrocnemius and quadriceps of immobilized limbs while *Myh7* levels were increased in the gastrocnemius of immobilized limbs. These results suggest an unloading-induced switch from slow- to fast-twitch fiber type, at least in the quadriceps. This is consistent with previous studies that revealed a switch from slow to fast fiber type associated with loss of skeletal muscle mass and strength<sup>26-32</sup>. Additionally, unloading from exposure to microgravity results in a switch from slow to fast fiber type<sup>8</sup>.

A/J mice lost the most percent body weight throughout the experiment and were the only strain that displayed decreased physical activity during casting. The reason for this is unclear. However, since A/J mice had the lowest physical activity after casting, it is possible that effectively both limbs were immobilized. The decrease in activity may have caused control limb atrophy during the first 7-14 days. Then the skeletal muscles reached homeostasis between days 15-21. Despite displaying a decrease in p-p70S6K1/total p70S6K1 ratio in the gastrocnemius of the immobilized limb, A/J mice appeared resistant to immobilization-induced gastrocnemius muscle atrophy. 129S1/SvImJ mice were resistant to immobilization-induced skeletal muscle atrophy. They lost a modest amount of body weight during the experiment but displayed no changes in physical activity as a result of casting. The immobilized limbs had similar levels, relative to control limbs, of markers of fast- and slow-twitch skeletal muscle fibers, atroгене levels, and markers of protein synthesis.

To better understand the role of genetics in the response to unloading, heritability for each property was calculated based on the percent difference between immobilized and control limbs. The results revealed a variety of impacts of genetics on these properties. Skeletal muscle mass normalized to body weight in both gastrocnemius and quadriceps displayed the greatest degree of heritability suggesting that this phenotype is the most affected by genetic variability. Interestingly the two markers of protein synthesis p-4EBP1/total 4EBP1 ratio and p-p70S6K1/total p70S6K1 ratio were influenced the least by genetic variability. This suggests a disconnect between skeletal muscle mass/body weight and protein synthesis, at least as regards the effects of genetic variability. We are unaware of literature reports of the effect



of disuse with casting in these strains of mice.

This study reveals differences in the response to unloading among the five mouse strains and suggests that DO mice, derived from these founders and three additional strains, would be powerful tools in identifying the mechanisms underlying disuse atrophy. However, this study has limitations that can be addressed with future studies. For instance, an examination of which genes are responsible for protecting skeletal muscle from unloading in the strains that showed minimal response to unloading would be insightful. This could be accomplished using RNA sequencing of skeletal muscle tissue samples. Additionally, our finding that immobilization did not reduce skeletal muscle mass in every strain but did affect at least one endpoint in all strains except 129S1/SvImJ, suggests that the duration of exposure to immobilization may differentially affect strain-dependent response to immobilization. Furthermore, we did not examine soleus muscle, which contains a majority of type 1 slow-twitch fibers and may be more responsive to disuse than gastrocnemius or quadriceps which have a majority of type 2 fast-twitch fibers<sup>33</sup>. For instance, 14 days of hind limb suspension of C57BL/6J mice resulted in a 21% vs 10% decrease in soleus and gastrocnemius wet weight, respectively<sup>33</sup>. Future studies will examine soleus muscle response to disuse.

This study is the first to report a differential skeletal muscle response of five DO founder strains of mice to immobilization. The results confirm our hypothesis that genetic variability plays an important role in the development of skeletal muscle atrophy, leading to differential, strain-dependent effects of unloading on skeletal muscles in these strains of mice. Additionally, we now have the basis for using DO mice in order to identify specific gene changes that protect skeletal muscle from disuse atrophy. In this way, a therapeutic strategy to limit the development of unloading-induced sarcopenia could be developed.

#### Funding

*This work was funded by NASA grant 80NSSC18K1473.*

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**Supplementary Table 1.** Amplicon Context Sequence used in the qPCR.

Gene	Amplicon Context Sequence
FBXO32	5'- AGTGGTTCATGTGCTGGGATTCAGA ACTTGAACAAGTTGATAAAGTCCTGGGGT GAAAGTGAAACGGAGCAGCTCTCTGGGTATTGGCAGTGCACGGATGGTCAGT CCCTTCCAGGAAAGGATGTGACAGTGTGCA-3'
TRIM63	5'-AGTGATGATGGTCTGCACACGGTCATTCCCCGCCACCAGCATGGAGATACAGTT ATTCAGTTCAGTCTTTTGTCCCTGGAAGACACTCTGCAATGGGGCCACCTCGCA GGCCTTGTTGGATCCCAAACACCTTGACATGGAGCAGGTGGGCACCTCACACG-3'
MYH4	5'-ACCAGAAATCCGGGTGAAGACTCTGGCTTCTCTATTTCTGGGGACAAGCTG CGGAAGCAGAGGGCGGGTGGAAAGAAAGGTGGCAAGAAGAAGGGTTCTTCT TTCCAGACCGTGTGAGCTCTCTCAGGGAGAATTTAAATAAGCTGATGACCAACT TGAAGAGCACCCACCCCACTTTGTCAGATGCCTCATTCCCAATGAAACTAAGAC TCCTGGTGCCATGG-3'
MYH7	5'-GCTTCGCTCGCTCCAGGTCCATGCGCACCTTCTCTCTGCTCCAGGGATCCTT CCAGATCATCCACTTGCTGCTCCAGCTTGACTTTGGCCTTAGTCAGGGTGTGAC CTTGCTCTCTCGCCTGAAGGTCATCCAGAGC-3'