THE EFFECT OF GROWTH-RESTRICTION ON VOLUNTARY PHYSICAL ACTIVITY ENGAGEMENT IN MICE.

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ABSTRACT

THE EFFECT OF GROWTH-RESTRICTION ON VOLUNTARY PHYSICAL ACTIVITY ENGAGEMENT IN MICE.

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INTRODUCTION. Current evidence suggests that early life growth restriction reduces physical activity engagement. Therefore, the purpose of this investigation was to examine the effects of early life growth-restriction on levels of wheel running in mice, and determine if known biological mechanisms regulate physical activity engagement. **METHODS.** Using a cross-fostering, protein-restricted nutritive model, mice were growth-restricted during either gestation (GUN; N=3 litters) or postnatal life (PUN; N=3 litters), along with a well fed control group (CON; N=3 litters). At 21 days of age, all mice pups were weaned and fed a non-restrictive healthy diet for the remainder of the study. At 45 days of age mice were individually housed in cages with free moving running wheels to assess physical activity engagement. At day 70, mice were euthanized, and the nucleus accumbens was analyzed for dopamine receptor 1 expression. Skeletal muscle fiber type and cross-sectional area of the soleus, extensor digitorom longus, and diaphragm were analyzed by immunohistochemistry. The soleus from the other hind leg was evaluated for calsequestrin 1 and annexin A6 expression. **RESULTS.** The PUN female mice had a reduction (P=0.0221) in wheel revolutions per day as compared to the GUN and CON females. PUN female mice also expressed significantly higher Drd1(P=0.0247) and Casq1 (P=0.0398) compared to the other groups. **CONCLUSION.** Growth-restriction during lactation reduced physical activity in female mice by reducing the central drive to be active and displayed a more fatigable skeletal muscle phenotype.

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KEY TO ABBREVIATIONS

A6	Annexin A6
Casq1	Calsequestrin 1
CON	Control Group
CSA	Cross-Sectional Area
DoHAD	Developmental Origins of Health and Disease
Drd1	Dopamine Receptor 1
Drd2	Dopamine Receptor 2
Drd3	Dopamine Receptor 3
Drd4	Dopamine Receptor 4
Drd5	Dopamine Receptor 5
EDL	Extensor Digitorum Longus
GUN	Gestationally Undernourished Group
PN	Postnatal Day
PUN	Postnatally Undernourished Group

INTRODUCTION

Growth-restriction as a result of early life undernutrition affects 7 million individuals per year (1), resulting in increased risk for cardiovascular disease (1), obesity (2, 3), hypertension (3), type II diabetes (4-6), and sarcopenia (7). Growth-restriction also leads to less leisure time physical activity in adulthood compared to non-growth-restricted individuals (8, 9). This is alarming as the decreased engagement in physical activity could further compound the chronic disease risk associated with growth-restriction (6, 10-14). Few studies have investigated the effect of growth-restriction on physical activity engagement, with no studies examining the mechanisms that regulate voluntary physical activity in growth-restricted individuals. Therefore, a gap in the literature is the understanding of mechanisms that regulate physical activity engagement in the growth-restricted population. Once mechanisms are identified, therapeutic countermeasures to increase physical activity and offset chronic disease risk can be developed.

Physical activity engagement is regulated by environmental, social, and biological factors, with evidence suggesting that biological factors are the primary contributor (15). The biological factors center on: 1) cardiorespiratory fitness, 2) the skeletal muscle capacity to be physically active, and 3) the drive to be physically active (15).

Cardiorespiratory fitness has been evaluated in the growth-restricted population by Salonen *et al.*, who found individuals with heavier body weights at 2 and 7 years old had a higher cardiorespiratory fitness (measured by a 2 Km walk) (16). Ferguson *et al.* examined cardiorespiratory fitness in growth-restricted mice at the cellular, tissue, and whole organism level, and determined that mice undernourished during the postnatal period (days 1-21) had a 20% reduction in VO_{2peak} due to impaired cardiac function during diastole, a higher proportion of mononucleated cardiomyocytes, and impaired calcium kinetics of isolated cardiomyocytes as compared to non-growth-restricted mice (17). Therefore, growth-restriction reduces cardiorespiratory fitness, which may partially explain the reduction in physical activity engagement (13, 17). The extent to which growth-restriction influences the (i) drive and (ii) capacity for physical activity, however, has not been evaluated.

Researchers studying the drive to be physically active have focused on dopamine pathways in the brain (18-21). The expression of dopamine receptor 1 (Drd1) modulates reward seeking behavior, specifically an increase in Drd1 leads to decreased physical activity levels in mice (20, 21).

The capacity to be physically active is influenced by the skeletal muscle's ability to produce force for locomotion, resist fatigue, and utilize substrates for ATP production (15). Previous investigators have demonstrated that mice with smaller fiber cross sectional area and more type I fibers have higher physical activity levels compared to control strains (22-24). Additionally, Ferguson *et al.* found higher expression of calcium regulatory proteins (Calsequestrin 1 and Annexin A6) in the mouse soleus was associated with reduced fatigue and increased physical activity engagement (25).

A variety of factors have been proposed to regulate physical activity engagement, with the prime candidates Drd1, Casq1, A6, and skeletal muscle phenotype consistently shown to play a role. Therefore, we hypothesize that growth-restriction alters expression of these key biological regulators of physical activity thereby influencing physical activity engagement. To test this hypothesis we have developed the following aims with specific sub hypotheses:

Aim 1: To determine the effect of gestational (GUN) and postnatal (PUN) undernutrition on physical activity engagement (wheel running) in mice. We hypothesized that both GUN and PUN mice will have lower levels of physical activity engagement compared to a control group.

Aim 2: To determine the effect of GUN and PUN on the expression of dopamine receptor 1 (Drd1), Calsequestrin 1 (Casq1), and Annexin A6 (A6). We hypothesize that both PUN and GUN mice will have lower expression of Casq1 and A6, and higher expression of Drd1 as compared to the control group.

Aim 3: To determine the effect of GUN and PUN on skeletal muscle cross-sectional area (CSA) and fiber type percentage. We hypothesized that PUN mice will have smaller CSA compared to GUN

and control mice, as well as a higher expression of glycolytic fibers compared to the GUN and control group.

There is a paucity of information on how early life growth-restriction influences physical activity engagement. Successful completion of these aims will determine if growth-restriction alters the primary biological mechanisms associated with the drive and capacity to be active. From this, investigators will be able to evaluate additional biological factors for physical activity engagement (biological candidates previously showing conflicted results), as well as the environmental/social factors (controlled for in this investigation), which will help accomplish the long-term goal to develop interventions to promote physical activity and reduce chronic disease risk in the growth-restricted population.

LITERATURE REVIEW

Physical activity engagement is a cost-effective preventative measure for chronic disease risk (26-30). Globally, 15 million babies are born each year experiencing growth-faltering (31) which results in increased risk for chronic diseases (41-43, 71, 91). Given this increased health risk, physical activity in this population could be an effective therapeutic countermeasure. However, there is evidence that early life growth-restriction may reduce physical activity engagement. This literature review will discuss the importance of physical activity, regulators of physical activity, chronic diseases associated with growth-restriction, and provide rationale for investigating the biological regulation of physical activity in the growth-restricted mouse model.

Physical Activity and Health

Common chronic diseases that burden the healthcare system include heart disease, type II diabetes, and metabolic syndrome (32-34). Evidence has shown that physical activity reduces the risk for these conditions. In a study of 5,000 individuals using self-reported physical activity data, Churilla and Fitzhugh found an inverse relationship between leisure time physical activity and incidence of metabolic syndrome (27), which is supported by a review by Lamonte *et al.* indicating physical activity reduces the incidence of Type II diabetes (28). Physical activity has also been extensively shown to reduce the risk for cardiovascular disease (33, 35). In 1987, Powell *et al.* conducted a meta-analysis of 43 studies to determine the influence of physical activity on incidence of cardiovascular disease. These researchers showed the relative risk of coronary heart disease was 50-104% increased due to physical inactivity (36). Importantly, researchers also demonstrated a dose dependent response, where individuals engaging in lower levels of physical activity showed higher levels of coronary heart disease (36). With the multitude of established benefits of physical activity, it is important to prescribe activity to at-risk populations for chronic disease.

The Developmental Origins of Health and Disease Hypothesis.

The Developmental Origins of Health and Disease (DOHaD) Hypothesis describes how early life exposures can increase the incidence of chronic disease (37). Many early investigations used retrospective epidemiological studies, with some of the most cited research conducted by Dr. David Barker. Specifically, these studies demonstrated growth-restricted individuals had higher rates of cardiovascular disease (38-40), Type II Diabetes (10, 11, 14), and obesity (12) in adulthood.

Animal models have been utilized in order to support the results from epidemiological investigations and develop mechanistic explanations for increased incidence of chronic disease. The current gold standard to model growth restriction is to utilize an undernutrition nutritive model (Figure 1), where mothers are fed a low protein diet, which restricts growth during gestation by decreasing amino acid availability (41). The low protein diet during lactation reduces milk production and thereby induces growth restriction (42). Researchers can utilize cross-fostering to isolate windows of growth-restriction to gestation and lactation (postnatal life).

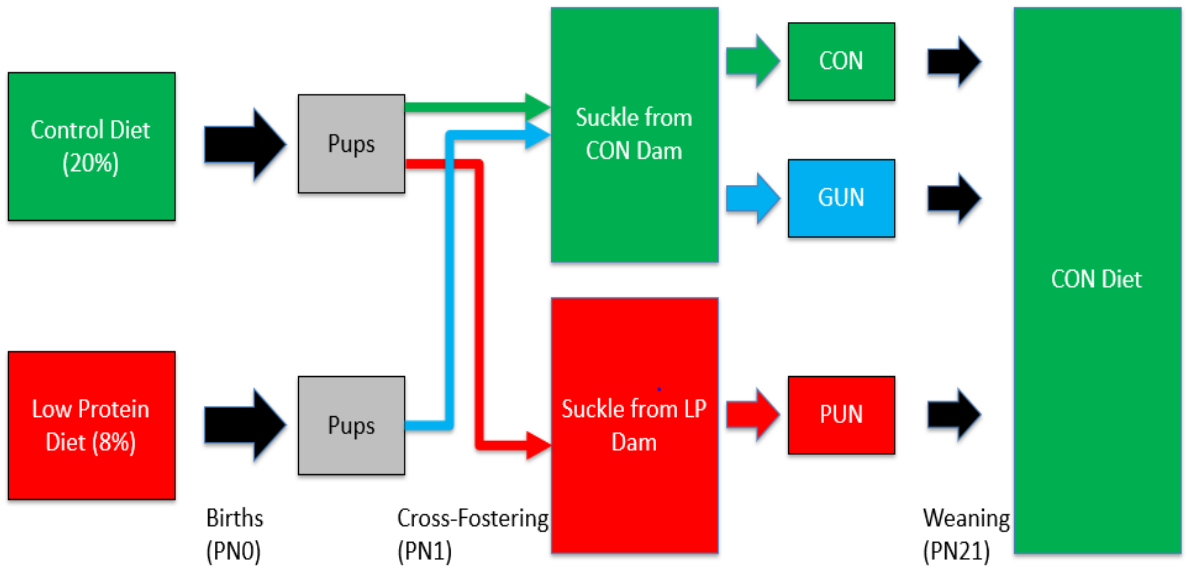

Figure 1. Nutritive Model.

Langley *et al.* utilized three levels of gestational undernourishment in rat dams to examine the blood pressure of their offspring at 9 weeks of age, and found all undernutrition groups had increased blood pressure compared to the controls (43). In a study by Zhu *et al.*, ewes were undernourished in mid-gestation, and weaned to a control diet shortly before birth. These researchers then examined the offspring at 8 months of age, and showed a reduced number of muscle fibers coupled with an increased percentage of type IIb fibers in the longissimus dorsi, as well as lower overall muscle mass to body weight ratio (44). Additionally, a reduction in maternal nutrition in ewes for the first 70 days of gestation resulted in significantly higher blood pressure in their offspring (45) and impaired glucose tolerance (46) in adulthood. Research in pigs has also demonstrated the effects of developmental programming, where undernourished offspring showed impaired levels of glucose tolerance at 12 months of age (47). Thus, early life growth-restriction has shown in human and animal literature to increase the incidence of chronic disease, specially heart disease and type II diabetes (48). To a lesser extent, the literature has shown growth-restriction to reduce muscle mass and potentially increase the incidence of sarcopenia (49).

Developmental Programming of Physical Activity

In addition to chronic disease risk, Davies *et al.* correlated birth weights of 25,874 individuals, with physical activity engagement in adulthood, and when we re-examined the data, found individuals born less than 2,000 grams engaged in less regular physical activity compared to babies born greater than 3,250 grams (50). Salonen and colleagues, utilizing the Helsinki Birth Cohort, examined 1,967 individuals born between 1934 and 1944, as well as their associated late adulthood physical activity levels, and demonstrated that higher birthweight correlated with higher intensity of physical activity engagement (8). Research conducted by Andersen *et al.* examined 13 Nordic cohorts consisting of 43,482 individuals and found similar results, where individuals born less than 2.75 Kg had reduced levels of physical activity levels compared to the normal birth weight infants (10). Thus, human epidemiological studies indicated that growth-restricted individuals have less physical activity engagement.

Results from an animal model study of physical activity support the human epidemiologic research, whereby mouse strains that produce low birth weight pups demonstrate that when pups reach adulthood, they engage in less physical activity as measured by wheel running (9). However, this particular experiment did not utilize a validated model of growth-restriction (i.e., undernutrition model). Despite this, the Baker et al. study provides evidence that growth-restriction programs mammals for reduced physical activity in adulthood (10). To advance our understanding in this area, it is necessary to understand the factors that influence physical activity engagement in order to promote physical activity in the growth-restricted population.

Environmental and Cultural Regulators of Physical Activity

A variety of psychological and environmental factors regulate physical activity engagement in adulthood. The landscape and built environment surrounding adults has been shown to have a large effect on their physical activity engagement (51-53). Perceived safety of the environment also influences physical activity engagement. This is exemplified in a study of 190 adults that showed those most physically active were from areas with the highest rating of social cohesion and perceived safety (54). Modes of transportation within the individual's environment, including footways or need for motor transportation, also affect physical activity engagement. Ewing *et al.* examined 206,992 adults from 448 counties and 83 metropolitan areas, and found that individuals in large sprawling counties engaged in less physical activity and tended to weigh more (55). Additionally, Troped *et al.* measured the effect of environmental perceptions on physical activity by asking participants how far they believed they lived from an exercise trail, and how many hills they would have to encounter to reach it. Distance from the trail showed an inverse relationship with trail use (56). Humpel *et al.* examined the perceived aesthetics of one's environment, and found that individuals who perceived a positive environment were twice as likely to increase their walking levels (57).

Personal factors account for a portion of physical activity engagement. Experiments designed to examine the social-cognitive aspects of physical activity engagement have found that high levels of self-

efficacy, regular participation with friends in physical activity, and perceived support from friends and family was positively associated with levels of physical activity (58, 59). Specifically, Sallis *et al.* evaluated 24 different variables, including dietary habits, self-efficacy, and perceived barriers to exercise, for their influence on physical activity engagement. These researchers found that the 24 variables in total accounted for 27% of the variance in physical activity engagement, with the strongest variables in the model being self-efficacy and support from friends. Overall, environmental and personal factors only account for a portion of the variance in physical activity engagement, which suggests a genetic component for this behavior.

Genetic Inheritance of Physical Activity

Many of the earliest studies examining physical activity inheritance used monozygotic and dizygotic twins in order to determine how the genetic code influences physical activity engagement. Put simply, the percent genetic heritability is the percentage of variability in physical activity engagement that can be explained by genetic factors. In 1981, 5,000 pairs of twins provided self-reported physical activity engagement and intensity levels, and researchers found the correlation coefficients of 0.57 for monozygotic twins, and 0.26 for dizygotic twins (60), indicating a moderate relationship for genetic inheritance within the monozygotic twins. Perusse and colleagues elucidated the genetic influence to account for 29% of the total variance in physical activity engagement (61), although the investigators examined only a three day self-reported physical activity span, limiting the strength of conclusion drawn. In 1991, researchers administered accelerometers to one hundred 4-7 year-old children, as well as their mothers and fathers. These researchers found that the odds ratio for children to be active from active mothers or active dads were 2.0 and 3.5 (indicating children were twice as likely to be active with active moms or 3.5 times more likely to be active with active dads), and if both were active, the odds ratio for active children was 5.8 (62). It should be noted active parents could have an environmental influence on children as well.

The magnitude by which genetics account for physical activity was characterized again in 2005, when Joosen and colleagues used accelerometers to measure physical activity in families, and found genetic heritability amongst family members to be an astonishing 78% (63). One year later, Butte *et al.* found heritability coefficients ranging from 0.32 to 0.69 using accelerometers, indicating a strong genetic influence (64). Despite the high numbers seen in these experiments, most studies report a much more modest inheritance. Studies using self-reported data have shown physical activity inheritance ranged from as low as 9% (65) and up to 35% in active families (66), demonstrating the vast range of genetic inheritance possibility, which might depend on the method utilized to collect physical activity data.

When evaluating the genetic influence on physical activity across the lifespan, children appear to have the lowest amount of physical activity explained by genetic inheritance at 20%, adolescents have the highest amount of physical activity explained by heritability at 70-80%, and adults have a medium amount explained at 50-60% (67). Human studies indicate genetics regulate physical activity, but due to the large variation caused by the environment, it is difficult to determine genetic mechanisms. Therefore, an animal model must be used to control for the environmental effects.

Animal Physical Activity Inheritance

Experiments utilizing mice operate under the fact that 1) there is a strong genomic similarity between the mouse and human genome (68), meaning genetic results observed in mice can be translated to humans, 2) the environment can be controlled, limiting environmental variability in the phenotype (69), and 3) mouse cohorts are genetically homozygous, thus there is no genetic variability among mice (70). The usual mouse model for physical activity is the running wheel. This is a close correlate to human physical activity (71), as mice experience similar cardiovascular responses to humans (72), activate similar neurotransmitters during wheel running as humans during physical activity engagement (73, 74), and self-select physical activity at similar intensity as humans (75, 76).

In an experiment examining wheel running behavior of 26 mouse strains, Festing found that genetic inheritance of physical activity ranged from 26 to 29% (70), indicating a weak relationship. More moderate estimates were found in an examination of 7 mouse strains, with genetic inheritances of 39% for wheel running distance and 42% for duration (77). Lightfoot *et al.* addressed the variability in mouse physical activity heritability by breeding different strains of mice to determine physical activity within strains (genetic inheritance), and physical activity levels of mice between strains (environmental regulations). These researchers showed that broad sense genetic heritability (the ratio of total genetic variance to phenotypic variance, a measure of the genetic contribution to an animals phenotype) of running distance varies between genders, with female heritability of 12-22% and males ranging from 31-48% (78). Duration of wheel running also had differences in heritability based on gender, with females again around 12-21%, and males around 44-61% (78). Turner and others examined the physical activity of 5 mouse strains as they aged across 26 weeks, and found that genetic variability accounted for 41-85% of differences in activity levels, depending on the mouse strain utilized (79). Therefore, while usage of the rodent model allows for a stronger degree of control and establishes similar results of genetic inheritance as humans, heritability studies demonstrates similarly low results for the genetic inheritance of physical activity, and as such the mechanisms regulating physical activity must be examined by a targeted genetic approach.

Regulators of Physical Activity

Although physical activity is a partially inherited trait, but there are limitations in understanding the mechanisms responsible for activity regulation based solely on heritability studies. To address genetic mechanisms for physical activity engagement, investigators have characterized the physical activity phenotype as either the "drive to be active" or the "capacity to be active". Specifically, Pomp *et al.* , Ferguson *et al*., and later Kelly defined the biological drive to be active as related to the motivation to engage in physical activity, and has been shown to be related to dopamine receptors within the brain (25, 80, 81)(detailed below). The capacity to be active is related to one's ability to engage and maintain

physical activity, which has been shown to be related to skeletal and cardiac muscle properties (detailed below). Expanding on this, Ferguson *et al.* hypothesized that wheel running behavior could reflect differences in drive and capacity to be active. The number of single (less than 4 seconds) and extended wheel running bouts initiated were used to assess the "drive to be active" while average speed determined the "capacity to be active". Bout duration and revolutions per day were considered aspects of both the drive and capacity to be active (25, 82).

Biological Drive to be Active

Previous research shows the drive to be physically active centers on the dopamine system. Dopamine is a neurotransmitter that is involved in the motivation of pleasure seeking activities, such as eating, sexual behaviors, addictive drug behavior, and exercise (19). Two families of receptors exist in the dopaminergic pathway: D1 like receptors include dopamine receptor d1 (Drd1) and dopamine receptor d5 (Drd5), while D2 like receptors include dopamine receptor d2 (Drd2), dopamine receptor d3 (Drd3), and dopamine receptor d4 (Drd4) (21). Both receptor families have been closely studied, with special attention paid specifically to Drd2 and Drd1. An experiment in 1992 showed administration of sulpiride, a Drd2 agonist, decreased dopamine levels and spontaneous physical activity (83). Bronikowski *et al.* used a gene array to examine the brains of mice given access to wheel running and those without, and found that mice with access to physical activity had 20% more Drd2 and Drd4 receptors (84). Furthermore, D2 receptor deficient mice showed significantly less spontaneous movements (85), as well as decreased amount of initiated locomotion (86). However, despite the differences in locomotion seen with D2 receptor modulation, recent literature has shown Drd1 may play a larger role in the biological drive to be physically active than D2 receptors.

In 2003, Rhodes & Garland elucidated the role D1 receptors play through the use of Ritalin (a dopamine agonist) and apormorphine (a dopamine antagonist) on mice with access to wheels (87). They found that manipulation of the D1 receptor reduced wheel running (87), and these results were later confirmed by Roberts and colleagues in 2012 (88). Knab *et al.* further clarified the role that the D1

receptors played in regards to physical activity in 2009, where they used 4 groups of mice: a high active strain with wheel access, a high active strain without wheel access, a low active strain with wheel access, and a low active strain without wheel access (20). The authors measured the mRNA expression of 7 dopamine genes: Drd1, Drd2, Drd3, Drd4, Drd5, tyrosine hydroxylase (used as an indicator of dopamine production), and DAT (a dopamine transporter). After excising the nucleus accumbens of the brain, they saw a significant decrease in the expression of dopamine genes in the high active mice compared to the low active cohort (20). Higher active mice showed significantly lower expression of Drd1, highlighting the role these receptors could play; high Drd1 expression could lead to quicker rewards from physical activity, leading to a decrease in engagement (20). Expanding upon these results, Knab and colleagues pharmaceutically modulated dopamine receptor activity in both a high-active strain (C57L/J) and low-active strain (C3H/HeJ) using a D1-like family agonist, a D1-family antagonist, a dopamine transporter antagonist, and a tyrosine hydroxylase inhibitor (89). Following serial administration of these drugs (with adequate washout time between), the authors found that the D1 like agonist reduced wheel running distance in the high-active C57L/J, and the dopamine reuptake inhibitor increased wheel running in the low-active C3H/HeJ mice. These findings provide additional evidence to suggest the importance of dopamine receptors in the drive to be physical activity engagement.

The endocannabinoid system has been hypothesized to be a target of interest in the drive to be active, as it is activated acutely during exercise and related to motor activity regulation through its relationship with the dopamine system (90). However, the specific effects of endocannabinoids on physical activity engagement is not clear, as some experiments show agonists promote physical activity (91), while others show these agonists decrease physical activity (92). Additionally, proteomic evaluation of the nucleus accumbens of the brain showed no differences of the endocannabinoid system between high and low active mice (82). Therefore, while other pathways have been hypothesized to be drive physical activity engagement, Drd1 shows consistent influences on physical activity when modulated.

Biological Capacity to be Active

The central regulators of physical activity, while important, do not explain physical activity engagement completely. As stated earlier, the peripheral body exerts influence on physical activity engagement. Amongst these peripheral regulators are skeletal muscle fiber type, fiber cross-sectional area, and proteins associated with calcium handling, like Annexin A6 (A6) and Calsequestrin 1 (Casq1), which influence force and frequency of contraction as well as fatigue (25).

The Garland lab has bred mice for high wheel running along with a moderately active control for over 96 generations (22). The high active mice have a marked reduction in the cross sectional area of type IIb fibers, despite no difference in overall body mass, as well as an increased proportion of Type I skeletal muscle fiber type (22). These genetic adaptations have been hypothesized to facilitate increased physical activity levels in this selectively-bred strain (23, 24).

The actual contractile properties of the muscle are important to consider when examining physical activity regulation, specifically, the role of calcium handling proteins A6 and Casq1. A6 is a protein primarily associated with the modulation of calcium from the sarcoplasmic reticulum (93), while Casq1 is associated with the proper release and reuptake of calcium from the sarcoplasmic reticulum (94). The importance of these two proteins was highlighted in a study by Ferguson *et al.*, in which the authors identified high active mice having a higher expression of these two proteins using 2D-DIGE (25). These researchers then confirmed Casq1 and A6's role in activity regulation by using vivo-morpholinos, a technique to block translation of genes (95), to reduce Casq1 and A6 protein levels in the high active mice to the levels of the low active mice. This protein level reduction coincided with a reduction in wheel running, highlighting the importance of these two candidates for physical activity regulation.

To support the skeletal muscles' capacity to be active, the cardiorespiratory system must function properly for an individual to maintain physical activity engagement. High active mouse strains present with increased $VO2_{peak}$, maximal treadmill running capacity, and cardiac output (75). However, it is not

clear if these cardiovascular properties are developed due to increase physical activity levels (due to drive and capacity detailed above) or if inherent high aerobic fitness allows for prolonged sustained activity. Lightfoot *et al.* highlighted the importance of cardiorespiratory fitness to support the skeletal muscle capacity to be active, and suggested without the proper functioning of the cardiovascular system, individuals will not maintain high amounts of physical activity even with sufficient drive (15).

Sex steroids and Physical Activity

It is necessary to note the role that sex hormones have in physical activity regulation. In humans, males have been shown to be more physically active than females (96). Interestingly, this trend tends to be reversed in many animal species, where females have significantly higher physical activity levels than males (97-99). Estrogen, as well as estrogen receptors, have been hypothesized to be related to this difference observed in physical activity (100), where Fahrback *et al.* administered estradiol to rats, and observed increased wheel running levels (101). In humans, individuals who received administration of estrogen hormone replacement therapies showed lower levels of physical inactivity compared to those not receiving the hormonal treatment (102). Investigators have also hypothesized estrogen to be related to the dopamine pathway. Becker *et al.* proposed estrogen helped enhance dopamine release (103), which Rhodes and Garland also demonstrated, as high-active female mice in their experiment had reduced expression of D1 family receptors (87).

Testosterone has not been studied as extensively as estrogen in physical activity regulation, but a study conducted by Roy and Wade demonstrate the differences surrounding these sex hormones. The authors administered both an androgen and estrogen to castrated mice in order to examine the specific sex hormone differences. Interestingly, they noted that while the androgen administration did increase wheel running levels, the estrogen treatment was 100 times more effective than the androgen supplement (104). Additionally, androgen supplementation in non-castrated mice showed no significant differences in wheel running activity (105). Work by Bowen *et al.* examined wheel running following surgical removal of the gonads, and again following administration of either 17B-estradiol or testosterone depending on sex of

the mouse (estradiol to females, testosterone to males). They demonstrated that removal of the gonads reduced distance run on the wheel by 10-30% of baseline levels, and reduced duration of wheel running by 20-47% of baseline levels. Interestingly, re-administration of testosterone resulted in mice fully recovering all parameters of wheel running, while the estrogen re-administration did not show this same result (106). Therefore, while sex hormones appear to play a role in physical activity regulation, the specific effects of these hormones in physical activity regulation are not entirely clear.

Conclusion and Implications for Physical Activity Regulation in Growth Restricted Individuals

Physical activity is important for health and prevention of chronic disease. There is clear evidence that environmental, social, and biological factors regulate physical activity levels, with strong evidence for biological factors being a primary factor. By evaluating these factors in specific populations that display reduced physical activity, therapeutic countermeasures can be developed.

Growth-restricted individuals engage in less physical activity, with minimal investigation into the biological mechanisms. Human and animal studies have shown that growth-restriction caused by early postnatal undernutrition reduces cardiorespiratory fitness in adulthood due to increased afterload, ventricular stiffness, and mononucleated cardiomyocytes with impaired contractile properties (17), but the skeletal muscle capacity and drive to be active have not been investigated. Therefore, the purpose of this study was to examine the effect of early life growth-restriction of physical activity engagement, as well as to elucidate the biological regulators for the drive to be active (Drd1) and the capacity to be active (Casq1, A6, skeletal muscle fiber type and CSA).

Introduction (*manuscript*)

Dr. David Barker reported that growth-restriction increases the incidence of cardiovascular disease, type II diabetes, and hypertension in adulthood. Barker termed this observation "Developmental Programing" also known as "The Developmental Origins of Health and Disease Hypothesis" (DoHAD) (4, 40, 107). Growth-restriction affects 7 million individuals per year leading to a 47% increased risk of chronic disease in adulthood (1). The beneficial effects of regular physical activity engagement decrease the incidence of cardiovascular disease, type II diabetes, hypertension, and obesity (108). However, human studies using self-reported physical activity questionnaires have indicated that growth-restriction in early life reduces cardiorespiratory fitness (8), along with leisure time physical activity engagement in adulthood (16). There is no mechanistic rationale for the observed decrease in physical activity engagement, but understanding such mechanisms could potentially lead to therapeutic countermeasures improving health outcomes from growth-restricted individuals.

The biological regulation of physical activity is influenced by the drive to be physically active, as well as the capacity to resist fatigue (15, 25, 109, 110). Studies examining the central drive to be active have recently focused on the dopaminergic system, where mouse strains bred for high wheel running have a decreased expression of dopamine receptor 1 (Drd1) in the nucleus accumbens as compared to low active strains (20, 89, 111, 112).

The study of the capacity to be physically active has investigated skeletal muscle fatigue resistance via alterations in fiber type and the expression of calcium regulatory proteins (24, 76). Mouse strains that are high active and display the mini-muscle phenotype have a decrease in the cross-sectional area (CSA) of locomotor muscles with an increased percentage of oxidative fibers (24). Additionally, high active mice have an increased expression of the calcium regulating proteins Calsequestrin 1 (Casq1) and Annexin A6 (A6) in the soleus (25).

We hypothesize that these established mechanisms relating to the biological regulation of physical activity could elucidate processes by which growth-restriction decreases physical activity engagement. Therefore, we evaluated the drive (Drd1 expression) and capacity (skeletal muscle fiber type, cross sectional area, and expression of Casq1 and A6) to be active in mice that were growth-restricted during gestation and lactation.

Methods

Animal Model

This study was approved by the Institutional Animal Care and Use Committee at Michigan State University and conducted according to the guide for the care and use of laboratory animals. FVB mice were obtained from Charles Rivers Laboratories (Wilmington, MA), and housed with food and water *ad libitum*. Cages were kept in a vivarium with 12 hour light and dark cycles beginning at 6 am and maintained at 18-21°C. The FVB mice are appropriate to model growth restriction by early life undernutrition (13, 17, 49) because large litters are birthed and the dams are accepting of pups following the requisite cross-fostering (detailed below). Lightfoot *et al.* showed FVB mice are active wheel runners and not statistically different in wheel running as compared to the commonly used C57BL/6J strain (76). Furthermore, anecdotal evidence from our lab revealed that C57BL/6J dams eat pups that are cross fostered to them, preventing their use in growth-restriction studies. Thereby, the FVB strain is an appropriate model for growth-restriction and physical activity engagement.

Nutritive Model

Two weeks prior to mating, FVB dams were assigned a diet consisting of either a low protein (LP; 8% protein; Research Diets, New Brunswick, NJ, USA) or an isocaloric, control protein diet (CON; 20% protein; Research Diets). The LP diet restricts growth in gestation by reducing amino acid availability and during lactation by decreasing milk volume produced by lactating dams (41, 42, 113-115).

Following the two week diet acclimation, males were introduced to dam cages for a 24-hour period only, thus all pups born were the same age throughout the study. Upon birth (PN1), pups were cross-fostered to form three groups: pups from a CON diet mother were cross-fostered to another CON diet mother (control-CON group), pups from a CON diet mother were cross-fostered to a LP diet mother (postnatal undernutrition-PUN group), or pups born from a LP diet mother were cross-fostered to a CON

mother (gestational undernutrition-GUN group). Each litter received the same number of pups and was standardized to equal sex ratio and litter size (4 male and 4 female pups per litter). A total of three litters per diet group were used in this study. Each pup was given an identifying tattoo and litter size was maintained by donor pups; donor pups were never studied. From PN1 to PN21 body mass were measured every 4 days. At PN21, all pups were weaned to the CON diet, thus limiting the window of undernourishment to either gestation (GUN) or lactation (PUN). Body mass was measured once a week until the end of the study.

Wheel Running Measurement

At PN45, pups were individually housed and given access to a free moving running wheel (Columbus Instruments, Columbus, OH). Wheel running was measured from PN45 to PN67, with the first seven days being an acclimation week. Thus, reported wheel data consists of PN52-59 (termed week 1 in results) and PN60-67 (termed week 2 in results). Wheel running variables that were analyzed were wheel revolutions per day, number of single wheel bouts less than 4 seconds, number of wheel running bouts longer than 4 seconds (termed extended wheel bouts), wheel running bout duration (for bouts over 4 seconds), and average speed per wheel running bout. The number of single and extended wheel running bouts initiated assessed the "drive to be active", whereas average speed determined the "capacity to be active". Bout duration and revolutions per day were considered aspects of both the drive and capacity to be active (15, 25, 110).

Euthanasia

At PN67 running wheels were removed from cages to limit transient effects of wheel running on protein expression (25). At PN70, mice were anesthetized using vaporized isoflurane followed by cervical dislocation. Tibia length was measured as a surrogate for body composition using Vernier calipers (116). The diaphragm, soleus and extensor digitorum longus (EDL) was removed for skeletal muscle histology

and western blotting. The nucleus accumbens of the brain was dissected for western blot analysis of Drd1.

Skeletal Muscle Fiber Typing

The procedure for fiber typing has previously been described by Wang *et al* (117). Briefly, after dissection, muscle samples were placed into cryomolds, and fibers aligned for proper sectioning. Samples were covered in Tissue Tek Optimal Cutting Temperature Compound (Sakura Finetek, Torrance, Ca) and flash frozen in 2-methylbutane chilled by liquid nitrogen, and stored at -80°C. Samples were then cut into 5 µm sections using a cryostat microtome (Leica, Wetzlar, Germany) and placed onto glass microscope slides. Slides were stored at -20°C.

For analysis, slides were brought to room temperature, and fixed in acetone for 15 minutes at 4°C. After a rinsing with phosphate buffered saline (PBS), sample cell membranes were permeabilized by incubating with 0.5 % Triton X-100/PBS solution for 15 minutes. Individual muscle samples were encircled using a "Pap Pen" and incubated with Image-IT FX signal enhancer for 30 minutes, followed by another PBS rinse. Samples were then incubated for an hour with AffiniPure Goat Anti-Mouse IgG. Samples were rinsed with PBS and blocked in 5% Normal Goat Serum (NGS) in 4% Bovine Serum Agent (BSA) for thirty minutes. Skeletal muscle sections were incubated in Laminin (cell membranes, Sigma Aldrich), BA-D5 (myosin heavy chain type I, Developmental Studies Hybridoma Bank), SC-71 (myosin heavy chain type IIa; Developmental Studies Hybridoma Bank) and BF-F3 (myosin heavy chain type IIb; Developmental Studies Hybridoma Bank) overnight at 4°C. The next day, sections were rinsed then probed using the secondary antibodies (Life Technologies) AF488 Goat Anti-Mouse IgG1 to bind to SC-71 (type IIa), AF546 Goat Anti-Mouse IgM to bind to BF-F3 (type IIb), AF647 Goat Anti-Mouse IgG2b (Laminin), and AF647 Goat Anti-Rabbit (type I) for one hour. Slides were then rinsed in PBS, and incubated in SlowFade Gold antifade mountant (Thermofisher Scientific, Waltham, Ma) followed by application of a cover slip.

Samples were imaged using a FluoView 1000 (Olympus, Center Valley, PA) inverted confocal laser microscope. A PlanFluor UPLFN 20X NA.50 objective collected images using a 488 nm Argon Laser or 543/643 HeNe laser for excitation of fluorescence. Single XY confocal images were taken using sequential excitation, and no changes were made to the images post acquisition. Cross-sectional area and muscle fiber type for each cell was quantified using ImageJ software (118). 300 cells were counted per sample. CSA was normalized for each mouse by dividing CSA by body weight (17).

Soleus fiber type expression was additionally evaluated by SDS PAGE and silver nitrate stain (119). Protein was extracted and separated by SDS PAGE (detailed in the western blot procedure section). Gels were then fixed (100 mL Glacial acetic acid; 300 mL ethanol; 600 mL water) for one hour, and rinsed for 20 minutes in 20% ethanol solution. After a 10 minute rinse with ultra-pure water, gels were sensitized (0.2 g Sodium thiosulfate; 1,000 mL water) for 1 minute, and rinsed again in ultra-pure water. Gels were stained using silver nitrate solution (2.0 g silver nitrate; 1,000 mL H2O), followed by a 5 second water rinse, and soaked in developer solution (15 g sodium carbonate; 25 mL sensitizer solution; 125 µL 37% formaldehyde to 500 mL water). Stop solution (50 g TRIS; 25 mL glacial acetic acid to 1,000 mL H2O) was applied to gels to terminate the procedure. Stained gels were visually inspected for differences in myosin heavy chain.

Western Blot Procedure

Protein extraction was performed by homogenizing the tissue using a tissuemizer (Thermo-Fisher), followed by the addition of a lysis buffer (9.9 mL H_20; 0.4 g CHAPS, Calbiochem; 100 µL TRIS 7.5; 1 protease inhibitor pellet, Roche). A Bradford assay was then used to determine protein concentration to allow for equal loading of proteins on SDS PAGE. Proteins were separated by SDS PAGE and transferred to a Polyvinylidene difluoride membrane. Membranes were blocked in nonfat dried milk and incubated with primary antibodies overnight at 4°C. The nucleus accumbens was probed for Drd1 at 1:3333 ratio (Abcam, Cambridge, Ma), while soleus was probed for Casq1 at a 1:1000 ratio

(Abcam, Cambridge, MA), and A6 at a 1:1000 ratio (Abcam, Cambridge, MA). For all western blots Gapdh (1:1000 ratio; Cell Signaling Technologies, Danvers, Ma) was used as a loading control.

Following overnight incubation membranes were rinsed in tris buffered saline with tween (TBST), and then incubated in secondary horseradish peroxidase antibody (Abcam, Cambridge, Ma) at a 1:2500 ratio for one hour. After rinsing with TBST, membranes were incubated in SignalFire ECL Reagent (Cell Signaling Technology, Danvers, Ma) for 6 minutes. Chemiluminescence images were collected using Kodak ImageStation 2000 (Kodak, Rochester, NY), and individual bands were analyzed by densitometry software comparing intensities of target protein standardized to loading control. Results are expressed as arbitrary units (AU).

Statistics

All data were analyzed in JMP Pro v. 13.0 (SAS, Cary, NC) using a repeated-measures ANOVA for growth data and a two-way ANCOVA comparing the main effects of diet (CON, PUN, and GUN) and sex (male and female) with body weight as a covariate for phenotypic data collected during adulthood with an alpha level of 0.05 set *a priori*. If significance was found a Tukey's HSD post hoc test for multiple comparisons was run. Furthermore, to evaluate the effects of body weight on wheel running a linear regression analysis was utilized. For all analysis litters were used as a statistical unit, as pups within a litter are not statistically different. Where the same variable was measured for more than one offspring in the same litter, data were averaged within the litter for each sex (13, 17, 49, 69). Results are presented as means ± standard deviation (SD).

Results

Growth

From PN1-21 (Figure 2a), growth rate was reduced (P<0.0001) in the PUN group (Male: 0.31±0.017 g·day^{-1}, Female: 0.31±0.015 g·day^{-1}) as compared to the other diet treatments (GUN male: 0.46±0.11 g·day^{-1}; GUN female: 0.45±0.17 g·day^{-1}; CON male: 0.46±0.012 g·day^{-1}; CON female: 0.48±0.15 g•day^{-1}). Additionally, individual days of the experiment elicited significant differences in body weight. At PN1, GUN mice (Male: 1.3±0.13 g, Female: 1.3±0.09 g) were born significantly (P=0.0001) smaller than both CON (Male: 1.8±0.17 g, Female: 1.6±0.10 g) and PUN (Male: 1.7±0.29 g, Female: 1.6±0.22 g) mice. Following PN1 the body mass pattern consisted of CON mice being the largest (P<0.001), followed by GUN and lastly PUN mice through PN21. At PN21, GUN mice (Male: 10.0±1.17 g, Female: 9.9±1.02 g) were significantly (P<0.0001) smaller than CON mice (Male: 11.8±0.68 g, Female: 11.2±0.52 g), but significantly larger than PUN mice (Male: 8.2±0.97 g, Female: 8.1±0.93 g). From PN1-21 there was no sex effect on body mass.

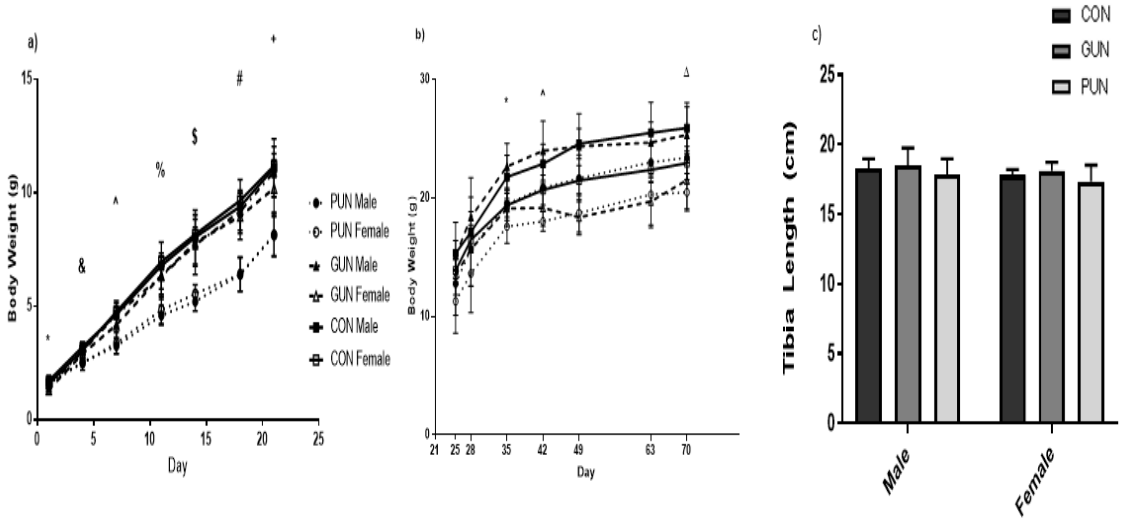

Figure 2. Growth and Body Composition. Panel A. Growth of mice subjected to gestational (GUN) and postnatal undernutrition (PUN) as compared to controls (CON) on days 1-21 following birth.

Values are expressed as mean ± SD. At PN1, GUN mice were born significantly (*P=0.0001) smaller than both CON and PUN mice. By PN4, CON mice were significantly (&P<0.0001) larger than both GUN and PUN mice. GUN mice were significantly (&P<0.0001) larger than PUN mice. GUN mice were significantly (^P<0.0001) smaller than CON mice but significantly larger than PUN mice at PN7. This continued at PN11, where GUN mice were significantly smaller (%P<0.0001) than CON mice but larger than PUN mice. At PN14, CON mice were significantly ($P<0.0001) larger than GUN and PUN mice. GUN mice were also significantly larger than PUN mice at this time point. GUN mice were significantly (#P<0.0001) smaller than CON mice at PN18, but significantly larger than PUN mice. At PN21, CON mice were significantly (+P<0.0001) larger than both GUN and PUN mice. GUN mice were significantly larger than PUN mice as well. Panel B: Growth of mice from PN21 to PN70. At PN35, when CON mice were significantly larger (*P=0.0006) than PUN mice. At PN42, CON and GUN were significantly larger (^P<0.0001) than PUN mice. At PN49, there was no difference between the GUN mice and PUN mice, but CON mice were still significantly larger (P=0.0425) than PUN mice. By PN70, CON mice were significantly larger (ΔP=0.0441) than PUN mice, but there were no differences with GUN mice between either groups Panel C: Tibia length measured at PN70 in mice. Values are expressed as mean ± SD.

After weaning to the control diet there was no difference (P=0.081) in growth rate (Figure 2b) between the groups. At PN35, CON mice (Male: 23.1±1.2 g, Female: 20.0±1.01 g) and GUN mice (Male: 22.1±2.25 g, Female: 18.9±1.38 g) were significantly larger (P=0.0006) than PUN mice (Male: 19.4±1.20 g, Female: 17.8±0.78 g). This pattern continued throughout the study. At PN70, CON mice (Male: 24.9±1.62 g, Female: 23.0±1.42 g) were significantly larger (P=0.0441) than PUN mice (Male: 22.6±2.7 g, Female: 20.0±2.26 g), with GUN mice (Male: 24.0±2.2 g, Female: 19.7±1.92 g) not different from either group. From PN21-70 there was no effect of sex on body mass. While there were differences in final body mass, there was no difference in tibia length at PN70 (Figure 2c).

Voluntary Physical Activity

Following the one-week wheel acclimation period, the PUN mice had significantly (P=0.005) shorter wheel running bout duration as compared to the rest of the groups (Figure 3a). Furthermore, PUN males ran significantly (P=0.025) slower than CON males, GUN males and GUN females (Figure 3b). There was no difference in running speed between PUN females, and CON females (Figure 3b). No differences were observed (P=0.9224) among the groups in the number of wheel running bouts initiated lasting less than 4 seconds in length (Figure 3c), nor number of bouts initiated longer than four seconds in length (Figure 3d). The PUN female mice had a reduction (P=0.0221) in wheel revolutions per day as

compared to the GUN females and CON females. There were no differences in wheel revolutions per day amongst the male groups (Figure 3e).

Unlike the previous week, week 2 of wheel running indicated a trend (P=0.061) for reduced bout duration in the PUN female mice as compared to the other groups (Figure 4a). Similar to week 1 there was no significance (P=0.224) in average running speed (Figure 4b), and wheel bouts lasting less than 4 seconds (Figure 4c). However, PUN females, PUN males, and CON males had significantly less (P=0.0243) extended wheel running bouts (longer than 4 seconds) than CON female, and GUN female. GUN male mice did not differ in terms of extended wheel running bouts (Figure 4d). GUN females and CON females had higher (P=0.0163) daily wheel revolutions than PUN females during week 2 of wheel running, with no difference between the male mice (Figure 4e). The linear regression analysis showed no effect (P=0.53; $R^2 = 0.02$) of body weight on wheel revolutions

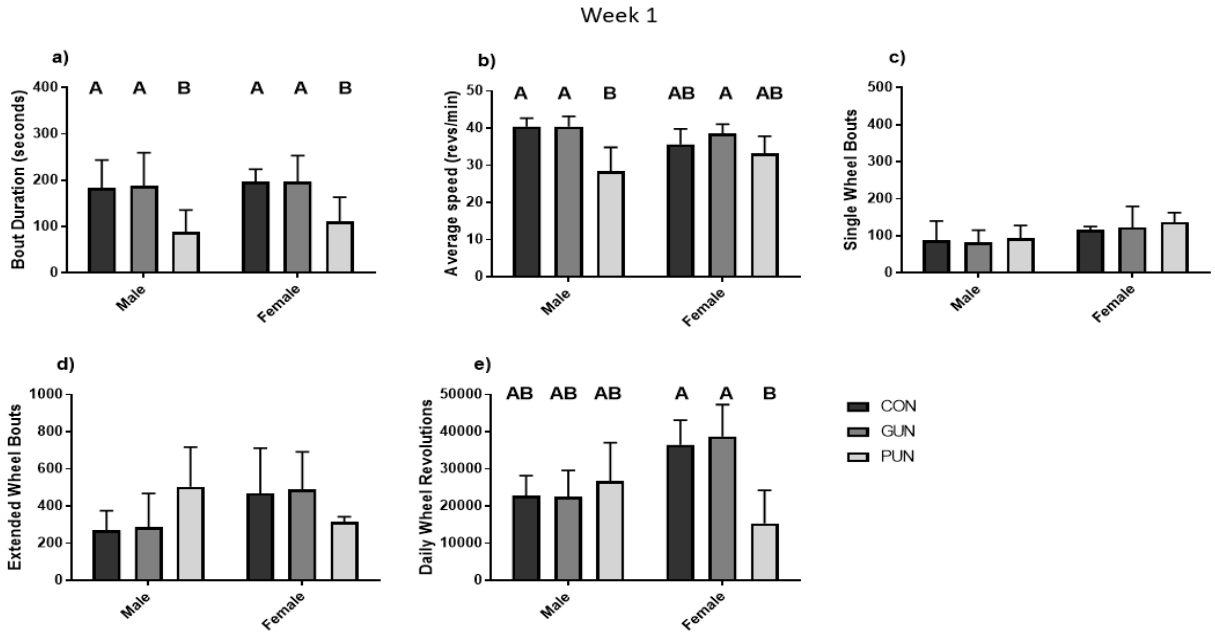

Figure 3. Week 1 of wheel running of mice subjected to gestational (GUN) and postnatal undernutrition (PUN) as compared to controls (CON). Values are expressed as mean ± SD and differing letters signify statistical difference between groups (P<0.05). Panel A: average duration of wheel running per day. Panel B: average speed during each wheel running bout. Panel C: average number of wheel running bouts per day lasting less than 4 seconds. Panel D: average number of wheel running bouts per day that lasted longer than 4 seconds. Panel E: average daily wheel spins.

Western blots

The PUN females expressed a significantly (P=0.0247) higher Drd1/Gapdh as compared to the other groups (Figure 5a). PUN females expressed more (P=0.0015) Casq1/Gapdh compared to the other groups (Figure 5b). There were no differences (P=0.490) in Annexin A6/Gapdh expression between groups (Figure 5c).

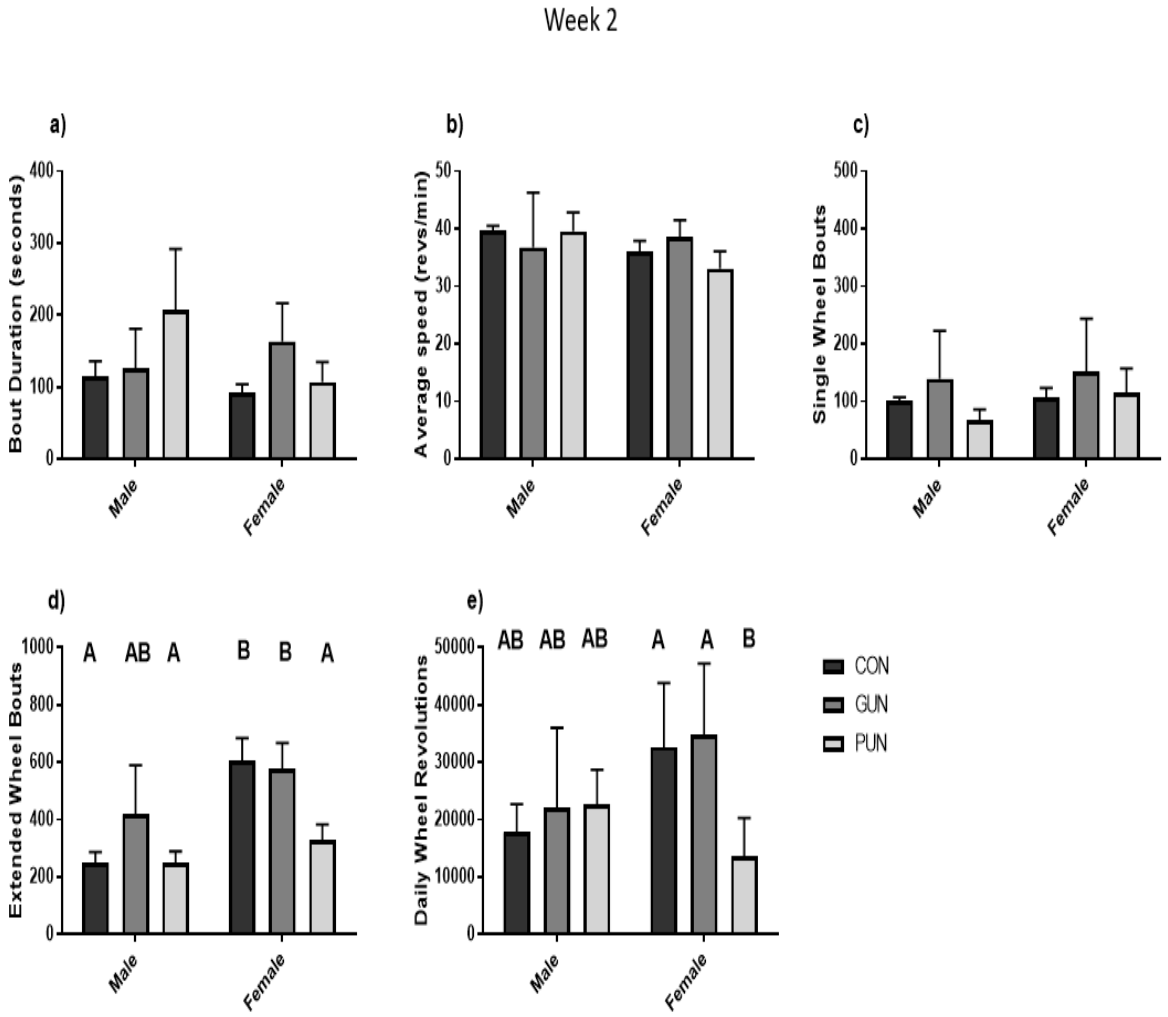

Figure 4. Week 2 of wheel running of mice subjected to gestational (GUN) and postnatal undernutrition (PUN) as compared to controls (CON). Values are expressed as mean ± SD and differing letters signify statistical difference between groups (P<0.05). Panel A: average duration of wheel running per day. Panel B: average speed during each wheel running bout per day. Panel C: average number of wheel running bouts per day lasting less than 4 seconds. Panel D: average number of wheel running bouts per day that lasted longer than 4 seconds. Panel E average daily wheel spins.

Skeletal Muscle Fiber Type and Cross-Sectional Area

A representative image of skeletal muscle histology is presented in Figure 6m (male) and 6n (female).

Diaphragm

No differences were observed in diaphragm type I (P=0.7177, Figure 6a), type IIa (P=0.3939, Figure 6b), type IIx (P=0.6419, Figure 6c), or type IIb (Figure 6d) fiber percentage. However, there was a trend (P=0.1398) for PUN mice to have more type IIb fibers compared to GUN and CON mice.

There was no difference in CSA of diaphragm type I (P=0.6561, Figure 7a), type IIa (P=0.9965, Figure 7b), type IIx (P=0.9508, Figure 7c), or type IIb (P=0.6792, Figure 7d) fibers between groups. GUN male type IIb fiber CSA had a trend (P=0.0660) to be larger than PUN and CON mice.

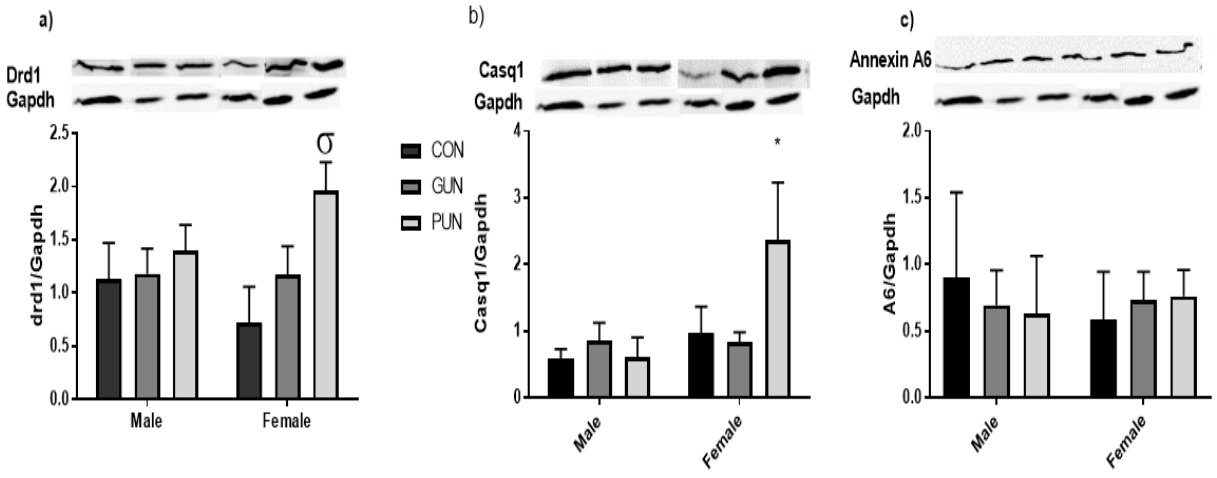

Figure 5. Western blots for proteins associated with physical activity engagement standardized to Gapdh. Values are expressed as means ± SD. Panel A. Dopamine receptor 1 expression in the nucleus accumbens of mice exposed to undernutrition during gestation (GUN) and lactation (PUN) as compared to controls (CON). PUN females expressed significantly (σP=0.0247) higher amount of Drd1 as compared to the other groups. Panel B. Calsequestrin1 expression in the soleus of mice exposed to gestational undernutrition (GUN), lactational undernutrition (PUN) compared to controls (CON). The PUN females had a significant (*P=0.001) higher expression as compared to the other groups. Panel C. Annexin A6 expression in the soleus of mice exposed to gestational undernutrition (GUN), lactational undernutrition (PUN) compared to controls (CON).

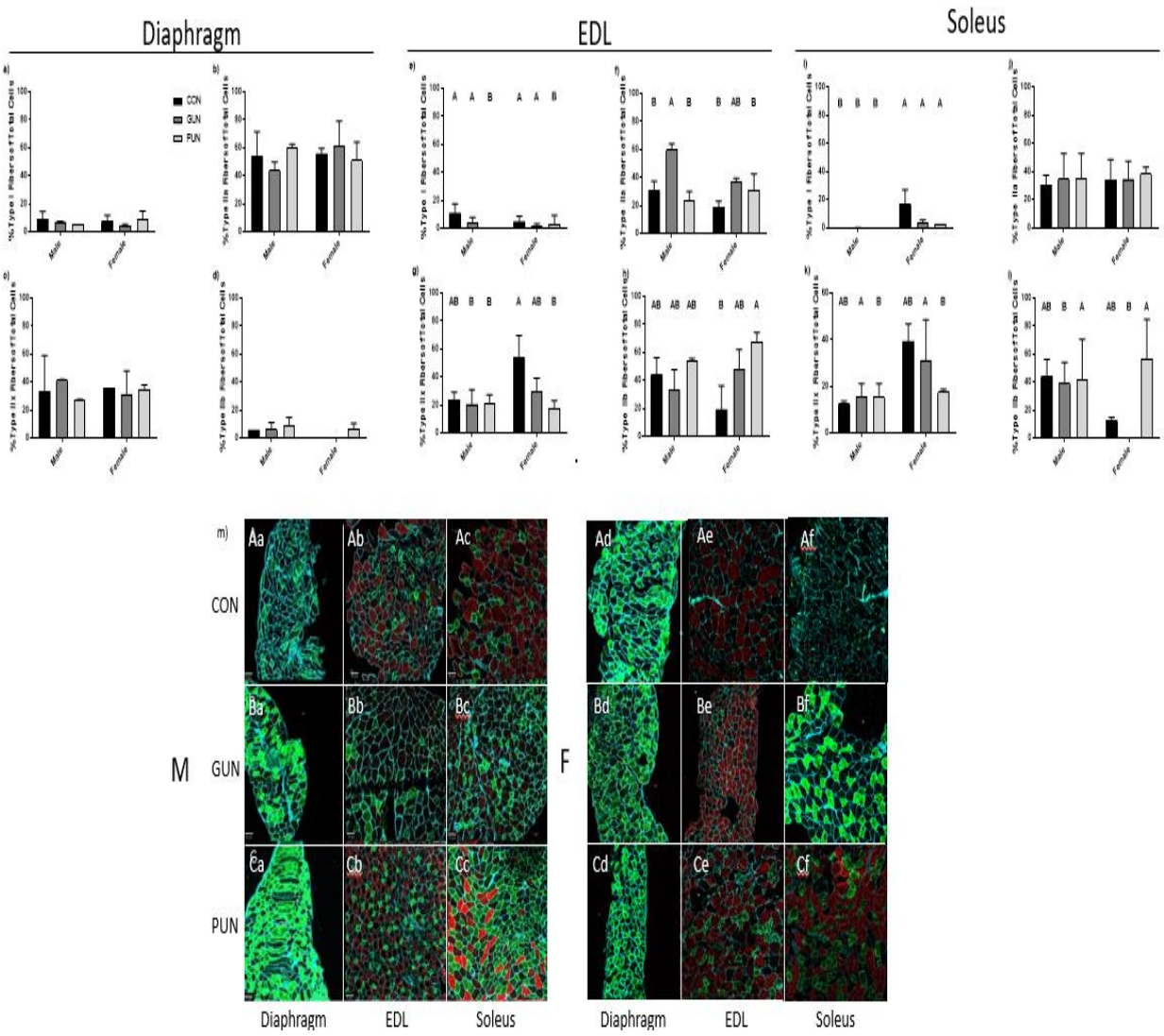

Figure 6. Skeletal muscle fiber type expressed as percent of total cells in the diaphragm, soleus and extensor digitorum longus of mice subjected to gestational (GUN) and postnatal undernutrition (PUN) as compared to controls (CON). Values are expressed as mean ± SD and differing letters signify statistical difference between groups ($P<0.05$). Panel A: percent type I fibers of the diaphragm. Panel B: Percent type IIa fibers of the diaphragm. Panel C: Percent type IIx fibers of the diaphragm. Panel D: Percent type IIb type fibers of the diaphragm. Panel E: Percent type I fibers of the EDL. Panel F: Percent type IIa fibers of the EDL. Panel G: Percent type IIx fibers of the EDL. Panel H: Percent type IIb fibers of the EDL. Panel I: Percent type I fibers of the soleus. Panel J: Percent type IIa fibers of the soleus. Panel K: Percent type IIx fibers of the soleus.. Panel L: Percent type IIb fibers of the soleus. If there are no bars shown, there were no cells for that diet group. Panel 6m represents muscles from control males (Diaphragm 6Aa, EDL 6Ab, and soleus 6Ac), GUN males (Diaphragm 6Ba, EDL 6Bb, soleus 6Bc), and PUN males (Diaphragm 6Ca, EDL 6Cb, soleus 6Cc). Panel 6n represents muscle from CON females (Diaphragm 6Ad, EDL 6Ae, soleus 6Af), GUN females (Diaphragm 6Bd, EDL 6Be, soleus 6Bf), and PUN females (Diaphragm 6Cd, EDL 6Ce, soleus 6Cf). Green cells represent type IIa myosin ATPase, blue cells represent type I myosin ATPase, black cells show cells without staining and represent type IIx myosin ATPase, and red cells represent type IIb myosin ATPase. Cells are outlined by laminin (blue stain).

EDL

PUN mice had less (P=0.0031, Figure 6e) type I fibers than CON and GUN mice. GUN male mice had more (P=0.0398) type IIa fibers compared to CON male, CON female, PUN male and PUN female mice. GUN females did not differ in type IIa fiber expression between groups (Figure 6f). Female CON mice had more (P=0.0438) type IIx fibers compared to PUN male, PUN female, and GUN male. GUN female and CON male type IIx fiber expression was not different from the other groups (Figure 6g). PUN female mice had increased (P=0.0409) type IIb fibers compared to CON females. There were no differences in type IIb fibers amongst the other diet treatment groups (Figure 6h).

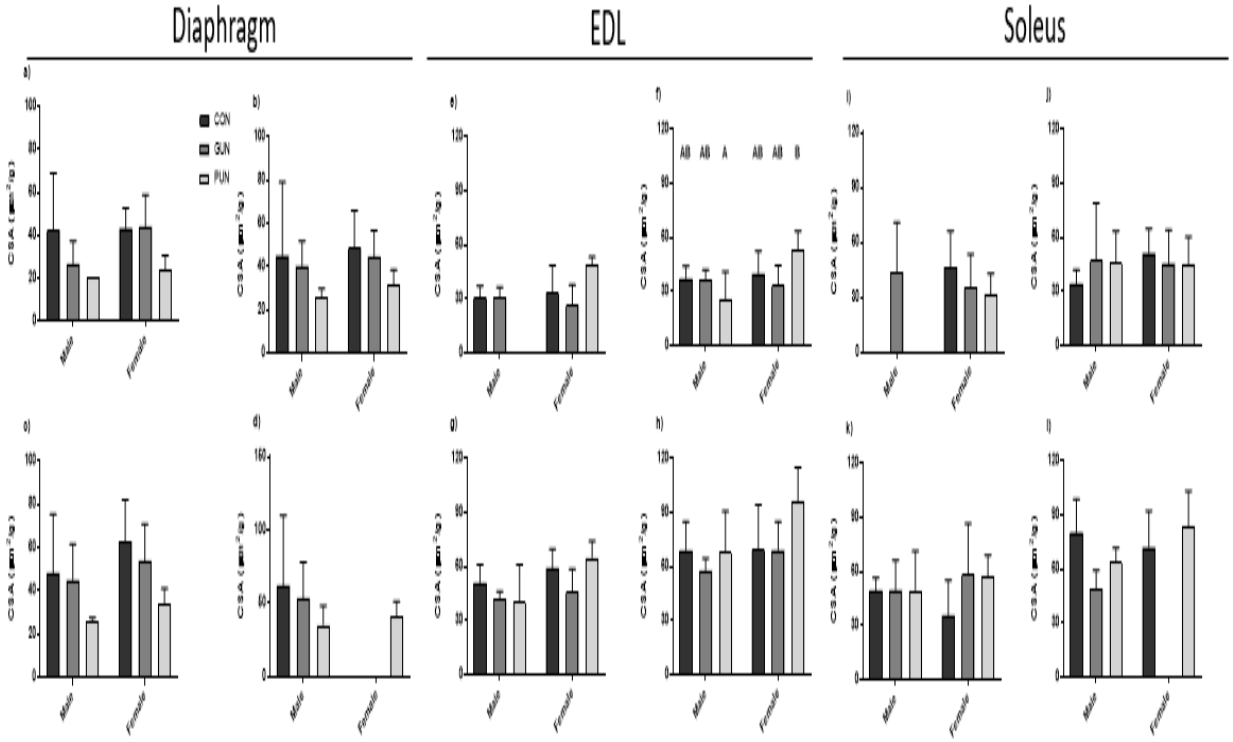

Figure 7. Skeletal muscle cross sectional area (CSA) in the diaphragm, soleus and extensor digitorum longus of mice subjected to gestational (GUN) and postnatal undernutrition (PUN) as compared to controls (CON). Values are expressed as mean ± SD and differing letters signify statistical difference between groups (P<0.05). Panel A: CSA of type I fibers in the Diaphragm. Panel B: CSA of type IIa fibers in the Diaphragm. Panel C: CSA of type IIx fibers in the Diaphragm. Panel D: CSA of type IIb fibers in the Diaphragm. Panel E: CSA of type I fibers in the EDL. Panel F: CSA of type IIa fibers in the EDL. Panel G: CSA of type IIx fibers in the EDL. Panel H: CSA of type IIb fibers in the EDL. Panel I: CSA of type I fibers in the soleus. Panel J: CSA of type IIa fibers in the soleus. Panel K: CSA of type IIx fibers in the soleus. Panel L: CSA of type IIb fibers in the soleus.

There were no differences (P=0.5240) in CSA of type I fibers between groups (Figure 7e). PUN females had larger (P=0.0398) type IIa fibers than PUN male mice, with no difference between the other groups (Figure 7f). No difference in CSA of type IIx (P=0.9508, Figure 7g) or type IIb (P=0.3775, Figure 7h) fibers were present amongst the diet groups.

Soleus

A significant (P=0.009) sex effect was observed, where females had more type I fibers compared to males, and a diet by sex trend (P=0.0676) for CON females to have more type I fibers than GUN males, CON males, and PUN males (Figure 6i). There were no differences (P=0.6949) amongst groups for type IIa fiber expression (Figure 6j). There was a significant (P=0.0376) diet effect on type IIx fiber distribution, where GUN mice had more type IIx fibers compared to PUN mice, with CON mice not being different from the other groups (Figure 6k). A sex effect showed female mice had more (P=0.0365) type IIx fibers compared to males. PUN mice had more (P=0.0314) type IIb fibers compared to GUN mice. CON mice type IIb fiber expression was not different from the other groups (Figure 6l). We confirmed our soleus fiber type expression using SDS PAGE and silver nitrate staining (Figure 8).

No significant differences were seen in the soleus CSA of type I (P=0.8696, Figure 7i), type IIa (P=0.7205, Figure 7j), type IIx (P=0.9564, Figure 7k), or type IIb fibers (P=0.3057, Figure 7l).

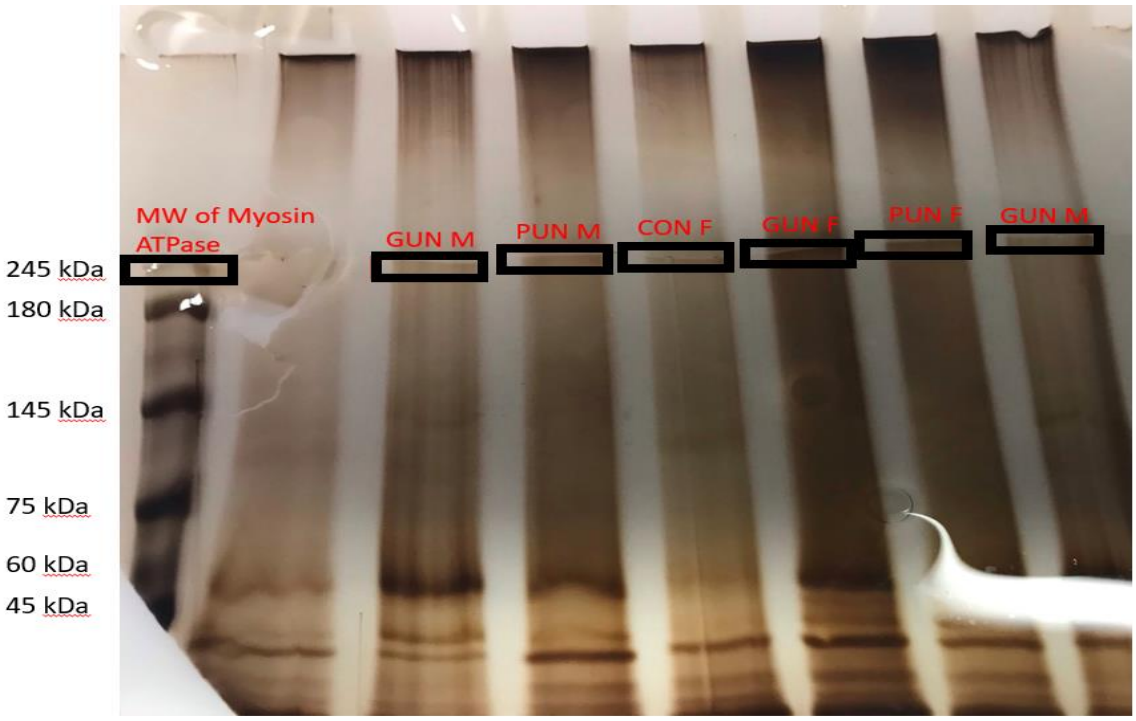

Figure 8. Silver nitrate stain to confirm soleus fiber type percentage.

Discussion

Regular physical activity engagement reduces the risk of chronic disease (120-122). Alarmingly, early life growth-restriction is associated with deceased physical activity in adulthood (8, 16). As growth-restricted individuals already have an increased risk for cardiovascular disease, type II diabetes, hypertension, and sarcopenia (1, 7, 38, 107), the fact that these individuals also experience less physical activity engagement could further increase their mortality risk. Thus, we characterized the biological mechanisms for physical activity engagement in a growth-restricted model so that future investigations could develop therapeutic countermeasures to reduce mortality risk.

Our results showed that mice that were undernourished during gestation (GUN) were born smaller as compared to the control group, indicating gestational growth-restriction was achieved. Once refeeding occurred, the GUN mice experienced catch-up growth, while undernutrition during lactation caused permeant growth-restriction (PUN, Figure 2). We and others have continually demonstrated growth-restriction due to PUN to be primarily a result of reduced adiposity and impaired skeletal muscle protein synthesis via a decrease in ribosomal abundance (17, 49).

Voluntary physical activity, as measured by total wheel revolutions per day, was reduced in female PUN mice by 59% and 53% as compared to CON on week 1 and 2, respectively. The male mice did not display a difference in total revolutions per day but did present with significant differences in isolated wheel running behavior. Specifically, during week 1, PUN male and female mice had shorter wheel running bouts (Figure 3a), with the indication that PUN mice potentially run at a slower speed (Figure 3b). There were no differences in the number of bouts initiated (Figure 3c and 3d).

The reduced wheel revolutions phenotype of the PUN female mice persisted through week 2 of measurement (Figure 4e). However, the reduction in wheel revolutions during week 2 were not due to a reduced session duration (Figure 4a) nor running speed (Figure 4a) as seen in week 1, but a reduced number of wheel running bouts initiated per day (Figure 4d). While literature has demonstrated a

negative correlation between body weight and wheel running levels (123), results from the regression analysis demonstrate that body weight was not a predictor for the differences in voluntary physical activity levels ($P=0.53$, $R^2=0.02$). Therefore, we conclude reduction in wheel running is due to developmental programming not body weight.

The capacity to be active is reflected in the speed of wheel running (ability to resist fatigue), while the drive to active is represented by the number of wheel running bouts initiated ("want" to be on the wheel). Revolutions per day and session duration could be influenced by both the drive and capacity to be active. Specifically, the drive to be active is associated with reward driven behavior, and can manifest as seeking out wheel exposure, or if "reward" is achieved the mouse will stop wheel running regardless of peripheral fatigue factors (15).

Knab *et al.* showed less physically active mouse strains have higher expression of Drd1 mRNA (20). PUN female mice, the least physically active group in our investigation, had increased Drd1 protein compared to the other diet groups (Figure 5a). Thereby, postnatal growth-restriction reduces the drive for physical activity engagement.

Interestingly, the PUN female mice had a higher expression of Casq1 in the soleus, which is typically expressed more in type II fibers (124). Ferguson *et al.* demonstrated that increased expression of Casq1 causes more frequent and forceful contractions of skeletal muscle leading to increased wheel revolutions (25). As the PUN female mice had reduced wheel running, in the growth-restricted model the biological drive to be active potentially overrides the capacity.

Expanding on skeletal muscle's role on the capacity to be active, the Garland lab has been selectively breeding mice for high levels of wheel running activity, and after 22 generations observed an increased proportion of oxidative fibers in all skeletal muscle (23, 24), allowing for a more fatigue resistant phenotype and sustained wheel running. Our results showed that PUN female mice had increased type IIx fibers in the soleus and EDL. The PUN female mice in this study potentially have a

reduced physical activity level due to a reduction in oxidative fibers. Consistent with this conclusion, the PUN male mice did not have reduced physical activity nor oxidative fibers compared to controls.

While there was a difference in fiber type, CSA differences between the groups were minimal: PUN females had larger type IIa fibers in the EDL compared to PUN males. Increased CSA increases oxygen diffusion transit time, thereby reducing oxidative capacity and potentially wheel running (23, 24).

The fiber type distribution for the EDL and diaphragm is consistent with existing literature (125, 126). However, the fiber type distribution for the soleus is small. Although atypical, others have reported a reduction in oxidative fibers using this nutritive model in the FVB strain (17). It should be noted that exposure to wheel running for three weeks has caused skeletal muscle fibers to shift to the type IIa phenotype (127). Three weeks of wheel running (1 acclimation week, 2 weeks of measurement) was used to limit wheel running as a result of exploratory behavior (novelty of the wheel), which has been done extensively in the literature (15, 20, 76, 108). Thus, although we were able to reliable characterize physical activity in the growth-restricted mouse model, a potential confound could be a fiber type shift. Although possible, the magnitude of a fiber type shift is minimal as the literature on growth-restricted mice indicates a similar fiber type phenotype to the data presented in this investigation (20, 49).

It is necessary to acknowledge the role of sex hormones on physical activity engagement. Female mice typically run more than male mice due to the effects of estradiol. Work by Bowen *et al.* demonstrated that removal of the ovaries reduced wheel running whereas replacement with 17β-estradiol restores wheel running to pre-surgical levels (128). We did not evaluate sex hormones in this study, but gestational growth-restriction studies indicate impairment in sex organ development, but upon refeeding, the impairment is recovered (129). However, growth-restriction during the postnatal period reduces expression of androgen receptors (130). Thus, it can be speculated that the PUN mice have physical activity alterations via androgen receptor expression. However, the literature on postnatal growth-restriction influencing sex hormone function is scant. Future studies should examine the role of growth-restriction during lactation on sex hormone concentration and physical activity engagement.

The aim of this investigation was to determine if isolated windows (gestation or lactation) of growth-restriction influenced physical activity via established biological mechanisms. Our results indicated that gestational undernutrition does not influence physical activity in adulthood, but undernutrition in lactation reduced wheel running in the female group. We propose that Drd1 expression is the primary factor, but also highlight the skeletal muscle peripheral factors that influence physical activity engagement. It should be noted that these are not the only biological mechanisms proposed to regulate physical activity. A recent review by Lightfoot *et al.* (15) in Medicine and Science in Sport and Exercise discussed the multitude of factors responsible for physical activity engagement, with specific examples related to Cannabinoid receptor 1 and melanocortin expression (131). Thus, future studies should investigate the variety of biological parameters that influence physical activity engagement in growth-restricted individuals.

APPENDIX

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REFERENCES

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