ABSTRACT

EFFECT OF EARLY LIFE PHYSICAL INACTIVITY LEVEL ON MUSCLE HEALTH DURING EARLY POSTNATAL DEVELOPMENT

by Austin Dean Smith

Background: Physical activity (PA) is a vital behavior to maximize health and wellness. Less is understood regarding the impact of muscle disuse on children, specifically during key stages of skeletal muscle development. The time frame between weaning and sexual maturation is known to be a critical period of development in mice. **Purpose:** We propose that, similar to malnutrition, exposure to different levels of physical inactivity (PIA) early in life will impair growth rate, muscular function and tissue composition. **Methods:** We exposed postnatal mice (3-4 weeks old) to 2 weeks of physical inactivity (PIA) in the form of hindlimb unloading (HU) and small mouse cage (SMC) or standard mouse cage activity (controls) after weaning. Grip strength and body composition were assessed before inactivity and after the inactivity period. Muscle weights were collected after completing PIA or 7D recovery. **Results:** Body weights and lean mass in PIA mice (SMC and HU) were significantly (CON>SMC, $P<0.001$; CON>HU, $P<0.0001$) attenuated compared to controls immediately following PIA. Fat mass was significantly ($P<0.0001$) higher in control and SMC mice compared to HU mice after PIA. Absolute maximum grip strength between weeks 3 and 5 was significantly ($P<0.05$) reduced in SMC and HU mice after PIA. All muscle weights, except the tricep, collected in SMC and HU mice weighed significantly less than control mice following PIA. **Conclusion:** Mice subjected to physical inactivity displayed lower body weights, maximum grip strength, and lean & fat mass compared to control mice. Comparing HU and SMC, HU mice appear to have lower body weight, grip strength and lean mass than SMC mice. Changes in lean mass and body weight suggest significant deficits in physical health that may have bearing on healthy development and aging. Additionally, since grip strength is a strong predictor of health status, reduced functionality, and early mortality, these findings of premature dynopenia (muscle weakness) as a result of early life muscle disuse are concerning.

EFFECT OF EARLY LIFE PHYSICAL INACTIVITY LEVEL ON MUSCLE HEALTH DURING EARLY POSTNATAL DEVELOPMENT

Thesis

Submitted to the

Faculty of Miami University

in partial fulfillment of

the requirements for the degree of

Master of Science

by

Austin Dean Smith

Miami University

Oxford, Ohio

2022

Advisor: Dr. Paul Reidy

Reader: Dr. Kyle Timmerman

Reader: Dr. Paul Schaeffer

©2022 Austin Dean Smith

This thesis titled

EFFECT OF EARLY LIFE PHYSICAL INACTIVITY LEVEL ON MUSCLE HEALTH DURING EARLY POSTNATAL DEVELOPMENT

by

Austin Dean Smith

has been approved for publication by

College of Education, Health, and Society

and

Department of Kinesiology, Nutrition, and Health

_____

Dr. Paul Reidy

_____

Dr. Kyle Timmerman

_____

Dr. Paul Schaeffer

Table of Contents

List of Tables

List of Figures

Dedication

This thesis is dedicated to my family. I am incredibly grateful for all of the love, support, and encouragement they have all provided along the way.

Acknowledgements

First, I am very thankful for a loving and supportive family, who encouraged me to jump on this opportunity to work with Dr. Reidy and move out to Ohio. To my faculty mentor, Dr. Paul Reidy, I am incredibly grateful for the opportunity to learn and grow by your example. I never envisioned the pursuit of a masters degree would be a stop along the way, but I am forever grateful for this opportunity and all of the support you have provided the past two years. You always provided guidance when necessary but also allowed me to figure things out on my own, and because of that I have seen tremendous growth in myself over these two years. I have learned so many practical skills that will not only translate to my career, but life in general as well. I cannot be grateful enough. To my thesis committee members, Dr. Paul Schaeffer and Dr. Kyle Timmerman, I greatly appreciate the time and effort you have put in to provide recommendations, advice, and words of wisdom throughout this process. Dr. Timmerman, I want to thank you for encouraging me to apply for the graduate teaching assistantship this year. As a quiet, shy undergraduate student, never in a million years did I think that I would ever become a teaching assistant. It's quite remarkable how far I've come since then, and I'm grateful for the encouragement to make that leap of faith. To all of the undergraduate students that help in Dr. Reidy's lab, Ben, Alex, Katie, Ty, Anthony, and Rachael, I want to thank you all for stepping in when necessary to help me out with experiments and also filling in the gaps when the mice needed to be weighed. I understand working with mice can be very labor-intensive at times, but all of your hard work did not go unnoticed. So, I thank you all.

Introduction

Physical activity (PA) is a critical component of childhood. It is known that active children sleep better, build strong peer relationships, improve cognitive ability and coordination, and promote strong muscle and bone growth. Overall, children who are consistently physically active maintain a state of overall well-being (UK Chief Medical Officers, 2021). This, also, encourages the formation of future exercise habits, so that a child can maintain their activity level into adolescence and adulthood (UK Chief Medical Officers, 2021). With that being said, what happens when a child remains sedentary for much of their childhood? Does this have imp;ications for health later on? If so, what would cause this? Generally, it is known that sedentary behavior can lead to an increase in all-cause mortality and double the risk for multiple chronic diseases such as cardiovascular disease and diabetes (World Health Organization 2002). On a skeletal muscle level, this reduced activity level can lead to losses in muscle mass and muscular strength. What impact does physical inactivity have at a young age on muscle and health outcomes?

To help answer this question, it is important to outline what is known about muscle development from embryo to adulthood. Skeletal muscle originates from embryonic somites which are transient mesodermal structures that form pairs on either side of the neural tube. There are cues from other tissues near the somites, including the neural tube, dorsal ectoderm and notochord that induce the formation of muscle precursor cells. The cells are committed to myogenesis and then migrate to target muscle groups (Zhao & Hoffmann 2004). Human muscle development starts at 6 to 8 weeks of gestation. At that time the primary fibers form followed by the secondary fibers that begin forming between weeks 8 and 18. At this time, secondary muscle fibers are sensitive to the prenatal environment including the influence of maternal diet and hormones. Prenatal malnutrition could be associated with a permanent reduction in muscle fiber size and number (Sayer et al. 2004). After week 24 of gestation myogenesis is finished, an increase in muscle fiber size (hypertrophy) is predominantly responsible for the development of muscle size. From that point forward, muscle fiber development relies on nutrients from the diet, hormone action, and growth factors that influence regulatory and structural genes necessary for myogenesis (Patel et al. 2012). From youth up through adulthood, muscle fibers grow by fusing

with additional myoblasts leading to the increase in fiber size (Zhao & Hoffmann 2004). Literature suggests that max cross-sectional area in myofibers is reached between the ages of 18 and 20 (Delhaas et al., 2013; Verdijk et al., 2014) In adulthood, skeletal muscle size and strength continues to steadily decrease over decades, especially in late adulthood. To compensate for physiological demands and muscle damage in adulthood, skeletal muscle has the ability to re-activate the myogenic mechanisms. There are many parallels that have been found between myogenesis during gestation and regeneration of mature skeletal muscle. Prenatal myogenesis and regeneration share common transcription factors and signaling molecules. Both maintenance and repair of skeletal muscle tissue are largely conducted by satellite cells that can proliferate, differentiate, generate new fibers, and repair old muscle fibers (Hernandez-Torres et al. 2017). Gomes et al. (2017) explains how muscle loss is not fully understood and multifactorial in nature, primarily affecting the geriatric population and those with systemic diseases. Some of the main causes listed in Gomes et al. (2017) include physical inactivity (PIA), changes in hormone levels, insulin resistance, genetics, loss of appetite, and nutritional deficiencies. The contribution of each factor to the aging process is poorly understood. Due to each of these factors playing a role in the aging process, it is safe to conclude that each individual likely ages differently, and physical activity or lack thereof during critical periods of development may have long-term health implications.

The use of animal models such as mice and rats allow us to observe both short-term and long-term changes due to physical inactivity in a compressed amount of time due to the lifespan of mice and rats. With there being similarities to humans in the process of skeletal muscle development, mice provide an excellent model to understand the changes that occur due to physical inactivity. A 2016 study done by Dutta & Sengupta conceptualized the parallels between the age of mice and the equivalent amount of time in humans since the lifespan of mice is typically 2 years. These comparisons were based on the weight of the eye lens, epiphyseal closure, tooth wear pattern and body weight patterns. Taking all of these factors into account, Dutta and Sengupta reviewed previous articles discussing the comparisons to humans regarding these 4 variables and were able to make age determinations in mice in order to relate to human age/life stages. In this study, mouse weanlings are used in which the mice are between 3 and 4 weeks old. Dutta and Sengupta (2016) approximated that the end of weaning in humans is roughly 6 months of age, on average. Based on this average, they were able to calculate how

many human days are equivalent to 1 mouse day. They concluded that 6.43 human days is equal to 1 mouse day. This means that 1 human year is roughly equivalent to 56.77 mouse days for mice this age. To put a period of physical inactivity into perspective, a 2-week period of inactivity in mice would be equivalent to subjecting a 6-month-old human baby to about 3 months of physical inactivity. A week of recovery in a standard mouse cage would be the equivalent of allowing the 6-month-old baby to recover for roughly 2 years. It is important to recognize that this approximated equivalence should not be taken in a literal sense. Human weaning does not stop at 6 months for all humans. Likewise, 6 month old children are not nearly as active as newly weaned mice are. Based on this, a better comparison to human age would likely be better depicted as an age range between 2-4 years of age since children become much more active at this stage. Relating this to physical inactivity in children, a study done in the UK by Davies et al. (2014) compiled common health conditions of children that essentially grew up in the hospital due to their poor health condition. All children were admitted in the hospital for 6 months or longer. Some of the health conditions included cancers, heart assistive devices, long term ICU stays, awaiting organ transplant or completed transplant to name a few. Children that grew up in these settings or experience an extended period of physical inactivity while in the hospital may encounter the effects of sedentary behavior much later in life; although, this has not been well-established in the literature yet and remains to be seen.

Sayer et al. (2006) describes how postnatal development is a key period of development in our lives that is highly likely to influence health later in life. There is literature to support that muscle mass in older people is positively associated with birth weight regardless of current size (Gale et al. 2001). Other studies have been able to replicate similar findings as Gale et al. For example, a United Kingdom (UK) birth cohort including both men and women, who were born in 1946, demonstrated strong relationships between size at birth and grip strength as a middle-aged adult (Kuh et al. 2002). Beyond birth weights and size at birth, Sayer (2006) demonstrates an association between poor growth in early life and risk of falls later in life. This association was established by looking at conditional infant weights which compares the infant's weight to the weight that is predicted based on previous weight. A lower conditional infant growth was significantly associated with a history of falls in old men. Relating to falls, a separate study done by Cooper et al. (2001) supported an association between poor growth in childhood and hip fracture risk later in life. Low grip strength in adulthood was another finding from this

study. This finding was supported by other studies as well (Kuh et al. 2002; Sayer et al. 1998; Sayer et al. 2004). These studies collectively found that there was a relationship between poor early life growth and reduced muscle strength during adulthood. According to Sayer (2006), these findings suggest that early environmental influences may result in long term impairment of muscle function as well as influence the incidence of falls later in life.

It has been well-established that poor growth leads to impaired muscle function and sarcopenia. Both factors as well as the factors mentioned previously (ie: heightened risk of falls and hip fractures) create a large burden on our healthcare system. Coinciding with poor skeletal muscle health and function, other chronic health conditions such as cardiovascular disease, osteoporosis and metabolic syndrome may also occur as a result of poor growth. Consequently, we believe that early life inactivity is a critical factor during early childhood development that may program the path toward poor health outcomes later in life.

Not only is postnatal development a critical component in our lives, but there are also a variety of factors that impact postnatal development. Dodds et al. (2015) described factors that contribute to muscle mass and function such as genetics, diet, physical activity, chronic disease, and lifestyle factors in youth. Therefore, there is a need to gain a better understanding of the impact of physical inactivity during periods of critical development. Previous literature suggests that longer periods of muscle disuse can lead to greater muscle loss (atrophy) (Elder & McComas 1987). Building off that, a few studies found that the younger the rodent is the greater the amount of disuse atrophy, leading to impaired muscle growth (Saitoh et al. 1999; Elder & McComas 1987; Simard et al. 1987; Steffen et al. 1990). In other words, younger rodents subjected to disuse atrophy displayed a greater amount of skeletal muscle mass loss compared to their older rodent counterparts. A study evaluating the impact of how concurrent catabolic conditions amplify disuse atrophy indicated the diminished ability to rebound to normal developmental levels of muscle mass and strength. (Wu et al. 2010; Wade et al. 2013). The inability to recover muscle mass and strength can lead to a lower quality of life and poorer health overall (Dodds et al. 2015). There is also a higher risk for chronic disease in sedentary individuals. Malnutrition is another factor that has been studied to influence stunted regrowth in children (Pitts 1986; Winick and Noble 1966; Winick 1989). In fact, Patel et al. (2012) looked at the effect of undernutrition on muscle fiber development and birth weight, and the resulting impact of fewer muscle fibers led to harmful effects on muscle mass, postnatally.

The above literature utilized mice to study the implications of sedentary behavior and disuse atrophy specifically. These disuse models utilize either hindlimb unloading (HU) using tail suspension, casting (immobilization), or denervation which are stressful but effective ways to induce muscle atrophy. These experiments are muscle disuse models as they typically isolate certain limbs to cause muscle loss and are not strictly considered physical inactivity models, although they do induce physical inactivity. Although originally designed to be ground-based analog to spaceflight, HU is considered a form of inactivity that mimics hospitalization since it induces stress and symptoms of depression while concurrently causing skeletal muscle to atrophy. Some more translatable forms of physical inactivity, like reducing step count, may not induce such prominent muscle atrophy, but may also lead to a plethora of negative health effects (Reidy et al., 2021). This may be more appropriately simulated with use of the less frequently used model of a small mouse cage (SMC). SMC was also used in mice in Roemers et al (2019) and Mahmassani et al (2020). Cage size reduction has also been used in rats in Marmonti et al. (2017). This is a novel way to induce physical inactivity and muscle atrophy by recreating step reduction with SMC. This model is less extreme and more generalizable to moderate physical inactivity (Reidy et al. 2021) and has not been conducted in early postnatal mice. All known prior studies using SMC have not been as restrictive as the SMC used in this study. Most of the studies examining the effects of muscle disuse listed above employed some variation of HU, a model for inducing muscle disuse. It remains unknown if less extreme models of physical inactivity such as SMC can also have similar atrophic effects during this important postnatal stage.

For this study, mice weanlings are subjected to a two-week period of physical inactivity (PIA) to induce muscle atrophy and growth restriction through SMC or HU. Two weeks of physical inactivity is a well-established standard used in previous literature to induce disuse atrophy and sedentary behavior. In addition, one week of recovery is an established method for evaluating short term recovery. Grip strength and body composition are assessed before and after physical inactivity to evaluate any changes due to the physical activity restriction. After two weeks of physical inactivity (PIA), mice were either euthanized at 5 weeks of age (5-week mice) or allowed to recover for 1 week and euthanized at 6 weeks of age (6-week mice). Upon euthanizing the mice, skeletal muscle tissues were collected and weighed.

Hypothesis

I hypothesize that early postnatal weanlings (3 weeks of age) subjected to inactivity for two weeks will experience a weaker grip strength, lower lean body mass, and a higher fat mass compared to control mice. Due to different dosages of inactivity being used, I hypothesize k that greater deficits in muscle development such as reduced grip strength and lower lean mass will be seen in HU mice compared to SMC mice. Following one week of recovery, I hypothesize that mice in the SMC will rebound better than mice in HU. In other words, mice in the SMC will have more similarities in grip strength, lean body mass and fat mass with control mice following recovery compared to hindlimb unloaded mice after one week of recovery.

Aims

Aim 1: To determine how different levels of inactivity as HU compared to SMC, between 3 weeks to 5 weeks of age, attenuates skeletal muscle development and postnatal growth by assessing muscle weights, grip strength and tissue composition.

Aim 2: To assess how the one-week of recovery, between 5 weeks and 6 weeks of age, restores the effects of differing levels of sedentary behavior (AIM 1) by assessing muscle weights, grip strength and tissue composition.

Methods

Animals

C57/BL/6 male and female mice were used in this experiment. The mice were generated by breeder pairs were obtained from The Jackson Laboratory (Bar Harbor, ME) and a colony was maintained in the Laboratory Animal Resource at Miami University between September 2021 and March 2022. Mice were removed from their breeder cage just after weaning (P21) and placed into one of six age-matched experimental conditions, SMC (7.5cm L x 7.5cm W x 6.7cm H) (n=15), HU (n=14), standard mouse cage (29.2 cm L x 18.4 cm W x 12.7 cm H) for control (CON) (n=13), SMC plus one-week recovery (n=13), HU plus one-week recovery (n=12), or standard mouse cage with one-week recovery (n=12). The one-week recovery period is defined as the removal of the mice from HU or SMC and placing them into a standard mouse cage for one-week. Control mice will remain in their standard cage an additional week following the 14-day physical inactivity period if they are placed into the CON group. All mice were housed at

a temperature of 22 degrees Celsius and 30-50% humidity in the animal care facility at Miami University. A 12:12 day:night cycle was used. Mice were identified by an ear notching number system. This is done by scruffing the mouse and using an ear punching instrument. Mice were anesthetized using isoflurane before ear notching if the mouse is older than 21 days.

Table 1: Mouse sample size by cohort

Mouse sample sizes				
Total mice: 79	Control	SMC	HU	Totals
5 week cohort	13	15	14	42
6 week cohort	12	13	12	37

Small Mouse cage (SMC)

Mice (n=25) were placed into the SMC. This was previously done by Mahmassani et al. (2020) with slightly less restrictive cage dimensions and other enrichment modifications. The SMC mice were placed into a rectangular box (L:7.5 x W:7.5 x H:6.7cm) for a period of 14 days. This cage was fabricated from acrylic plastic constructed by the Miami University instrumentation lab. Specialized feeders were adapted to hold the food directly above the mouse. Hydropacks with a lixit inserted were used to dispense water ad libitum. 12 air holes at the top of each side of the rectangular box were placed to allow for air flow. In addition, holes were added to enable the hydropack lixit to fit into the SMC. Paper bedding (Teklad TEK-fresh Laboratory Animal bedding and Teklad Laboratory Grade Pelleted Paper Bedding, Envigo, Indianapolis, IN) was used. Bedding was filled to the brim just before the air holes and weighed to determine the proper amount of bedding for each mouse. The mouse's body weight is then subtracted from the remaining total to determine the amount of bedding placed in the SMC. Based on this weight, 2 grams were subtracted to allow for growth. Bedding changes were performed by students working in the research lab. Bed changes occurred every 2-3 days to ensure a clean environment for the mice and to allow room for growth (Mahmassani et al. 2020).

Hindlimb unloading (HU)

Mice (n=26), who were randomly assigned to HU, were placed in a cage adapted for HU for 14 days. This was accomplished by having a cross bar constructed that slides into a rat cage (Reidy et al. 2019). A 3.5 inch screw with 3-4 metal bobbin spools screws into the crossbar to set up HU by tail suspension. The crossbar and related parts were adapted for use by the Miami University Instrumentation Lab. The mice are set up in the HU apparatus by placing athletic tape at the base of the tail of the mouse. On the first day of HU, the mice are placed in the cage but all four limbs remain loaded to ensure the mouse knows how to access food and water. Each day following, the hook attached to the tape on the tail is raised one rung higher on a metal bobbin spool until the mouse's hindlimbs are fully unloaded. This is achieved by day 3 of the inactivity period. Food and water were provided in a way that the mouse could easily access them while preventing the mouse from climbing on the feeders or water bottle. Solid bedding mats were used to maintain cleanliness of the cage. The bedding mats were changed every 3-4 days.

Recovery

After mice were placed in the SMC, HU, or a standard mouse cage for 2 weeks, a little under half of the mice (n=34) from each group were placed into a standard mouse cage for one week to allow for recovery. During this time, mouse body weight, food intake and water intake were recorded each day.

Weighing

Mouse body weight was recorded on days 0, 1, 2, 3, 4, 6, 8, 10, 12, and 14. Mice were weighed daily during the one-week recovery. For the 2 weeks of inactivity, food and water was weighed to determine food intake on day 0 and 14. During one-week recovery, food and water intake was recorded every day.

Grip strength

Dual grip strength was assessed using an automated Grip Strength Meter (Grip Strength Meter, no. 160163; Columbus Instruments, Columbus, OH) measured by a force transducer. A mouse is handled by grabbing near the base of the tail and placing the mouse on the pull bar (force transducer), 3" x 2" (76mm x 50mm). Once the mouse reflexively grasps the pull bar, the investigator applies even tension to the mouse's tail parallel to the transducer. The transducer

registers the maximal force produced until the mouse's grip releases. The grip strength meter is zeroed and the procedure is repeated. Three trials per mouse were performed and the maximum effort was recorded. Grip strength was assessed in all mice at 3 weeks of age (the beginning of physical inactivity, after 14 days (at the end of physical inactivity), and after the one week recovery period in mice that continue to that time course (Castro & Kuang 2017). This assessment could be repeated longitudinally on the same mice allowing for stronger statistical comparisons. Data are reported as maximum absolute grip strength in grams of force and maximum grip strength in grams of force divided by body weight.

Tissue composition

Lean body mass and fat mass was assessed with EchoMRI-SuperFLEX™ whole body composition analyzer (Echo Medical Systems, Houston, TX). The mice are placed in a plexiglass restraint tube and inserted into the MRI machine. The mice remain conscious throughout the test (Tinsley et al. 2004). Body composition of the mice were assessed before physical inactivity, again at day 14 of inactivity, and at the end of one week of recovery. This assessment could be repeated longitudinally on the same mice allowing for stronger statistical comparisons

Dissection

Prior to dissection, the mice are fasted for 6 hours and placed into a cleaned cage with new bedding. Mice were euthanized at 5 weeks of age or 6 weeks of age, depending on whether the mouse was allowed one week of recovery. Carbon dioxide inhalation followed by cervical dislocation was conducted to euthanize the mice. After euthanization, blood, skeletal muscle, and liver tissue were weighed and collected for further analysis. Skeletal muscles that were collected from the mouse include triceps, tibialis anterior/extensor digitorum longus (TA/EDL), gastrocnemius, plantaris, and soleus. Both muscles from each limb were collected. One muscle being used for histology. Upon collection, the muscles are placed into labeled cryovials and stored in a container in liquid nitrogen until all of the tissues could be placed into a -80 degree Celsius freezer for future analysis. Skeletal muscle weights were expressed as absolute weight in milligrams (mg) and tissue weight relative to body weight (mg/BW).

Statistical Analysis

Results were reported as means ± standard error of the mean with individual data point in some figures. Five statistical models were used to analyze tissue composition and grip strength data. A one-way analysis of variance (ANOVA) was used on all 5 models. Model one involved comparing the change between 3 weeks of age and 5 weeks of age in all mice. Model two looked at differences between control, SMC, and HU mice at baseline (3 weeks of age). Models one and two utilized all the mice. Model three compared the changes during 7D recovery between 5 weeks and 6 weeks of age. Model four analyzed differences at 3 weeks of age (baseline) for the 6 week mice. Model five compared the changes in body composition and grip strength from 3 weeks to 6 weeks in 6 week mice only. Only the mice that completed the one week of recovery were included in model three, four and five (6 week mice).To analyze muscle and liver weights, a two-way ANOVA, with factors of recovery (PIA vs 7D recovery) and level of inactivity (CON vs SMC vs HU), was utilized. When comparing the effects of inactivity level within PIA or 7D recovery, multiple comparisons were tested with a Tukey analysis. Šídák's multiple comparisons were tested when comparing the difference between PIA and 7D within control, SMC or HU groups. Changes in body weight over time were analyzed by performing a two-way ANOVA with factors of time and level of physical inactivity. The statistical significance was set at $p < 0.05$ for all analysis. Statistical analysis was performed using GraphPad Prism 9.3.1 (GraphPad Software Inc., La Jolla, CA). P-values and degrees of freedom (DFn, DFd) are depicted in tables for each outcome.

Figure 1: Outcome timeline describes at what age each outcome is performed

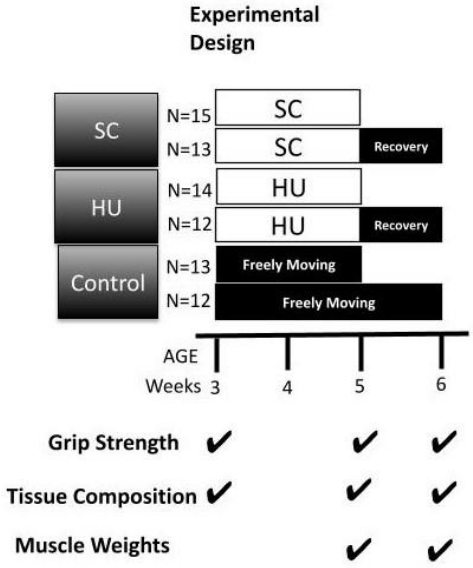
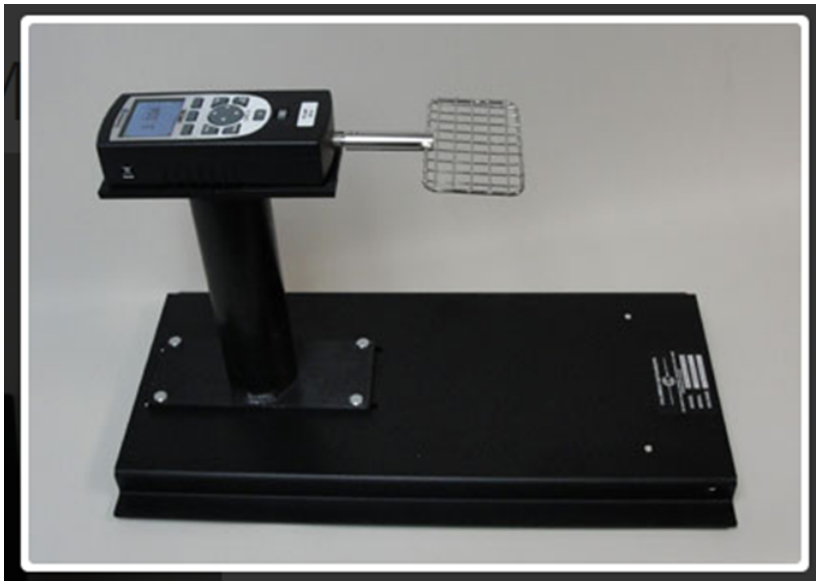


Figure 2: Grip strength meter (Columbus Instruments)



Results

Mice completing PIA only may be referred to as 5-week mice. Mice completing PIA and 7D recovery may be referred to 6-week mice. The mice were all 5 weeks of age by the end of PIA. All mice completing PIA plus 7D recovery were all 6 weeks of age.

Body weight

Throughout PIA and recovery, all mice continued to grow significantly compared to their weights at day 0 (week 3). Body weight, body weight change from day 0 (growth rate), and body weight change per day for PIA and 7D recovery are depicted in figure 4. P-values and degrees of freedom (DFn, DFd) are depicted in tables 2, 3, and 4

Body weight during PIA

From days 6-14 of PIA, the body weights of HU and control mice differ significantly. Comparing body weights between SMC and control mice, weights began to significantly differ by days 12-14 of PIA. The change in body weight from day 0 was significantly higher in Control mice vs HU mice and SMC mice compared to HU mice. Body weight change from day 0 was significantly different between SMC and HU mice and between Control and HU mice from days 5-14 of PIA. From days 10-14 of PIA, control mice had a higher body weight change from day 0 compared to SMC mice. For the average change in body weight per day, control mice showed the highest average body weight change per day followed by SMC mice, and HU mice displayed the lowest average body weight change per day. This stepwise trend regarding average body weight change per day is shown in figure 4.

Body weight during recovery

By the end of one week recovery, HU mice showed significantly lower body weights compared to CON mice. Consistent differences in body weight were maintained throughout the entire week of recovery. Body weight differences were found in SMC mice up until day 2 of recovery. By day 3 of recovery, no differences in body weight were observed between SMC mice and control mice. For change in body weight compared to day 0 of recovery, HU mice displayed a significantly higher growth rate compared to control mice, meaning the HU mice added a

significant amount of body weight during 7D recovery that was restricted during PIA. For average body weight change per day during recovery, there were no differences between groups.

Grip strength

All mice at baseline (3 weeks of age) showed no differences for both grip strength measurements (max grip strength, max grip strength/BW) between control, SMC and HU mice. Maximum grip strength and Maximum grip strength/ body weight can also be referred to as absolute and relative maximum grip strength, respectively. Results for maximum grip strength and maximum grip strength/ BW are depicted in figure 4. P-values and degrees of freedom (DFn, DFd) are depicted in table 5.

Max grip strength change

Compared to baseline (3 weeks of age), all mice significantly increased their maximum grip strength following PIA and 7D recovery. In terms of group differences after PIA, SMC and HU mice displayed a significantly ($p<0.05$) lower max grip strength change compared to control mice and there were no differences in the changes between SMC and HU mice. There were slight differences in the increase in max grip strength during the recovery period from weeks 5 to 6 between groups; however, those differences were not significant. From 3 weeks to 6 weeks of age, all mice significantly improved their max grip strength regardless of group

Max grip strength/body weight (BW) change

There were no significant differences in change in max grip strength relative to body weight between groups from weeks 3 to 5 (PIA period), 5 to 6 (recovery period or from weeks 3 to 6 (total intervention) suggesting grip strength is proportional to body size.

Body Composition

Lean mass, fat mass and total mass at baseline between control, SMC, and HU mice were non-significant. Lean mass and fat mass changes are depicted in figure 5. P-values and degrees of freedom (DFn, DFd) are depicted in table 6.

Lean mass change

Comparing the change in lean mass from baseline (3 weeks) to after PIA (5 weeks), all groups increased lean mass and there were significant differences across all three experimental groups. The increase in lean mass in control mice was significantly higher than SMC ($P<0.001$) and HU ($P<0.0001$) mice . SMC mice had significantly higher ($P<0.05$) lean mass change than HU mice.

Between week 5 and week 6 (the recovery week), there were significant increases in lean mass across all groups. Only HU mice demonstrated a greater increase in lean mass compared to control mice during this recovery week. Although not a given, this significant increase in lean mass during recovery may be due to the fact that PIA restricted growth and had the most to gain to catch up to the control mice during 7D recovery. Between week 3 and week 6, all mice showed a significant increase in lean mass with no differences between groups.

Fat mass change

All groups increased fat mass from baseline to after PIA, and there were significant differences between experimental groups. Control and SMC mice had significantly higher ($P<0.0001$) fat mass than HU mice. Between week 5 and week 6, the recovery week only the HU mice increased fat mass and that change in fat mass in HU mice was significantly greater than the change in Control and SMC mice. Similar to lean mass, fat mass followed a similar trend in which the HU mice had a greater amount to gain during 7D recovery. Between week 3 and week 6, all mice showed a significant increase in fat mass with no differences between groups.

Muscle weights

Muscle weights from males (n=43) and females (n=36) were pooled together to assess significance. Absolute muscle weights (mg) and muscle weights relative to body weight (mg/BW) for soleus, plantaris, gastrocnemius, and triceps surae are depicted in figure 6. Tibialis anterior, extensor digitorum longus, tricep, total muscle mass, and liver mass are all depicted in figure 7. P-values and degrees of freedom (DFn, DFd) are depicted in tables 7-14.

Soleus

Soleus muscle weights displayed an interaction and main effects for recovery and level of early life inactivity. Soleus weights in SMC and HU mice were significantly lower than control mice ($p<0.05$) with no significant differences between SMC and HU mice at PIA. Soleus weights taken after recovery (7D Recovery) were significantly greater than following PIA for HU ($p<0.05$) and a trend for SMC ($p=0.051$) mice. There were no significant differences in soleus weights at recovery between control, SMC and HU mice.

When looking at soleus weight relative to body weight (mg muscle /grams BW), there was an interaction and a main effect for recovery. At PIA, the HU mice displayed a trend ($p=0.056$) for smaller normalized solei compared to control. At 7D Recovery, HU mice had significantly higher soleus weights relative to body weight compared to SMC mice ($P<0.05$).

Plantaris

Plantaris (PLA) muscle weights displayed an interaction and main effects for recovery and level of early life inactivity. At PIA, PLA weights in HU mice were significantly lower than control and SMC mice ($p<0.05$). PLA weights taken after recovery (7D Recovery) were significantly greater versus PIA for HU mice ($p<0.05$).

When looking at PLA weight relative to body weight (mg muscle /grams BW), there was an interaction and a main effect for recovery. At PIA, The HU mice displayed smaller PLA weights relative to body weight compared to control and SMC mice ($p<0.05$). At 7D Recovery,

HU mice had significantly higher PLA weights relative to body weight compared to SMC mice ($P<0.05$).

Gastrocnemius (GAS)

Gastrocnemius (GAS) muscle weights displayed main effects for recovery and level of early life inactivity. At PIA, GAS weights in HU mice were significantly lower than control and SMC mice ($p<0.05$). No differences were noted between control and SMC mice. GAS weights taken after recovery (7D Recovery) were significantly greater versus PIA for SMC and HU mice ($p<0.05$).

When looking at GAS weight relative to body weight (mg muscle /grams BW), there were main effects for recovery and level of early life inactivity. At PIA, the HU mice displayed smaller GAS weights relative to body weight compared to control and SMC mice ($p<0.05$). At 7D Recovery, HU mice had significantly higher GAS weights relative to body weight compared to PIA ($P<0.05$).

Triceps surae

Tricep Surae (TS) muscle weights displayed main effects for recovery and level of early life inactivity. At PIA, TS weights in HU mice were significantly lower than control and SMC mice ($p<0.05$). TS weights taken after recovery (7D Recovery) were significantly greater versus PIA for SMC and HU mice ($p<0.05$).

When looking at TS weight relative to body weight (mg muscle /grams BW), there were main effects for recovery and level of early life inactivity. At PIA, the HU mice displayed smaller TS weights relative to body weight compared to control and SMC mice ($p<0.05$). At 7D Recovery, HU mice had significantly higher TS weights relative to body weight compared to PIA ($P<0.05$).

Tibialis anterior/extensor digitorum longus

Tibialis Anterior and Extensor Digitorum Longus (TA/EDL) muscle weights displayed main effects for recovery and level of early life inactivity. At PIA, TA/EDL weights in HU mice were significantly lower than control mice ($p<0.05$). TA/EDL weights taken at recovery (7D Recovery) were significantly greater for control versus HU mice ($p<0.05$) TA/EDL weights

taken after recovery (7D Recovery) were significantly greater versus PIA for SMC and HU mice ($p<0.05$).

When looking at TA/EDL weight relative to body weight (mg muscle /grams BW), there were main effects for recovery and level of early life inactivity. At PIA, the HU mice displayed smaller TA/EDL weights relative to body weight compared to control mice ($p<0.05$). At 7D Recovery, HU mice and SMC mice had significantly higher TA/EDL weights relative to body weight compared to PIA ($P<0.05$).

Tricep

Tricep (TRI) muscle weights displayed main effects for recovery and level of early life inactivity. TRI weights taken after recovery (7D Recovery) were significantly greater versus PIA for SMC mice ($p<0.05$).

When looking at TRI weight relative to body weight (mg muscle /grams BW), there was an interaction, but no other specific effects.

Total muscle

Total muscles pooled (TMP) weights displayed main effects for recovery and level of early life inactivity. At PIA and at 7D Recovery, TMP weights in HU mice were significantly lower than control mice ($p<0.05$). TMP weights taken after recovery (7D Recovery) were significantly greater versus PIA for SMC and HU mice ($p<0.05$).

When looking at TMP weight relative to body weight (mg muscle / grams BW) there were main effects for recovery and level of early life inactivity. At 7D Recovery, the HU mice displayed smaller TMP weights relative to body weight compared to control mice ($p<0.05$). At 7D Recovery, control mice had significantly higher TMP weights relative to body weight compared to PIA ($P<0.05$).

Liver

Liver weights displayed main effects for recovery and level of early life inactivity. At PIA, liver weights in HU mice were significantly lower than control and SMC mice ($p<0.05$). Liver weights taken after recovery (7D Recovery) were significantly greater versus PIA for SMC and HU mice ($p<0.05$).

When looking at liver weight relative to body weight (mg tissue /grams BW) there was a main effect level of early life inactivity with a trend ($p=0.059$) for a main effect of recovery. Following PIA, liver weights differed slightly with SMC mice having a higher liver weight relative to body weight compared to control and HU mice. However, there were no statistically significant differences noted. After 7D recovery, SMC still had a higher liver weight relative to body weight compared to control and HU. These results were also non-significant.

Figure 3: body weight, growth rates (body weight change from day 0), average change body weight change per day for 14 day PIA and 7D recovery. *, ^, or # indicates significant ($p<0.05$) differences of means between groups (Control, SMC or HU). # HU vs. CON and SMC; ^ SMC vs. CON; % HU vs. CON; * SMC vs HU. SMC=small mouse cage and HU= hindlimb unloading. Data are Means ± SEM**.**

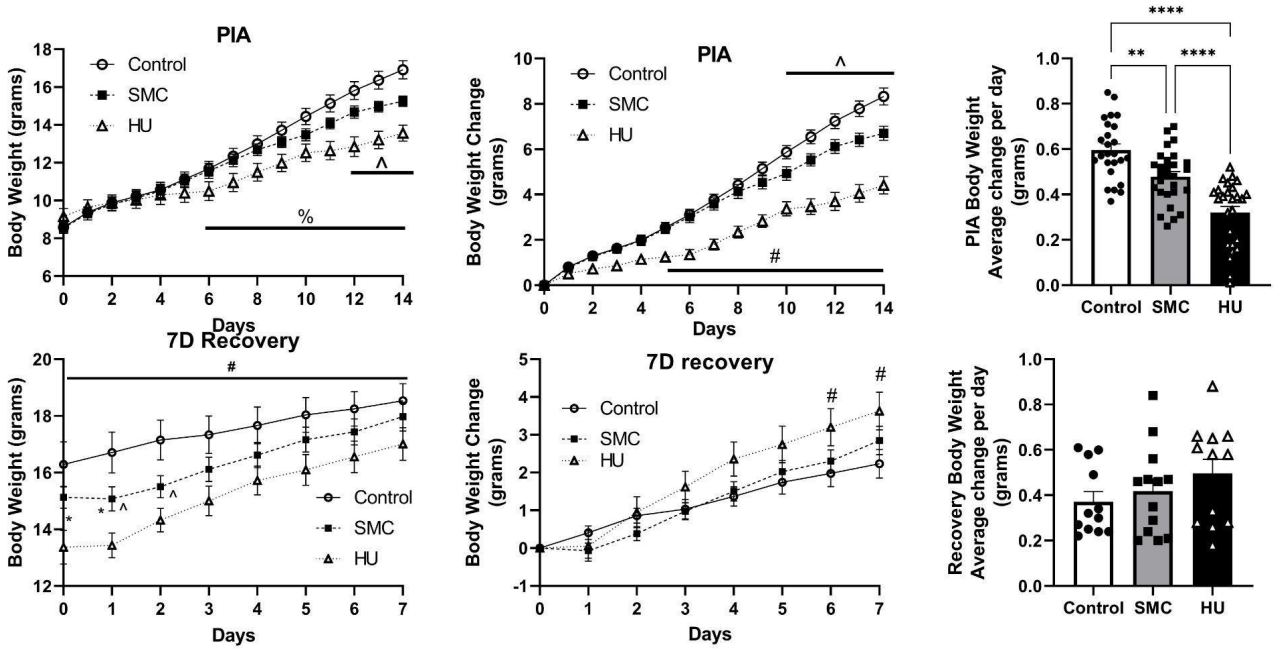


Table 2: This table displays the degrees of freedom and P-values for body weight during PIA and 7D recovery.

Body weights: PIA			Body weights: 7D recovery		
ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value
Time x Experiment	F (28, 994) = 16.33	P<0.0001	Time x Experiment	F (42, 609) = 6.429	P<0.0001
Time	F (14, 994) = 461.9	P<0.0001	Time	F (21, 609) = 324.8	P<0.0001
Experiment	F (2, 71) = 6.626	P=0.0023	Experiment	F (2, 29) = 5.675	P=0.0083
Mouse	F (71, 994) = 63.00	P<0.0001	Mouse	F (29, 609) = 47.66	P<0.0001

Table 3: This table displays the degrees of freedom and P-values for body weight change from day zero (growth rates) during PIA and 7D recovery.

Growth rate: PIA			Growth rate: 7D recovery		
ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value
Time x Experiment	F (28, 994) = 16.29	P<0.0001	Time x Experiment	F (42, 609) = 6.436	P<0.0001
Time	F (14, 994) = 462.1	P<0.0001	Time	F (21, 609) = 324.8	P<0.0001
Experiment	F (2, 71) = 31.76	P<0.0001	Experiment	F (2, 29) = 13.87	P<0.0001
Mouse	F (71, 994) = 19.34	P<0.0001	Mouse	F (29, 609) = 25.27	P<0.0001

Table 4: This table displays the degrees of freedom and P-values for body weight change per day during PIA and 7D recovery.

Average change per day: PIA	Average change per day: 7D

			recovery		
ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value
Treatment	F (2, 39) = 17.46	P<0.0001	Treatment	F (2, 60) = 0.9739	P=0.3835

Figure 4: Max grip strength and max grip strength/BW at 3 weeks of age (baseline). Change in max grip strength and max grip strength/BW depicted between 3 weeks and 5 weeks, 5 weeks and 6 weeks, and 3 weeks and 6 weeks of age. * ($p<0.05$) and *** $P<0.01$ indicates significant differences of means between groups (Control, SMC or Recovery). SMC=small mouse cage and HU= hindlimb unloading. Data are Means +- SEM.

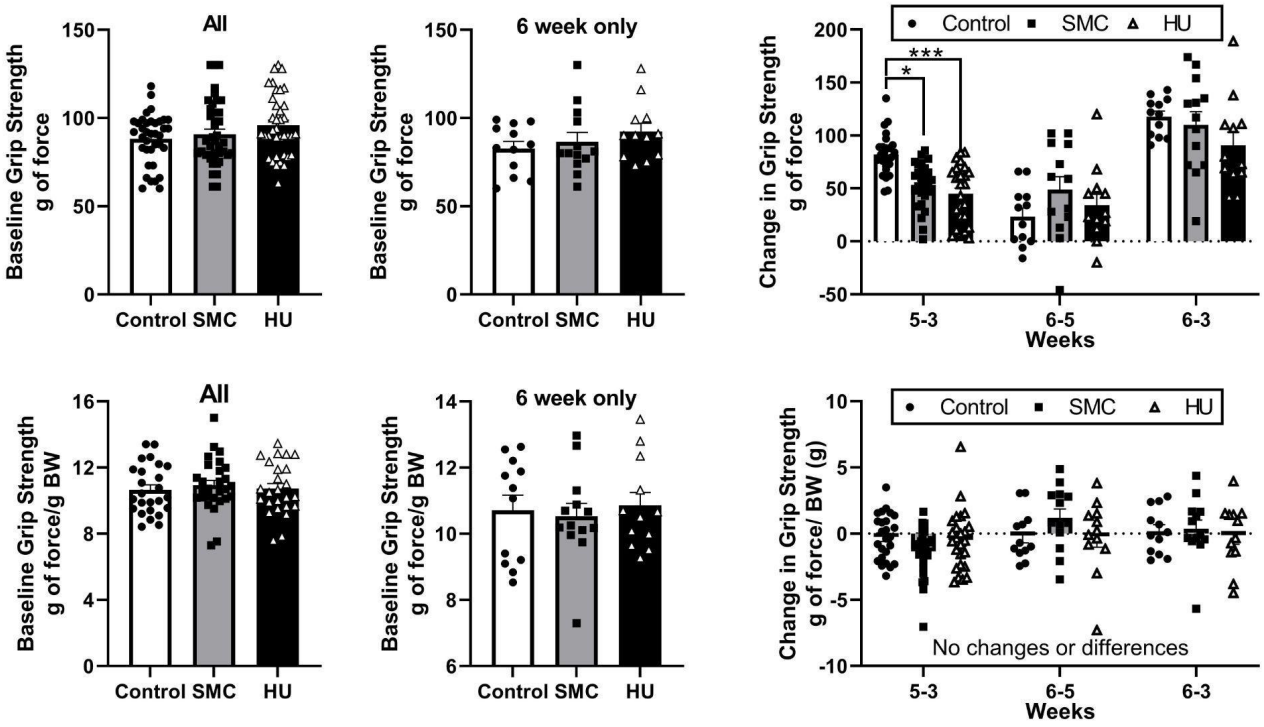


Table 5: This table displays the degrees of freedom and P-values for maximum grip strength and maximum grip strength relative to body weight during PIA and 7D recovery.

Maximum grip strength	F (DFn, DFd)	P value	Maximum grip strength/body weight	F (DFn, DFd)	P value
Model one	F (2, 75) = 18.40	P<0.0001	Model one	F (2, 39) = 1.168	P=0.3216
Model two	F (2, 75) = 1.107	P=0.3357	Model two	F (2, 70) = 0.1369	P=0.8723
Model three	F (2, 34) = 1.471	P=0.2440	Model three	F (2, 28) = 1.304	P=0.2875
Model four	F (2, 34) = 1.025	P=0.3696	Model four	F (2, 28) = 0.4388	P=0.6492
Model five	F (2, 34) = 1.654	P=0.2064	Model five	F (2, 28) = 0.4161	P=0.6636

Figure 5: Lean and fat mass at 3 weeks of age (baseline), lean and fat mass change between 3 and 5 weeks, 5 and 6 weeks, and 3 and 6 weeks. *P<0.05, ***P<0.001, ****P<0.0001 indicates significant differences of means between groups (Control, SMC or Recovery). SMC=small mouse cage and HU= hindlimb unloading. Data are Means +- SEM.

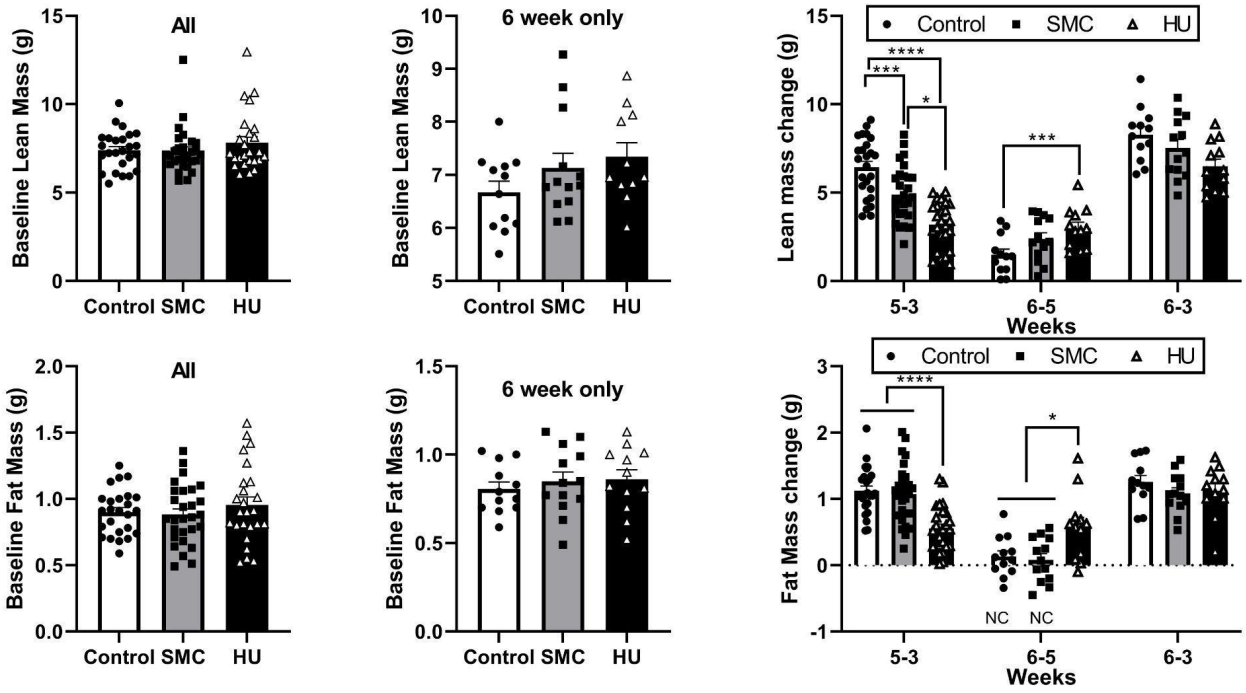


Table 6: This table displays the degrees of freedom and P-values for lean mass and fat mass during PIA and 7D recovery.

Lean mass	F (DFn, DFd)	P value	Fat mass	F (DFn, DFd)	P value
Model one	F (2, 75) = 18.56	P<0.0001	Model one	F (2, 75) = 17.41	P<0.0001
Model two	F (2, 75) = 0.1786	P=0.8368	Model two	F (2, 75) = 0.6779	P=0.5108
Model three	F (2, 34) = 5.016	P=0.0123	Model three	F (2, 34) = 5.931	P=0.0062
Model four	F (2, 34) = 0.3785	P=0.6877	Model four	F (2, 34) = 0.3477	P=0.7088
Model five	F (2, 34) = 1.195	P=0.3151	Model five	F (2, 34) = 0.8452	P=0.4383

Figure 6: Muscle weights and muscle weights/BW collected at 5 weeks (PIA) and 6 weeks (7D recovery). Differing letters within each time period (PIA or 7D Recovery) indicate significant ($p<0.05$) differences of means between groups (Control, SMC or Recovery). # indicates a significant difference ($p<0.05$) of means between PIA and 7D recovery for that group. Data are Mean ± SEM.

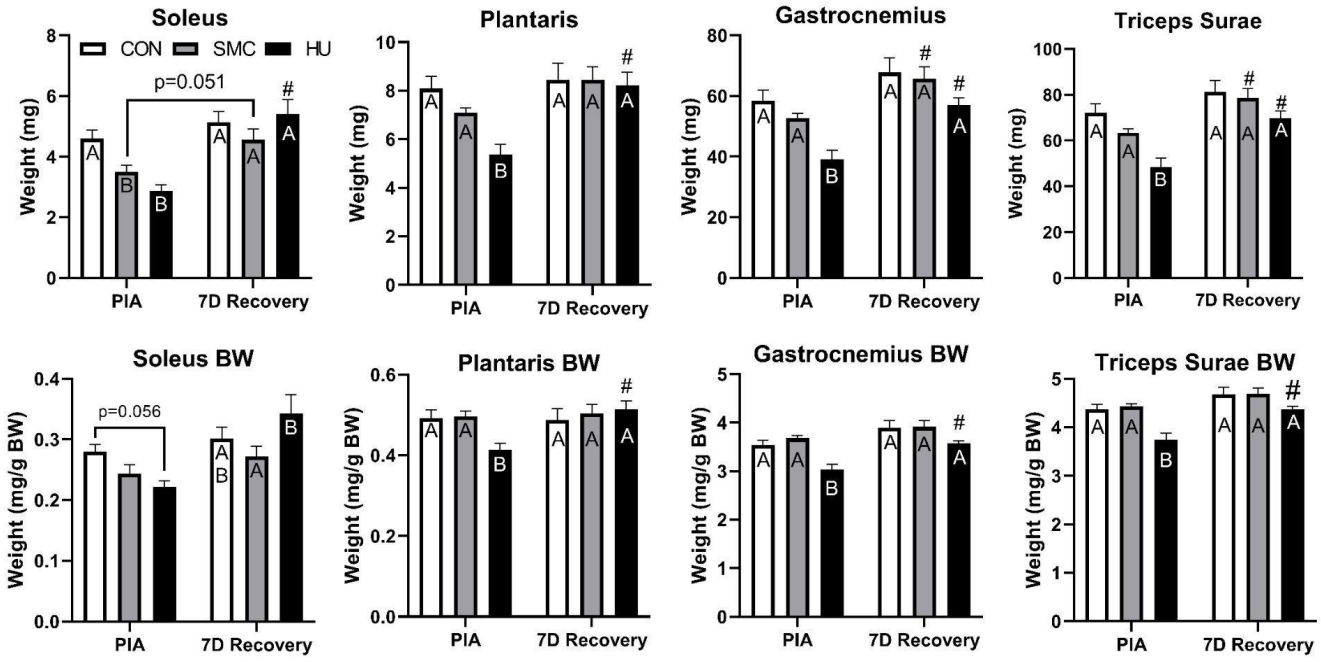


Table 7: This table displays the degrees of freedom and P-values for soleus and soleus relative to body weight

Soleus	F (DFn, DFd)	P value	Soleus/ BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 5.30	P=0.0071	Interaction	F (2, 73) = 4.89	P=0.0101
Recovery	F (1, 73) = 28.5	P<0.0001	Recovery	F (1, 73) = 15.3	P=0.0002
Early Life PIA	F (2, 73) = 4.11	P=0.0203	Early Life PIA	F (2, 73) = 1.93	P=0.1527

Table 8: This table displays the degrees of freedom and P-values for plantaris and plantaris relative to body weight.

Plantaris	F (DFn, DFd)	P value	Plantaris/ BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 3.25	P=0.0443	Interaction	F (2, 73) = 4.07	P=0.0212
Recovery	F (1, 73) = 15.0	P=0.0002	Recovery	F (1, 73) = 4.27	P=0.0424
Early Life PIA	F (2, 73) = 4.65	P=0.0126	Early Life PIA	F (2, 73) = 1.68	P=0.1942

Table 9: This table displays the degrees of freedom and P-values for gastrocnemius and gastrocnemius relative to body weight.

GAS	F (DFn, DFd)	P value	GAS/BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 0.833	P=0.4390	Interaction	F (2, 73) = 1.19	P=0.3110
Recovery	F (1, 73) = 25.2	P<0.0001	Recovery	F (1, 73) = 20.4	P<0.0001
Early Life PIA	F (2, 73) = 11.3	P<0.0001	Early Life PIA	F (2, 73) = 13.5	P<0.0001

Table 10: This table displays the degrees of freedom and P-values for TS and TS relative to body weight.

TS	F (DFn, DFd)	P value	TS/BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 1.29	P=0.2818	Interaction	F (2, 73) = 1.64	P=0.2005
Recovery	F (1, 73) = 25.1	P<0.0001	Recovery	F (1, 73) = 20.1	P<0.0001
Early Life PIA	F (2, 73) = 11.3	P<0.0001	Early Life PIA	F (2, 73) = 13.0	P<0.0001

Figure 7: Muscle weights, muscle weights/BW and liver weights collected at 5 weeks (PIA) and 6 weeks (7D recovery). Differing letters within each time period (PIA or 7D Recovery) indicate significant ($p<0.05$) differences of means between groups (Control, SMC or Recovery). # indicates a significant difference ($p<0.05$) of means between PIA and 7D recovery for that group. Data are Mean ± SEM.

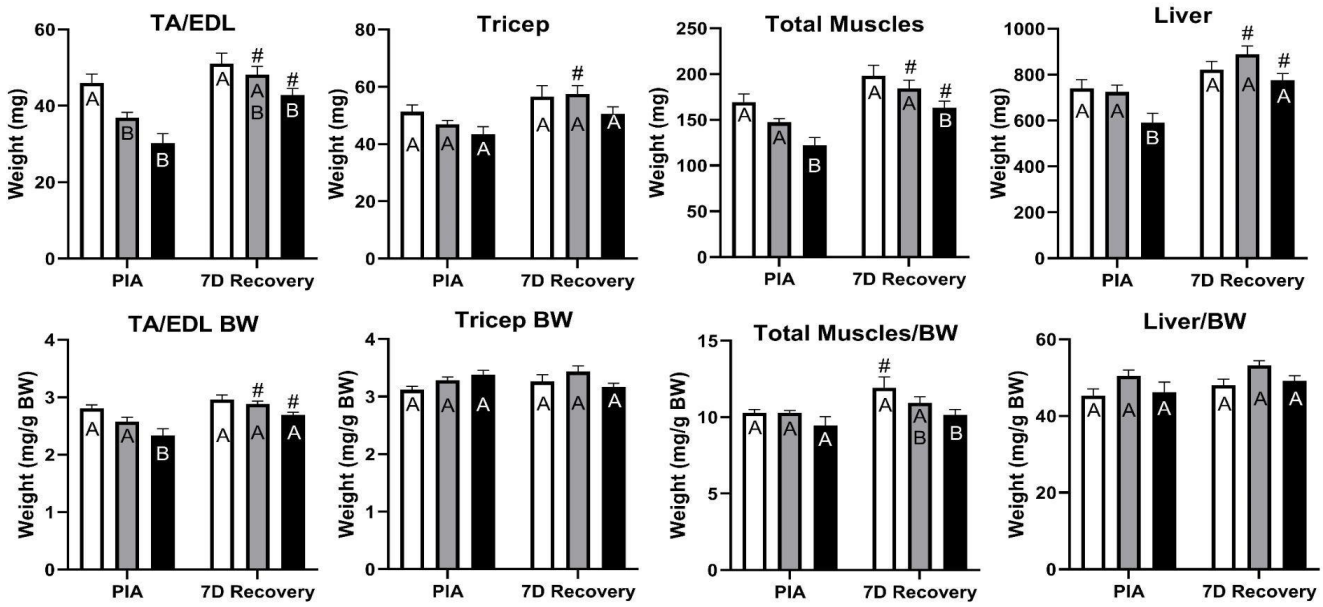


Table 11: This table displays the degrees of freedom and P-values for TA/EDL and TA/EDL relative to body weight.

TAEDL	F (DFn, DFd)	P value	TAEDL/BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 1.75	P=0.1816	Interaction	F (2, 73) = 0.838	P=0.4368
Recovery	F (1, 73) = 30.1	P<0.0001	Recovery	F (1, 73) = 17.8	P<0.0001
Early Life PIA	F (2, 73) = 15.1	P<0.0001	Early Life PIA	F (2, 73) = 10.5	P=0.0001

Table 12: This table displays the degrees of freedom and P-values for tricep and tricep relative to body weight.

Tricep	F (DFn, DFd)	P value	TRI/BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 0.521	P=0.5962	Interaction	F (2, 73) = 3.36	P=0.0403
Recovery	F (1, 73) = 12.7	P=0.0007	Recovery	F (1, 73) = 0.189	P=0.6654
Early Life PIA	F (2, 73) = 3.70	P=0.0294	Early Life PIA	F (2, 73) = 2.30	P=0.1070

Table 13: This table displays the degrees of freedom and P-values for total muscle mass and total muscle mass relative to body weight.

Total muscle mass	F (DFn, DFd)	P value	Total muscle/ BW	F (DFn, DFd)	P value
Interaction	F (2, 71) = 0.277	P=0.7590	Interaction	F (2, 73) = 0.819	P=0.4448
Recovery	F (1, 71) = 28.1	P<0.0001	Recovery	F (1, 73) = 7.82	P=0.0066
Early Life PIA	F (2, 71) = 12.1	P<0.0001	Early Life PIA	F (2, 73) = 4.51	P=0.0142

Table 14: This table displays the degrees of freedom and P-values for liver and liver relative to body weight.

Liver	F (DFn, DFd)	P value	Liver/BW	F (DFn, DFd)	P value
Interaction	F (2, 73) = 1.19	P=0.3103	Interaction	F (2, 73) = 0.00188	P=0.9981
Recovery	F (1, 73) = 24.2	P<0.0001	Recovery	F (1, 73) = 3.68	P=0.0589
Early Life PIA	F (2, 73) = 6.70	P=0.0021	Early Life PIA	F (2, 73) = 4.95	P=0.0096

Discussion

In this study, the main objectives were to assess deficits in grip strength, body composition and muscle weights after 2 weeks of differing levels of physical inactivity (between 3 and 5 weeks of age) and to assess how well mice can restore those deficits from PIA after 7 days of recovery (between weeks 5 and 6 of age). The differing levels of physical inactivity were an extreme form of inactivity as hindlimb unloading and a more moderate form of inactivity with a small mouse cage. These were compared to cage control mice.

When subjecting mice to 2 weeks of different levels of physical inactivity, we found that body weight, maximum grip strength, lean mass, fat mass, and muscle weights were all significantly attenuated in HU and SMC mice immediately after PIA. However, when the mice were allowed to recover (7D recovery), the mice were capable of catching up to their control counterparts in terms of grip strength, lean mass and fat mass as well as muscle weights. The only exception was found in HU mice in which they were unable to fully recover normal body weight and total muscle mass following 7D recovery.

Following 2 weeks of inactivity, we saw that normal body weight gain was attenuated in both HU and SMC mice. However, the reduction in growth in HU mice occurred much sooner and was much more drastic compared to SMC mice during PIA. It is still to be determined whether the decrease in SMC mice was due to activity restriction from the SMC itself or a reduction in cage space due to uneaten food that falls into the bedding. Varying amounts of uneaten food in the SMC may influence the level of activity restriction each SMC mouse experiences within the SMC. For instance, a mouse that left more uneaten food in the SMC would lead to a reduction in cage volume compared to a mouse that did not leave as much uneaten food. The changes in body weight fit a similar trend along with the other outcomes measured in this study. This is likely a manifestation of the SMC mice remaining loaded throughout the entire inactivity period and their ability to burrow into the paper/pellet bedding. Nonetheless, their ability to be active was still restricted compared to mice in a standard mouse cage. This is clearly reflected with the attenuation of body weight gain. Additionally, we found that both growth rate and the average change in body weight held a similar trend to control mice. Control mice showed the highest growth rate and average body weight change per day followed by SMC mice and then HU mice having the greatest attenuation of body weight gain of all three

experimental groups. These findings would also support the idea mentioned in the introduction that small mouse cage and hindlimb unloading are different dosages of physical inactivity. After one week of recovery, we observed that all mice continued to grow significantly during this period. Although, the degree of growth varied between control, SMC, and HU mice. Even after the week of recovery, the HU mice were still unable to recover what was lost from inactivity. On the other hand, SMC mice regained the deficits in weight by the end of recovery such that there were no differences in body weight compared to control mice. During this week of recovery, we observed that SMC and HU mice experience a rapid rebound in growth following PIA. This is supported by HU mice having the highest growth rate in the first two days of recovery, and in terms of average body weight change per day, HU mice gained weight much faster than control and SMC mice due to a significant growth restriction during PIA. We observe this rebound potentially due to the HU mice having the most body mass to gain during 7D recovery; although, it was uncertain whether this outcome would occur. For the SMC mice, this is a positive finding that the lower dose form of physical inactivity did not lead to deficits in growth/weight gain. While it is important to recognize that mice are not humans, this positive finding in mice may be true for humans when activity is reduced for a prolonged period of time. However, human studies such as step reduction studies are necessary to support the findings in mice.

We observed that both SMC and HU mice had a markedly lower max grip strength compared to control mice following PIA with no differences between SMC and HU mice. Based on this outcome, it appears to support the idea that a period of PIA has a significant impact on maximum grip strength. Decrements in maximum grip strength following cage reduction and hindlimb unloading were also observed in Roemers et al. (2019) and Chacon-Cabrera & Barreiro (2017). However, mice, during one week of recovery, were able to gain additional strength such that there were no longer major differences in max grip strength by the end of recovery compared to controls. This would be another positive finding that the young mice are capable of catching up to their control counterparts with no significant deficits in maximum grip strength. Max grip strength relative to body weight was not significant following PIA or recovery. Potentially, the deficits seen in absolute max grip strength after PIA may not be as serious as they initially lead on to be or just an indication of how tightly coupled grip strength is to body size.

Looking at lean mass change as a result of PIA, it is quite clear that control, SMC, and HU mice were affected to varying degrees with control mice having the highest lean mass

change. SMC mice were significantly lower in lean mass than control mice, and HU mice were significantly lower than SMC mice. Similar findings described in Reidy et al. (2021) support the outcomes of this study regarding lean mass and fat mass. Another study saw the same response to lean mass change after 8 days of PIA with a significant drop in lean mass from pre to post PIA (Mahmassani et al., 2020). This outcome would support the idea that the ability to gain lean mass depends on loading of extremities and level of activity at least to a degree.

During recovery, HU mice saw the greatest regrowth of lean mass. This finding is not all that surprising given that HU mice during recovery also experienced the greatest amount of average weight body weight change per day and the greatest loss during PIA. Similar to what was seen in body weight changes, HU mice had the most to regain in lean mass. At this time, the HU mice experienced significant growth in lean mass to catch up to their control counterparts. By the end of recovery, no major differences in lean mass were found, so it appears that both SMC and HU mice were able to add lean mass without permanent deficits due to PIA.

Fat mass following PIA was not severely impacted in SMC mice compared to control mice. Surprisingly, HU mice experienced a much lower fat mass change after PIA compared to SMC and control mice. In Wall et al. (2013), they explain how during disuse not only is there a loss of skeletal muscle but there's also a reduction in basal metabolic rate leading to the accumulation of additional fat as a result of disuse. I thought a similar result in mice would occur as a result of physical inactivity. Due to differences between mice and humans, the metabolic rate in mice may not have changed significantly enough to cause the accrual of fat (Mahmassani et al. 2020). We also know that younger mice have higher energy expenditure than older mice, so an increase in fat mass due to PIA could be seen in older mice (Azzu & Valencak 2017). However, that was not the case in this study. Thus, PIA acts by restricting growth in all facets of body composition measures and strength. Throughout the week of recovery, HU mice regained a significant amount of fat mass compared to SMC and control mice. Similar to the results seen with lean mass and body weight, the HU mice are rapidly growing due to re-loading, heightened levels of activity, and a reduction in stress. By the end of recovery, there were no deficiencies in fat mass, comparing all experimental groups.

Interestingly, all muscles including total muscle weight and liver weight showed significant main effects for recovery and level of physical inactivity. Following PIA, both HU and SMC mice had significantly lower absolute muscle weights of most muscles and liver weight

compared to control mice. A plethora of literature would support the muscle atrophy seen in HU, but this is the first to show muscle loss with the small mouse cage. This suggests that even the loading activity of burrowing is not sufficient to maintain some muscle mass. We did not observe statistical differences between HU and SMC mice, although a visual pattern was clear for several muscles for HU to have more atrophy than SMC. Interestingly, the hindlimb muscles were most affected by both levels of PIA and not the triceps forelimb muscle we collected. One thought that may explain this phenomenon is the fact that both SMC and HU mice are still capable of loading their triceps throughout the inactivity period. SMC mice may use their forelimbs to burrow and HU mice use their forelimb to navigate to their water and food. In fact, Roemers et al. (2018) explained how burrowing improved forelimb grip strength in mice by having the mice dig out different materials out of a tube.

Unlike PIA, following recovery, there were no major differences in absolute muscle weights between all experimental groups for the plantar flexors, the triceps forelimb muscle and liver weights. However, total muscle mass and hindlimb dorsiflexors were significantly reduced in HU mice compared to control mice at the end of recovery. This may be a result that the dorsi-flexors were in a shortened position during HU and muscles placed in shortened position during disuse experience more atrophy (Jokl & Konstadt, 1983). Thus, HU mice were unable to regain the muscle mass lost during 2 weeks of PIA despite 1 week of recovery.

The absolute weights of the gastrocnemius, triceps surae, tibialis anterior/extensor digitorum longus and liver collected after recovery were much higher than those after PIA for both HU and SMC mice. This shows that these muscles experienced regrowth in both levels of physical inactivity. However, only HU mice demonstrated regrowth with both the soleus and plantaris. This may be a reflection of the robust decrease in growth of these muscles during PIA. We could hypothesize that these increased muscle weights during the recovery period may be inflated by muscle damage-induced edema and swelling (Aihara et al. 2017). Although not significant, the absolute soleus weights in SMC mice displayed a trend to recover, suggesting increased use of the soleus with return to the normal cage.

Ultimately, there seems to be a level of resilience in the skeletal muscle of very young mice to rebound after a short period of physical inactivity, and this period of inactivity may not lead to changes in normal skeletal muscle turnover and regeneration at least in the short-term. Despite a known decline in satellite cells following HU, muscle progenitors play a key role in

muscle regeneration process during reloading and may partially explain additional muscle mass added during 7D recovery (Guitart et al. 2018; Schultz et al. 1994). Alternatively, muscle tissue swelling and edema may partially explain the gains in body mass post-PIA. Musacchia et al. (1990) describes how it took 7 days of reloading to restore wet muscle mass after 7 days of HU in rats. However the increase in dry muscle mass is likely small (Litvinova et al. 2007; Kachaeva et al. 2010) Those findings suggest that the increase in muscle mass may be attributed to edema and not necessarily an accumulation of muscle protein mass. They warrants further investigation.

There were a few limitations that should be pointed out in this study. HU mice would sometimes be temporarily reloaded when they climbed onto their water bottles and feeders. Cage adjustments were constantly made to mitigate the incidence of this, but provide a level of variability. As alluded to before, the SMC mice were still capable of burrowing into the bedding of the small mouse cage leading to additional activity and loading of extremities.

Future studies should adjust the duration of inactivity and recovery period to parallel human early life inactivity as close as possible. In Sayer (2006), they alluded to repeated bouts of inactivity that were problematic for the elderly relating to the incidence of sarcopenia and risk of falls. Future studies could choose to focus on short repeated bouts of inactivity throughout early life to better understand the long-term consequences of inactivity on skeletal muscle health and risk for chronic disease later in life. Additional analysis of the skeletal muscle could also investigate muscular edema, myofiber size, infiltrating immune cells or the extracellular matrix within skeletal muscle to provide further characterization of the immediate effects of early life inactivity and recovery. .

Conclusion

Immediately after PIA, grip strength, tissue composition, body weight and muscle weights in SMC and HU mice were significantly impacted due to inactivity to varying degrees. By the end of recovery, SMC mice were largely able to recover the deficits from PIA; while, HU mice were unable to recover overall body weight and total muscle mass despite 7 days of recovery. These findings ultimately support the original hypothesis that both SMC and HU would be affected by PIA, but HU mice were affected to a greater degree compared to SMC

mice and SMC mice were capable of rebounding better in comparison to HU mice after 7D recovery.

References

Aihara, M., Hirose, N., Katsuta, W., Saito, F., Maruyama, H., & Hagiwara, H. (2017). A new model of skeletal muscle atrophy induced by immobilization using a hook-and-loop fastener in mice. Journal of physical therapy science, 29(10), 1779–1783.

Andrianjafiniony T., Dupre-Aucouturier S., Letexier D., Couchoux H., Desplanches D. Oxidative stress, apoptosis, and proteolysis in skeletal muscle repair after unloading. *Am. J. Physiol. Cell Physiol.* 2010;299:C307–C315. doi: 10.1152/ajpcell.00069.2010.

Asmussen, G., Miersch, H., & Soukup, T. (1989). The influence of suspension hypokinesia on contractile properties of slow and fast twitch muscles of young growing and adult rats. *Biomedica Biochimica Acta*, *48*(5–6), S426-431.

Assets.publishing.service.gov.uk. (2021). Retrieved 6 October 2021, from https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/829882/1-physical-activity-for-early-years-birth-to-5.pdf.

Azzu, V., & Valencak, T. G. (2017). Energy Metabolism and Aging in the Mouse: A Mini-Review. Gerontology, 63(4), 327–336.

Blume, S. W., & Curtis, J. R. (2011). Medical costs of osteoporosis in the elderly Medicare population. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*, *22*(6), 1835–1844.

Burns, E. R., Stevens, J. A., & Lee, R. (2016). The direct costs of fatal and non-fatal falls among older adults - United States. *Journal of safety research*, *58*, 99–103.

Cameron-Smith, PhD, Impaired Ribosome Biogenesis and Skeletal Muscle Growth in a Murine Model of Inflammatory Bowel Disease, *Inflammatory Bowel Diseases*, Volume 22, Issue 2, 1 February 2016, Pages 268–278

Castro, B., and Kuang, S. 2017. Evaluation of Muscle Performance in Mice by Treadmill Exhaustion Test and Whole-limb Grip Strength Assay. Bio Protoc 7(8): e2237. doi:10.21769/BioProtoc.2237.

Centers for Disease Control and Prevention (2005). Web-based injury statistics query and reporting system (WISQARS). *National Center for Injury Prevention and Control, Centers for Disease Control and Prevention*.

Chacon-Cabrera, A., Gea, J., & Barreiro, E. (2017). Short- and Long-Term Hindlimb Immobilization and Reloading: Profile of Epigenetic Events in Gastrocnemius. *Journal of cellular physiology*, *232*(6), 1415–1427. https://doi.org/10.1002/jcp.25635

Cooper, C., Eriksson, J. G., Forsén, T., Osmond, C., Tuomilehto, J., & Barker, D. J. (2001). Maternal height, childhood growth and risk of hip fracture in later life: a longitudinal study. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*, *12*(8), 623–629.

Delhaas T, Van der Meer SF, Schaart G, Degens H, Drost MR. Steep increase in myonuclear domain size during infancy. *Anatomical record (Hoboken, N.J. : 2007)*. 2013;296(2):192-7.

Dodds, R. M., Roberts, H. C., Cooper, C., & Sayer, A. A. (2015). The Epidemiology of Sarcopenia. *Journal of Clinical Densitometry: The Official Journal of the International Society for Clinical Densitometry*, *18*(4), 461–466. https://doi.org/10.1016/j.jocd.2015.04.012

Domingues-Faria C, Vasson MP, Goncalves-Mendes N, Boirie Y, Walrand S. Skeletal muscle regeneration and impact of aging and nutrition. Ageing Res Rev. 2016 Mar;26:22-36.

Dutta, S., & Sengupta, P. (2016). Men and mice: Relating their ages. *Life sciences*, *152*, 244–248.

Elder, G. C., & McComas, A. J. (1987). Development of rat muscle during short- and long-term hindlimb suspension. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *62*(5), 1917–1923.

Gale CR, Martyn CN, Kellingray S, Eastell R, Cooper C. Intrauterine programming of adult body composition. *J Clin Endocrinol Metab.*2001;86:267-272.

Gluckman P, Bier D, Challis J, et al. Report on the 2nd World Congress on Fetal Origins of Adult Disease, Brighton, U.K. June 7-10, 2003. *Pediatr Res.*2004;55:1-4.

Gomes, M. J., Martinez, P. F., Pagan, L. U., Damatto, R. L., Cezar, M., Lima, A., Okoshi, K., & Okoshi, M. P. (2017). Skeletal muscle aging: influence of oxidative stress and physical exercise. *Oncotarget*, *8*(12), 20428–20440.

Guitart, M., Lloreta, J., Mañas-Garcia, L., & Barreiro, E. (2018). Muscle regeneration potential and satellite cell activation profile during recovery following hindlimb immobilization in mice. *Journal of cellular physiology*, *233*(5), 4360–4372.

Heidenreich, P. A., Trogdon, J. G., Khavjou, O. A., Butler, J., Dracup, K., Ezekowitz, M. D., Finkelstein, E. A., Hong, Y., Johnston, S. C., Khera, A., Lloyd-Jones, D. M., Nelson, S. A., Nichol, G., Orenstein, D., Wilson, P. W., Woo, Y. J., American Heart Association Advocacy Coordinating Committee, Stroke Council, Council on Cardiovascular Radiology and Intervention, Council on Clinical Cardiology, … Council on Cardiovascular Surgery and Anesthesia, and Interdisciplinary Council on Quality of Care and Outcomes Research (2011). Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation*, *123*(8), 933–944.

Hernandez-Torres, F., Rodríguez-Outeiriño, L., Franco, D., & Aranega, A. E. (2017). Pitx2 in Embryonic and Adult Myogenesis. *Frontiers in Cell and Developmental Biology*, *5*, 46.

Hsia, A. W., Jbeily, E. H., Mendez, M. E., Cunningham, H. C., Biris, K. K., Bang, H., Lee, C. A., Loots, G. G., & Christiansen, B. A. (2021). Post-traumatic osteoarthritis progression is

diminished by early mechanical unloading and anti-inflammatory treatment in mice. *Osteoarthritis and cartilage*, S1063-4584(21)00932-8. Advance online publication.

Janssen I, Shepard DS, Katzmarzyk PT, Roubenoff R. The healthcare costs of sarcopenia in the United States. *J Am Geriatr Soc.*2004;52:80-85.

Jokl, P., & Konstadt, S. (1983). The effect of limb immobilization on muscle function and protein composition. *Clinical orthopaedics and related research*, (174), 222–229.

Kachaeva E.V., Turtikova O.V., Leinsoo T.A., Shenkman B.S. Insulin-like growth factor 1 and the key markers of proteolysis during the acute period of readaptation of the muscle atrophied as a result of unloading. *Biofizika.* 2010;55:1108–1116.

Kuh D, Bassey J, Hardy R, Aihie Sayer A, Wadsworth M, Cooper C. Birth weight, childhood size, and muscle strength in adult life: evidence from a birth cohort study. *Am J Epidemiol.*2002;156:627-633.

Litvinova K.S., Tarakin P.P., Fokina N.M., Istomina V.E., Larina I.M., Shenkman B.S. Reloading of rat soleus after hindlimb unloading and serum insulin-like growth factor 1. *Rossiiskii fiziologicheskii zhurnal imeni IM Sechenova.* 2007;93:1143–1155.

Mahmassani, Z. S., Reidy, P. T., McKenzie, A. I., Petrocelli, J. J., Matthews, O., de Hart, N. M., Ferrara, P. J., O'Connell, R., Funai, K., & Drummond, M. J. (2020). Absence of MyD88 from skeletal muscle protects female mice from inactivity-induced adiposity and insulin resistance. *Obesity (Silver Spring, Md.)*, *28*(4), 772–782.

Marmonti, E., Busquets, S., Toledo, M., Ricci, M., Beltrà, M., Gudiño, V., Oliva, F., López-Pedrosa, J. M., Manzano, M., Rueda, R., López-Soriano, F. J., & Argilés, J. M. (2017). A Rat Immobilization Model Based on Cage Volume Reduction: A Physiological Model for Bed Rest?. *Frontiers in physiology*, *8*, 184.

Mozdziak, P. E., Pulvermacher, P. M., & Schultz, E. (2001). Muscle regeneration during HU results in a reduction in muscle size after reloading. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *91*(1), 183–190.

Musacchia X.J., Steffen J.M., Fell R.D., Dombrowski M.J. Skeletal muscle response to spaceflight, whole body suspension, and recovery in rats. *J. Appl. Physiol.* 1990;69:2248–2253. doi: 10.1152/jappl.1990.69.6.2248.

Patel, H. P., Jameson, K. A., Syddall, H. E., Martin, H. J., Stewart, C. E., Cooper, C., & Sayer, A. A. (2012). Developmental influences, muscle morphology, and sarcopenia in community-dwelling older men. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *67*(1), 82–87.

Perkin, O., McGuigan, P., Thompson, D., & Stokes, K. (2016). A reduced activity model: A relevant tool for the study of ageing muscle. *Biogerontology*, *17*(3), 435–447.

Pitts GC. Cellular aspects of growth and catch-up growth in the rat: a reevaluation. *Growth*. 1986;50(4):419-36.

Reidy, P. T., McKenzie, A. I., Mahmassani, Z. S., Petrocelli, J. J., Nelson, D. B., Lindsay, C. C., Gardner, J. E., Morrow, V. R., Keefe, A. C., Huffaker, T. B., Stoddard, G. J., Kardon, G., O'Connell, R. M., & Drummond, M. J. (2019). Aging impairs mouse skeletal muscle macrophage polarization and muscle-specific abundance during recovery from disuse. *American journal of physiology. Endocrinology and metabolism*, *317*(1), E85–E98.

Reidy, P. T., Monnig, J. M., Pickering, C. E., Funai, K., & Drummond, M. J. (2021). Preclinical rodent models of physical inactivity-induced muscle insulin resistance: challenges and solutions. *Journal of applied physiology (Bethesda, Md. : 1985)*, *130*(3), 537–544.

Roemers, P., Mazzola, P. N., De Deyn, P. P., Bossers, W. J., van Heuvelen, M., & van der Zee, E. A. (2018). Burrowing as a novel voluntary strength training method for mice: A comparison of various voluntary strength or resistance exercise methods. *Journal of neuroscience methods*, *300*, 112–126.

Roemers, P., Hulst, Y., van Heijningen, S., van Dijk, G., van Heuvelen, M. J. G., De Deyn, P. P., & van der Zee, E. A. (2019). Inducing Physical Inactivity in Mice: Preventing Climbing and Reducing Cage Size Negatively Affect Physical Fitness and Body Composition. *Frontiers in Behavioral Neuroscience*, *13*, 221.

Saitoh, A., Okumoto, T., Nakano, H., Wada, M., & Katsuta, S. (1999). Age effect on expression of myosin heavy and light chain isoforms in suspended rat soleus muscle. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *86*(5), 1483–1489.

Sayer, A. A., Cooper, C., Evans, J. R., Rauf, A., Wormald, R. P., Osmond, C., & Barker, D. J. (1998). Are rates of ageing determined in utero?. *Age and ageing*, *27*(5), 579–583.

Sayer, A. A., Syddall, H. E., Gilbody, H. J., Dennison, E. M., & Cooper, C. (2004). Does sarcopenia originate in early life? Findings from the Hertfordshire cohort study. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *59*(9), M930–M934.

Sayer, A. A., Syddall, H. E., Martin, H. J., Dennison, E. M., Anderson, F. H., & Cooper, C. (2006). Falls, sarcopenia, and growth in early life: Findings from the Hertfordshire cohort study. *American Journal of Epidemiology*, *164*(7), 665–671.

Schultz, E., & McCormick, K. M. (1994). Skeletal muscle satellite cells. *Reviews of physiology, biochemistry and pharmacology*, *123*, 213–257.

Sharples AP, Stewart CE, Seaborne RA. Does skeletal muscle have an 'epi'-memory? The role of epigenetics in nutritional programming, metabolic disease, aging and exercise. *Aging cell*. 2016;15(4):603-16.

Simard C, Lacaille M, Vallieres J. Effects of hypokinesia/hypodynamia on contractile and histochemical properties of young and old rat soleus muscle. *Experimental neurology*. 1987;97(1):106-14.

Steffen JM, Fell RD, Geoghegan TE, Ringel LC, Musacchia XJ. Age effects on rat hindlimb muscle atrophy during suspension unloading. *Journal of applied physiology (Bethesda, Md. : 1985)*. 1990;68(3):927-31.

Tinsley, F. C., Taicher, G. Z., & Heiman, M. L. (2004). Evaluation of a quantitative magnetic resonance method for mouse whole body composition analysis. Obesity Research, 12(1), 150–160

Vandre C. Figueiredo, MSc, James F. Markworth, PhD, Brenan R. Durainayagam, MSc, Chantal A. Pileggi, BS, Nicole C. Roy, PhD, Matthew P. G. Barnett, PhD, David

Verdijk LB, Snijders T, Drost M, Delhaas T, Kadi F, van Loon LJ. Satellite cells in human skeletal muscle; from birth to old age. Age (Dordrecht, Netherlands). 2014;36(2):545-7.

Wade CE, Baer LA, Wu X, Silliman DT, Walters TJ, Wolf SE. Severe burn and disuse in the rat independently adversely impact body composition and adipokines. *Critical care (London, England)*. 2013;17(5):R225.

Wall, B. T., Dirks, M. L., & van Loon, L. J. (2013). Skeletal muscle atrophy during short-term disuse: implications for age-related sarcopenia. Ageing research reviews, 12(4), 898–906.

Wang, Y., & Pessin, J. E. (2013). Mechanisms for fiber-type specificity of skeletal muscle atrophy. Current opinion in clinical nutrition and metabolic care, 16(3), 243–250. https://doi.org/10.1097/MCO.0b013e328360272d

WHO. (n.d.). *Physical inactivity a leading cause of disease and disability, warns who*. World Health Organization. Retrieved April 9, 2022

Winick M, S. Noble A. *Cellular Response in Rats During Malnutrition at Various Ages*. 1966, 300-6 p.

Winick, M. (1989). The role of early nutrition in subsequent development and optimal future health. *Bulletin of the New York Academy of Medicine*, *65*(10), 1020–1025.

Wu X, Baer LA, Wolf SE, Wade CE, Walters TJ. The impact of muscle disuse on muscle atrophy in severely burned rats. *The Journal of surgical research*. 2010;164(2):e243-e51.

Yung, H. W., Hemberger, M., Watson, E. D., Senner, C. E., Jones, C. P., Kaufman, R. J., Charnock-Jones, D. S., & Burton, G. J. (2012). Endoplasmic reticulum stress disrupts placental morphogenesis: implications for human intrauterine growth restriction. The Journal of pathology, 228(4), 554–564.

Zhao, P., & Hoffman, E. P. (2004). Embryonic myogenesis pathways in muscle regeneration. *Developmental dynamics : an official publication of the American Association of Anatomists*, *229*(2), 380–392