

The Effects of Resistance Wheel Running on Skeletal Muscle Function and Adaptation in
C57BL/10SnJ Mice

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Abstract

Background: Resistance wheel running (RWR) can promote resistance-like training adaptations in mouse skeletal muscle (SkM), but its endurance-training effects are lesser known. **Methods:** Voluntary RWR was modulated as an exercise model to increase mouse hind-limb plantar-flexor torque and to promote endurance-training adaptations. Thirty male mice (cohort 1, n= 16; cohort 2, n= 14), were trained on a prototype RWR system that applied resistance relative to body mass (BM). Mice were sequentially, (1) screened for running ability (screening; 3-days); (2) trained with incremental adjustments to wheel loads (pre-training; 8-weeks); (3) grouped into cage-activity only (CA), and constant Low-0%, Med-15%, or High-25% BM resistance conditions (static training; 5-weeks); (4) trained with resistance adjusted in real-time (dynamic training; cohort 1, 7-weeks; cohort 2, 10-weeks); and (5) sacrificed for various assays. Plantar-flexor torque was determined during each training phase. After dynamic training, resistance runners in each cohort were sub-grouped *post-hoc* by work tertiles. **Results:** Wheel running distance varied between cohorts (cohort 2 > 1). During dynamic training, wheel running ($\pm$ added-resistance) improved plantar flexor torque normalized to BM by 19% only in cohort 2 ($p= 0.007$). Muscle mass and cross-sectional area were unchanged. Runners in both cohorts ($\pm$ added-resistance) improved maximal running capacity vs. CA-controls (+69% and +115%; both $p < 0.05$), but metabolic training adaptations were less evident. **Conclusions:** Wheel running promoted SkM strength and endurance, but there was a greater increase in endurance capacity than strength. This outcome may be due to adaptive signaling interference.

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List of Important Abbreviations

AMPK- Adenosine Monophosphate-activated Kinase
ANOVA- Analysis of Variance
BHAD- Beta-Hydroxyacyl-CoA Dehydrogenase
BM- Body Mass
CA- Cage-Activity control mice
CK- Creatine Kinase
CS- Citrate Synthase
CSA- Cross-sectional Area
EDL- Extensor Digitorum Longus muscle
FAO- Fatty Acid Oxidation
FG- Fast-Glycolytic fiber
FOG- Fast-Oxidative-Glycolytic fiber
FWR- Free-Wheel Running
GAS- Gastrocnemius muscle
GO- Glucose Oxidation
HFES- High-Frequency Electrical Stimulation
MHC- Myosin Heavy Chain
mTOR- Mammalian Target of Rapamycin
p70^{S6K}- Ribosomal p70 S6 Kinase
PFT- Plantar Flexor Torque
PGC-1 α - Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 α
PLN- Plantaris muscle
QUAD- Quadriceps femoris muscle
RWR- Resistance Wheel Running
SC- Satellite Cell
SED- Sedentary
SkM- Skeletal Muscle
SO- Slow-Oxidative fiber
SOL- Soleus muscle
TA- Tibialis Anterior muscle
Tri- Triceps muscle

Chapter 1: Introduction

Section 1.1: Statement of Problem

There are few skeletal muscle (SkM) hypertrophy models in mice that easily translate to SkM hypertrophy in humans (e.g., hypertrophy following resistance training). Some current mouse models of SkM hypertrophy include high-frequency electrical stimulation (1-3), synergistic ablation (4, 5), insulin-like growth factor 1 gene administration/modulation (6-8), and myostatin gene modulation (e.g., by follistatin viral vectors (9)). While each of these models has strengths and weaknesses, as a group it would be difficult to-translate/implement these hypertrophy models to humans.

One mouse exercise model, which has been understudied despite its potential relevance to humans, is the application of resistance to running wheels. The results of several studies suggest that resistance wheel running (RWR) may increase SkM size and strength in mice (10-13). However, these studies had various limitations. For example, only one of these studies in mice investigated how RWR affects markers of endurance training in SkM (13) and none of these studies monitored the effects of RWR on contractile properties of prime movers over time.

The work described herein aimed to develop a new RWR system and exercise program for C57BL10/SnJ mice, and to determine how RWR affects markers of endurance and resistance training adaptation in SkM. The broader goal of this work was to develop/characterize a RWR exercise model in wild-type mice, with the idea that the system could be subsequently used in mouse models of neuromuscular disease.

Section 1.2: Specific Aims and Sub-Aims

1. To develop a novel resistance running wheel system.
 - a. To develop an exercise program for mice, which is computer-controlled and can set wheel resistance as a function of animal body mass, can limit work output, and can be manipulated in real-time.
2. To determine if RWR promotes functional and physiological adaptations similar to endurance and/or resistance training.
3. To determine how RWR alters various physiological parameters over time *in-vivo*.
 - a. To assess the contractile properties of hindlimb plantar flexor muscles in C57BL/10SnJ mice over time (between ages 1 and 7 months), relative to cage-activity/detrained controls and free-wheel runners.
 - Method: hindlimb plantar flexor muscles were stimulated *in-vivo* and contractile properties (e.g., torque and temporal data) were obtained by computer program.
 - b. To determine if RWR improves body composition (i.e., increased lean mass and decreased fat mass).
 - Method: lean and fat masses were quantified by nuclear magnetic resonance.
 - c. To determine if RWR and free-wheel running increase treadmill running fitness and hanging wire endurance.
4. To determine how RWR alters various physiological parameters in isolated tissues.

- a. To determine if wheel running decreases plasma creatine kinase activity, relative to cage-activity controls.
 - Method: plasma creatine kinase activity kit.
- b. To determine if RWR increases muscle mass and/or increases cross-sectional area of primary running muscles, relative to cage-activity controls and free-wheel runners.
 - Method: muscle masses were obtained on a scale and immunofluorescence was performed to label cell membranes for determination of cross-sectional area.
- c. To determine if RWR promotes metabolic adaptations in skeletal muscle similar to those caused by free-wheel running.
 - Method: quadriceps muscles were assayed for glucose oxidation, fatty acid oxidation, and oxidative enzyme activities *ex vivo*.
- d. To determine if free-wheel running and RWR decrease central nucleation of primary running muscles, relative to cage-activity controls.
 - Method: immunofluorescence was performed to label cell membranes and to determine relative positions of myonuclei.
- e. To determine if free-wheel running and RWR promote satellite cell activity that is characteristically different from cage-activity controls.
 - Method: immunocytochemistry was performed to quantify the differentiative and proliferative capacities of harvested satellite cells.

- f. To determine if free-wheel running increases the proportion of type I-positive fibers and if RWR increases the proportion of IIA-positive fibers, compared to cage-activity controls.

- Method: immunohistochemistry was performed to stain for type I and IIA myosin heavy chain.

Section 1.3: Hypotheses

1. Relative to cage-activity controls and free-wheel runners, RWR will develop stronger plantar flexor muscles.
2. Relative to cage-activity controls, RWR and free-wheel runners will similarly increase whole-body lean mass and decrease fat mass over time.
3. Relative to cage-activity controls, RWR and free-wheel running will similarly increase treadmill running fitness to exhaustion, but RWR will exhibit better hanging wire endurance than cage-activity controls and free-wheel runners.
4. Relative to cage-activity controls, RWR and free-wheel runners will have lower plasma CK activity at sacrifice.
5. Relative to cage-activity controls and free-wheel runners, RWR will increase mass and cross-sectional area of gastrocnemius, soleus, tibialis anterior, and triceps muscles.
6. Relative to cage-activity controls, free-wheel running and RWR will similarly increase glucose and fatty acid oxidative capacities, as well as beta-hydroxyacyl CoA dehydrogenase and citrate synthase activities in quadriceps muscles.
7. Relative to cage-activity controls, free-wheel running and RWR will similarly decrease central nucleation.

8. Relative to cage-activity controls, free-wheel running and RWR will increase the differentiative and proliferative capacities of satellite cells in skeletal muscles.
9. RWR will increase type IIA-positive fibers relative to cage-activity controls and free-wheel runners, but free-wheel runners will express more type I-positive fibers (in gastrocnemius, soleus, tibialis anterior, and triceps muscles).
10. RWR will promote SkM adaptations similar to both endurance and resistance training, whereas FWR will only promote endurance training adaptations.

Chapter 2: Background and Literature Review

Section 2.1: Skeletal Muscle Biology

2.1.1 Introduction

Skeletal muscle (SkM) is a specialized tissue that undergoes cycles of contraction and relaxation to produce tensile forces and thereby facilitate skeletal movement. SkM exhibits a range of functional characteristics such as conductivity, excitability, contractility, elasticity, and adaptability (14). SkM can conduct and transduce electrical signals received from the central and peripheral nervous systems; these stimuli modulate SkM's cycles of contraction and relaxation (14). Following contraction or externally imposed stress, SkM can elastically rebound to its original size/shape, ready for its next task (14). Finally, if SkM is activated enough and pushed to a certain threshold via exercise, it can adapt to improve performance when conducting similar tasks in the future (14). These characteristics are essential for SkM to effectively carry out its locomotor functions.

2.1.2 Skeletal Muscle Structure and Arrangement

2.1.2.1 Skeletal Muscle Structure

During maturation, SkM develops into a structural hierarchy, including the whole muscle, fascicle, myofiber, myofibril, and the sarcomere; the sarcomere is the fundamental unit of muscular contraction (Figure 1).

Sarcomeres are primarily composed of thick (myosin) and thin (actin) protein filaments, which interact with one another in a sliding motion to produce tensile forces. Sarcomeres in series form a myofibril; bundles of myofibrils form and span the length of a myofiber, or individual muscle cell (14).

Myofibers are closely packed together in SkM. Bundles of myofibers (oriented in parallel) form fascicles, and bundles of fascicles form whole muscle. Individual myofibers, fascicles, and whole muscles are surrounded by layers of fibrous connective tissue, or fascia, known as the endomysium, perimysium, and epimysium, respectively. SkM fascia integrates itself into sheaths that form the tendons and/or aponeuroses on either end of a muscle (originating on, or inserting into, bone). The connection between myofibers and the surrounding fascia is crucial for longitudinal and lateral force transduction (15, 16). SkM-fascial connections are primarily due to costamere structures, which are formed by proteins in the dystrophin-glycoprotein complex (DGC) that link sarcomeres to the extracellular matrix. Severe muscular dystrophy and locomotor impairment occur when SkM lacks functional dystrophin (or related DGC proteins) (15, 17).

2.1.2.2 Sarcomere Structure and Arrangement

Sarcomeres in SkM are $\sim 2.0 - 3.65\mu\text{m}$ long (14) and can contract $\sim 20\text{-}50\%$ because thin actin filaments interdigitate with and slide along thick myosin filaments (14). The extent of overlap between thick and thin filaments influences the sarcomere's ability to generate force. Optimal overlap for force production, or L_0 , usually occurs when sarcomere length is $\sim 2.0 - 2.2\mu\text{m}$ long; force production potential decreases outside of this range (14).

Myosin filaments in sarcomeres are $\sim 1.65\mu\text{m}$ long (18). These filaments are composed of two heavy chain subunits, which form a central tail and two terminal heads, and two pairs of light chain subunits that support and regulate the activity of the myosin heads (one pair for each head) (18, 19). The central tail region takes the shape of a rod-like coiled-coil complex that is formed by the C-terminal sections of each myosin heavy chain, also known as light meromyosin (20). Each end of the tail section is connected to a flexible neck region (one on either side of the

myosin molecule); which associates with a pair of myosin light chains (regulatory and essential). The light chains provide structural support for the neck and modulate contractile activity (19). The N-terminus of the myosin heavy chain molecules form the globular myosin heads, which interact with actin filaments to produce sarcomeric contraction (20). Actin filaments are $\sim 2.0\mu\text{m}$ long helical structures formed by fibrillar F-actin, which is a polymer of globular G-actin proteins (20). F-actin interacts with, among other proteins, nebulin, for longitudinal stability, which serves as a molecular template for the thin filament (18).

Electron microscopy reveals the overlap of thick (myosin-dominated) and thin (actin-dominated) filaments form a striated structure of alternating dark and light bands with several distinct regions. Within the light bands, the Z-discs can be observed; sarcomeres are located between Z-discs (adjacent sarcomeres share a Z-disc). From the center-outward, the characteristic regions of a sarcomere are: the M-line, H-zone, A-band, and I-band (Figure 1).

The Z-disc is (mostly) made of actinin protein (20). Actinin in the Z-disc interacts with a spring-like protein called titin to connect the Z-disc to the M-line (21). With its spring-like properties, titin is thought to be responsible for SkM passive tension (18). The M-line marks the center of the sarcomere and is contained within the H-zone, which is primarily made of myosin protein. The H-zone is a subsection of the A-band. The A-band is entirely composed of thick (myosin) filament. Finally, the I-band is the region between the A-bands of two adjacent sarcomeres (Figure 1) (14, 22).

During contraction, the A-band remains the same length (i.e., the thick filament) while the H-zone shortens because the thin (actin) filaments are pulled towards the M-line; whereas the thick (myosin) filaments do not change position. When the sarcomeres in a myofiber

simultaneously contract, the entire myofiber contracts; when enough myofibers contract they produce enough tension to shorten the whole muscle.

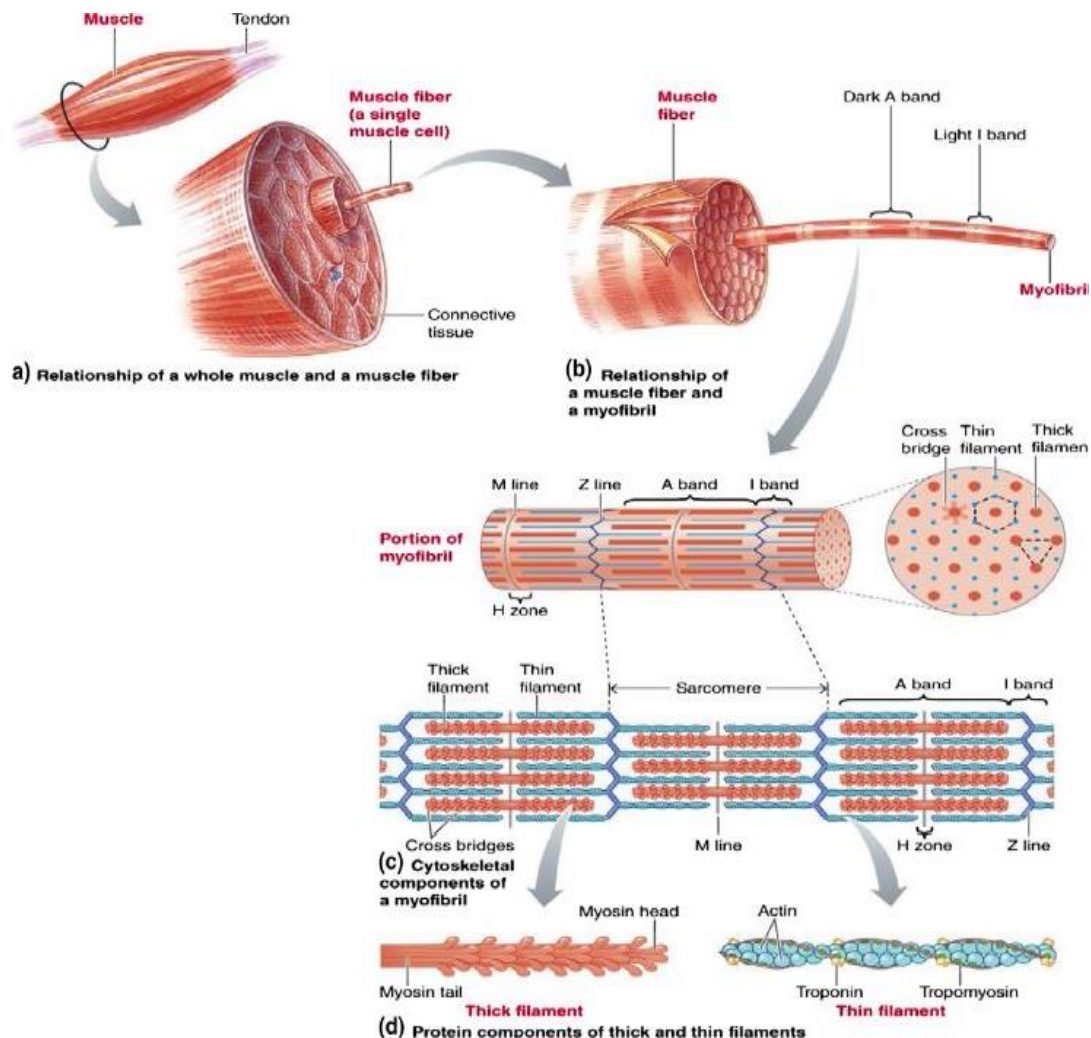


Figure 1. Skeletal Muscle Hierarchy and Sarcomere Structure.

a) Whole muscles are composed of bundles of fascicles, and ultimately of bundles of myofibers that contain myofibrils. **b)** Myofibrils are formed by sarcomeres connected end-to-end at Z-lines (or Z-discs); serial sarcomeres create banding patterns with dark (A) and light (I) bands. **c)** The sarcomere is made of thick and thin filaments, which interact to form cross-bridges for contraction. **d)** Thick (myosin) filaments have a central tail and two polar heads. The central tails of myosin proteins are coiled together, while the myosin heads radiate outward to cross-bridge with thin (actin) filaments. Actin filaments are made of actin and regulatory and structural proteins like troponin and tropomyosin. Image reprinted with kind permission from Springer Science and Business Media: Springer and Calcified Tissue International, 2014, Skeletal Muscle: A Brief Review of Structure and Function, Frontera WR and Ochala J, Figure 3.

2.1.2.3 Myofiber Development and Satellite Cells

SkM tissue arises from the embryonic mesoderm and ultimately composes 40-60% of total body mass (23). During SkM development, myoblasts either mature into myofibers or remain quiescent. During maturation, myoblasts fuse together end-on-end to form myotubes, which are multinucleated and develop into mature myofibers (22, 24). Each nucleus in a myofiber is responsible for the transcriptional/translational activities required for its specific cytoplasmic domain (22, 24).

Under conditions of growth, repair, or adaptation to exercise, myofibers can incorporate more nuclear and cytoplasmic materials from local pools of quiescent myoblasts (muscle stem cells also known as satellite cells (SC)) (22, 24). SC number varies depending on the muscle but satellite cell reserves are generally low in healthy SkM (<2% of total nuclei) (25-27) and decrease with age, due to inherent limitations of their self-regenerative capacity (28). When sufficient mechanical strain is imposed on SkM, satellite cells are activated and they begin to proliferate; these events are mediated by significant changes in gene expression (29). For example, quiescent SCs robustly express paired box 7 (Pax7) transcription factor, but do not express the transcription factors: myogenic differentiation (MyoD) or myogenic factor 5 (Myf5) (25, 30-32). Upon activation, SCs proliferate and begin to differentiate as they express MyoD and/or Myf5. Then, most of the partially differentiated satellite cells begin to express myogenin, which triggers terminal differentiation and fusion with a local myofiber (25, 30). However, proliferated satellite cells also have the option of down-regulating MyoD to return to quiescence (25, 33-35). During adulthood, satellite cells are recruited in response to injury (36) and SkM overload (4, 36, 37). When satellite cells are recruited in response to overload, they promote

SkM hypertrophy by increasing the fiber's capacity to produce myofibrillar proteins (4, 36, 37). When more myofibrillar protein is synthesized, myofiber volume and anatomical cross-sectional area (CSA) increase. Muscles with larger volume and CSA can produce more force (38).

2.1.3 Skeletal Muscle Bioenergetics and Metabolism

To power the contractile structures in SkM, myocytes must efficiently produce adenosine triphosphate (ATP), the 'energy currency' of the cell. Hydrolysis of the gamma and beta-phosphodiester bonds in ATP are highly exergonic (i.e., thermodynamically favorable); hence, ATP is the dominant energy source for cell functions, such as metabolism and SkM contraction (39).

SkM can generate ATP anaerobically (i.e., O₂ not required) and aerobically (i.e., O₂ required). Key anaerobic mechanisms for ATP production include the ATP-phosphocreatine (ATP-PC) system and anaerobic glycolysis. The key aerobic ATP-producing system is known as oxidative phosphorylation. During exercise, contractile proteins in SkM rapidly use significant quantities of ATP; hence SkM metabolism is also highly responsive to fluctuating conditions to ensure that ATP-supply meets ATP-demand (39). The primary ATP-producing systems are summarized in table 1. Following the table, there are descriptions of the each system, and a summary schematic is provided at the end of this section (Figure 2).

Table 1. Summary of ATP Production Mechanisms Used during Exercise			
Properties	Mechanism		
	ATP-PC	Anaerobic Glycolysis	TCA cycle/Oxidative Phosphorylation
Dominant Exercise Time Period	1-5 seconds	Up to 2 minutes	Beyond 2 minutes
Requires O ₂	No	No	Yes
Carbon Substrate(s)	n/a	Glucose	Glucose or Fatty Acids
NADH Produced	0	2	3

FADH ₂ Produced	0	0	1
Net ATP Yield	1	2	~10 per acetyl-CoA

During an exercise bout the ATP-PC system (first 1-5 seconds) and anaerobic glycolysis (up to 2 minutes) initially supply enough ATP to meet contractile demands before oxidative phosphorylation can be sufficiently engaged to supply the majority of SkM ATP (39, 40). However, as exercise duration increases, all of the ATP-producing mechanisms are engaged: aerobic metabolism is just more heavily used than anaerobic mechanisms to supply ATP.

The ATP-PC system involves the enzymatic transfer of inorganic phosphate (P_i) from phosphocreatine to a molecule of adenosine diphosphate (ADP) via creatine kinase (CK), to form one molecule of ATP. This system provides quick energy because it can be rapidly engaged, but it is limited by cytosolic phosphocreatine. Hence, for the first ~1-5 seconds, the ATP-PC system is the dominant bioenergetic mechanism used to supply ATP during exercise (39).

Anaerobic glycolysis is the next system used to supply the majority of ATP up to ~120 seconds of exercise (39). During anaerobic glycolysis, glucose (from local muscle glycogenolysis and blood) is catabolized and oxidized in a series of enzymatic reactions to produce 2 pyruvate molecules and 2 ATP molecules (39).

During glycolysis, metabolic intermediates other than pyruvate and ATP are produced, such as reduced nicotinamide adenine dinucleotide (NADH; referred to as NAD^+ when oxidized). When oxygen supply is low, lactate dehydrogenase (LDH) reduces pyruvate to lactate with its electrons on NADH; this reaction allows SkM to maintain glycolytic flux (and therefore maintain ATP supply) despite low O_2 supply (39, 40).

Under aerobic conditions (ample O_2 available), pyruvate and NADH are shuttled into a mitochondrion for aerobic ATP production (39). In SkM, mitochondria form two

subpopulations: ~20-25% reside just beneath the sarcolemma, while the remaining ~75-80% form networks that surround the contractile apparatus to allow for quick and easy ATP delivery (39, 41).

In the mitochondrion, pyruvate is converted to acetyl-coenzyme A (CoA) by the pyruvate dehydrogenase complex (PDC). Acetyl-CoA in the mitochondrial matrix (inner luminal portion) can enter the tricarboxylic acid (TCA) cycle, where acetyl-CoA is enzymatically combined with a molecule of oxaloacetate to produce citrate via citrate synthase (CS). During the TCA cycle, citrate is enzymatically metabolized by a series of reactions that ultimately produce 3 NADH, 1 ATP, and 1 reduced flavin adenine dinucleotide (FADH₂; referred to as FAD when oxidized) (39). To accomplish aerobic respiration, the electrons transferred from reactions in the TCA cycle (and from glycolysis) must be transported to the inner mitochondrial membrane on NADH and FADH₂ to drive oxidative phosphorylation (39) (Figure 2).

NADH and FADH₂ donate their electrons to specific protein complexes in the electron transport chain (ETC) of the inner mitochondrial membrane to initiate a series of electron transfers that results in the reduction of O₂ to H₂O and the formation of ATP (by cytochrome C oxidase and ATP synthase, respectively). Each turn of the TCA cycle ultimately yields ~10 ATP (39).

Overall, oxidative phosphorylation can produce ~32 net ATP from a single molecule of glucose, making oxidative phosphorylation much more efficient than anaerobic mechanisms. Hence, oxidative phosphorylation produces the majority of ATP during steady-state exercise when metabolic demands are relatively constant (i.e., steady-state heart rate and VO₂) (39). However, the production of ATP via oxidative phosphorylation is relatively slow compared to anaerobic mechanisms. So ATP-PC and anaerobic glycolysis are also required to maintain ATP

status, particularly during the early stages of exercise when O₂ delivery rate does yet not meet the acute metabolic demands of contraction.

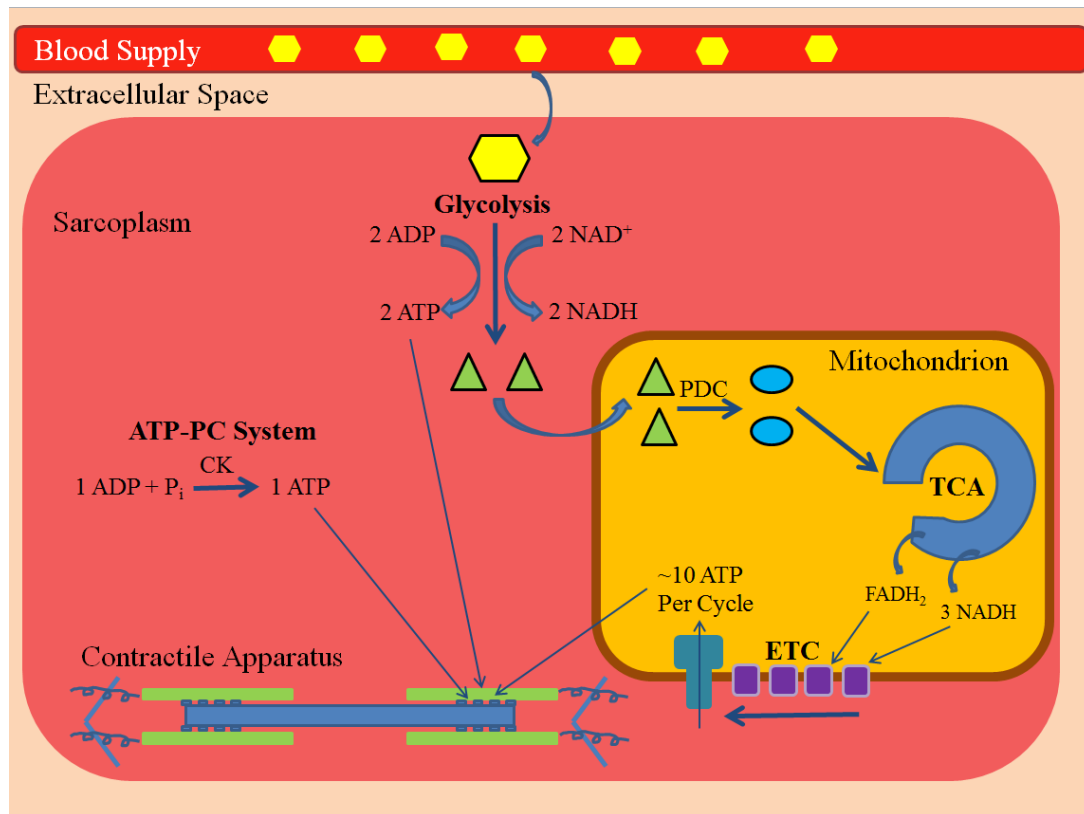


Figure 2. Schematic of ATP Production Systems in Skeletal Muscle.

The basic steps and outcomes involved in the ATP-PC system, glycolysis, and oxidative phosphorylation (TCA & ETC) are shown in their respective cellular compartments. Several colored shapes represent important molecules in SkM metabolism: yellow hexagons (glucose), green triangles (pyruvate), blue circles (acetyl-CoA). Ultimately, ATP is produced by each energy system and delivered to the contractile apparatus to power locomotion. CK= Creatine kinase; TCA= Tricarboxylic Acid cycle; ETC= Electron Transport Chain.

2.1.4 Skeletal Muscle Excitation and Contraction

2.1.4.1 Mechanism of Skeletal Muscle Excitation and Contraction

SkM contraction is initiated by an action potential (AP). APs originate in the brain (e.g., motor cortex) or spinal cord (i.e., motor neuron pools) and are delivered to specific target

muscles via axons. Each muscle fiber is innervated by a single motor neuron. Motor neurons originate in the spinal cord and their axons form branches that synapse with a group of myofibers; together, the motor neuron and its group of innervated myofibers are a motor unit. When a motor neuron is activated, it activates all of the myofibers in its motor unit (14).

Communication between the motor neuron and its myofibers is achieved by the action of a neurotransmitter known as acetyl-choline (ACh). When a motor neuron is activated by an AP, the AP is propagated to the end of the motor neuron, known as the axon button. When the AP reaches the axon button, a series of calcium (Ca^{2+})-induced biochemical events transduces the electrical signal (AP) to a chemical signal by triggering the release of acetyl-choline (ACh) from vesicles in the axon button. ACh molecules diffuse across the synaptic cleft and bind to the ACh receptor on a special section of the sarcolemma, known as the motor endplate. When the ACh receptor is activated, the receptor interacts with and activates sodium (Na^+)/potassium (K^+) ion channels, which triggers an influx of Na^+ . The influx of Na^+ depolarizes the post-synaptic membrane (from -90mV to +30mV) and the AP spreads across the sarcolemma (39). When the AP reaches and depolarizes the transverse or t-tubule networks, the dihydropyridine receptor (DHPR) is activated (14). The DHPR is physically linked to the ryanodine receptor (RyR) at the sarcoplasmic reticulum (SR; which serves as an intracellular storage site of calcium) (14). The RyR is a voltage-gated Ca^{2+} channel, which facilitates Ca^{2+} release from the SR (42). When Ca^{2+} is released from the SR via the RyR, Ca^{2+} diffuses through the sarcoplasm to initiate sarcomere contraction (14).

As the concentration of sarcoplasmic Ca^{2+} increases, Ca^{2+} ions accumulate on binding sites on the actin-regulating protein, troponin-C. Troponin-C co-localizes/complexes with several regulatory proteins: troponin-I (a contraction inhibitor), troponin-T (tropomyosin-binding

troponin), and tropomyosin. These proteins form the troponin-tropomyosin complex. When Ca^{2+} binds to troponin-C, the troponin-tropomyosin complex undergoes a conformational shift that exposes actin's binding sites for globular "myosin heads", which are located at the N-terminus of myosin's heavy chain (14, 20). The myosin head serves as an ATP hydrolase that can bind to F-actin. Myosin ATPases convert the chemical energy in ATP's gamma-phosphodiester bond into mechanical energy to enable movement of actin and myosin filaments (20, 43) (Figure 3).

When ATP is bound to the myosin head, the myosin head has weak affinity for actin, but when ATP is hydrolyzed (forming the products ADP and P_i), chemical energy is released and strong myosin-actin binding results. When P_i is released from the myosin head, the power stroke occurs which pulls actin (and the Z-disc) towards the M-line on each side of the sarcomere, which results in muscle shortening. This cycle of myosin-actin binding and release is called cross-bridge cycling. As ATP-powered cross-bridge cycles simultaneously occur on either side of the M-line, power strokes are generated that pull actin filaments along the myosin filaments towards the center of the sarcomere in a ratchet-like motion, thus producing tensile force (14, 20).

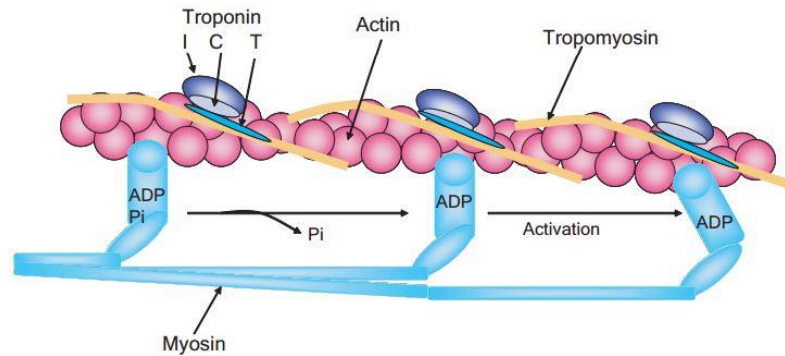


Figure 3. Actin-Myosin Cross-bridge Formation.

Calcium ions (not shown) bind to troponin C, which promotes a conformational shift in the troponin-tropomyosin complex; the conformational shift exposes myosin-head binding sites (on actin) for cross-bridge formation. After ATP is cleaved into ADP and P_i by the myosin head ATPase, the P_i is removed, which promotes a ratchet-like motion (power stroke) that pulls on actin and causes the sarcomere to contract. Image reprinted from Philip M. Hopkins, *Skeletal Muscle Physiology, Continuing Education in Anaesthesia, Critical Care & Pain*, Vol. 6, pg. 4, 2006, by permission of Oxford University Press.

2.1.4.2 Muscle Relaxation

When APs from the nervous system cease to stimulate a myofiber, the sarcolemma begins to repolarize, which inhibits the activities of DHPR and RyR to promote relaxation. These events slow down Ca^{2+} release from the SR, and sarcoplasmic Ca^{2+} is concomitantly sequestered via several mechanisms; these events decrease overall Ca^{2+} concentration in the sarcoplasm. Depending on the muscle, Ca^{2+} is sequestered by the activities of a combination of sarco-endoplasmic reticulum calcium ATPase (SERCA) pumps, parvalbumin (in fast fibers), and/or calsequestrin (in the SR) (44, 45). As Ca^{2+} concentrations fall, troponin-C's affinity for Ca^{2+} decreases; as Ca^{2+} is released from troponin-C, a reverse conformational shift of the troponin-tropomyosin complex occurs, and myosin heads can no longer bind to sites on actin (14, 20).

2.1.4.3 Types of Muscular Contraction

The primary purpose of SkM contraction is to move the skeleton for locomotion. However, skeletal muscles can produce force when changing length or remaining static (46). The three major types of SkM contractions that occur during typical daily activities are concentric, isometric, and eccentric. Concentric muscular contractions are defined as force production during muscle shortening (e.g., curling a free-weight). Isometric contractions are defined as force production with no change in muscle length (e.g., pushing or pulling against a fixed object). Eccentric contractions are defined as force production while the muscle is lengthening (i.e., external load exceeds muscle force output).

2.1.4.4 Contractile Properties

SkM contractile properties include functional and temporal properties. Contractile measures can be obtained at the level of individual excised fibers or whole muscles *in-vitro*, or in whole muscle groups *in-vivo*. Several important *in-vivo* contractile properties include active torque (AT), time to peak torque (TPT), and half-relaxation time (HRT) (Figure 4). AT is defined as maximum torque minus baseline torque (also known as resting torque). TPT is defined as the time elapsed between baseline torque and maximum. HRT is defined as the time elapsed between maximal torque and one-half maximal torque (during relaxation) (47, 48).

When stimulated by a single AP or artificial electrical stimulus (delivered as a square pulse), muscle(s) produce low (minimal) torque and then relax back to baseline; this response is called a twitch (48) (Figure 4).

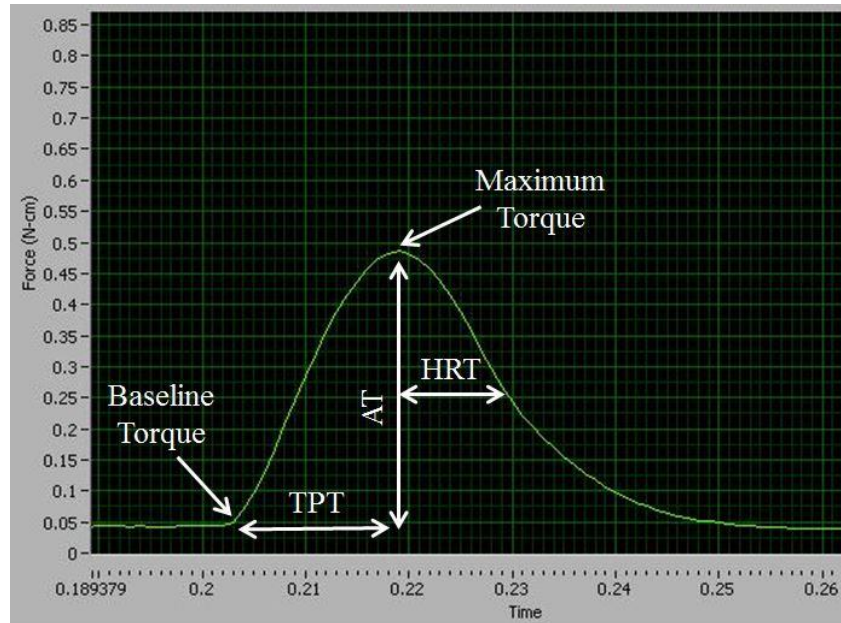


Figure 4. Representative Isometric Twitch Profile.

This twitch was produced by a mouse's hindlimb plantar flexor muscles when activated by needle electrodes *in-vivo*. The profile of the twitch illustrates that, upon stimulation, muscles produced active torque (AT; maximum torque minus baseline torque), and promptly relaxed. Time to peak torque (TPT) and half-relaxation time (HRT) were also recorded. Screenshot was taken while analyzing the twitch profile, using Dynamic Muscle Analysis software version 5.2.1.1 (Aurora Sci., Aurora, ON). Note: Y-axis is Torque (in N*cm; labeled "Force" in program); X-axis is Time (in seconds).

When electrical stimuli are delivered at increasing frequencies, the stimulated muscles do not have enough time between stimulations to sequester all the Ca^{2+} released from the SR, which prevents relaxation and results in continued torque production. As a result torque production increases with increased stimulation frequency; this process is known as temporal summation (48). Plotting torque as a function of stimulation frequency reveals how summation occurs in a roughly sigmoidal fashion until maximal torque production occurs, which is referred to as fused tetanus (Figure 5). Assessing the profile of torque-frequency curves can reveal information both about the nature of the myofibers involved in contraction (e.g., fast, powerful muscles vs. slow,

endurance muscles; further described in section 2.A.5) and the functional capacity of a group of muscles (e.g., comparing the area under the curve or steepness of the slope) (Figure 5).

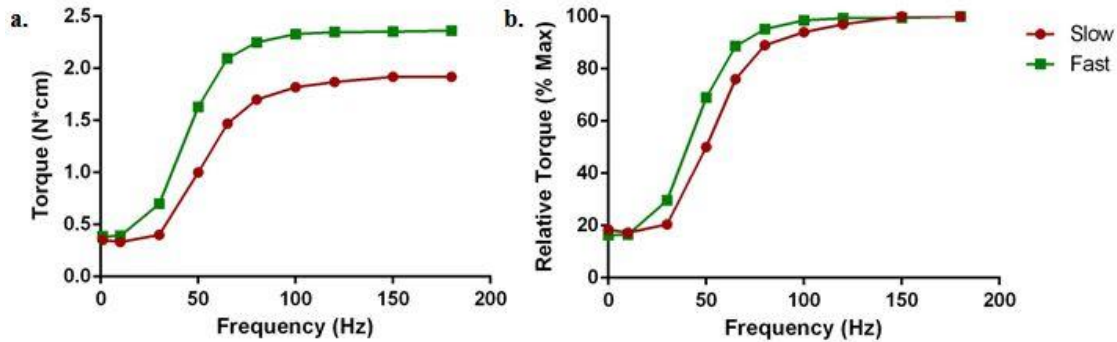


Figure 5. Representative Profiles of Absolute and Relative Torque-Frequency

a) Theoretical examples of absolute torques produced by characteristically slow and fast muscles when stimulated at increasing stimulation frequencies. Typically, slow muscles produce less absolute torque than fast muscles. **b)** The profiles of torque produced (as a percent of maximum) when stimulated at increasing frequencies. Typically, fast muscles reach their maximum faster than slow muscles.

2.1.5 Fiber Types

In rodents, SkMs express a distribution of characteristically different types of myofibers, but some muscles are predominantly composed of a single fiber type. Myofiber types range on a continuum from slow-oxidative (SO or type I), to fast-oxidative glycolytic (FOG or type-IIA), to fast glycolytic (FG or type-IIX/B). These classifications refer to 1) a fiber's maximal contractile speed, which is determined by a fiber's predominant expression of myosin heavy chain (MHC) isoforms, and 2) a fiber's capacity to oxidize different fuel sources (Table 2). The different MHC isoforms contain different ATPases which differ in their rates of ATP hydrolysis (49). MHC isoform expression also varies across species (40, 49). Mice express the following MHC isoforms in SkM: type I → IIA → IIX → IIB (listed in order of increasing enzyme reaction rate). Humans do not express type IIB (14).

According to the size principle of motor unit recruitment, small (SO) motor units are first recruited to handle SkM load, but as intensity and load increase, the larger (FOG, then FG) motor units are recruited (39, 49).

Generally, SO fibers contract and relax slower, generate less power, have a greater capacity for aerobic metabolism, have higher myoglobin content, and are more resistant to fatigue than the other fiber types (40, 49). Within SO fibers, the sarcomeres are densely surrounded by mitochondria, which help these fibers to resist fatigue in times of energy demand. Additionally, SO fibers produce large quantities of myoglobin, which serves as an O₂ reserve and contributes to their distinct red color (14). SO fibers are highly expressed in postural muscles (e.g., soleus), which are very frequently active. In contrast, FG fibers contract and relax more quickly, generate the most power, have greater anaerobic capacity, and fatigue faster than the other fiber types. FG fibers do not produce as much myoglobin as other types of fibers; hence they appear whiter in color. FG fibers are typically needed for high intensity activity, such as weight lifting or sprinting, and are the last fibers recruited by motor units (49). FOG fibers exhibit physiologic characteristics in-between SO and FG fibers.

Table 2. General Properties of Skeletal Muscle Fiber Types			
Properties	Fiber Type Classification		
	Slow-Oxidative (SO)	Fast-Oxidative-Glycolytic (FOG)	Fast-Glycolytic (FG)
Predominant MHC	Type I	Type IIA	Type IIX or IIB
Contractile Velocity	Slow	Intermediate	High
Glycolytic Potential	Low	Intermediate	High
Oxidative Potential	High	Intermediate	Low
Mitochondrial Density	High	Intermediate	Low
Myoglobin Content	High	Intermediate	Low
Resistance to Fatigue	High	Intermediate	Low

Although fiber types are distinct from one another, SkM fibers can actually shift their fiber type under certain conditions, such as artificially imposing alternate innervation patterns, hindlimb suspension, and synergistic ablation (49-51). Additionally, fiber type shifts can occur with exercise (52-55), where endurance exercise promotes an FG→FOG→SO shift, while resistance exercise may promote the opposite shift (39, 54, 55). Two additional points: basal fiber type distribution is largely determined by genetics (56, 57) while exercise-induced fiber type shifts appear to be limited (49, 56, 57). Generally, high SO fiber expression is positively correlated with aerobic fitness, while individuals with high FG fiber expression are likely to be either inactive or demonstrate propensity for sports that require high muscle power output (e.g., track or football) (49, 58).

Section 2.2: Exercise and Skeletal Muscle

2.2.1 Introduction to Exercise

Exercise can be classified on a continuum ranging from endurance (aerobic) to resistance (strength and power) training. Endurance exercise is characterized by submaximal, prolonged, and rhythmic whole-body activity that stresses the cardiorespiratory system to maintain metabolic demands of working muscles (e.g., running, swimming, cycling). Resistance exercise stresses muscle groups with high-intensity, high-power, short-duration contractions (e.g., weight lifting or sprinting exercises). Chronic endurance training promotes characteristically different SkM adaptations than those of chronic resistance training; these changes are mediated by different molecular signaling events that modulate gene transcription and translation. Adaptations to exercise help muscles to limit future disturbances in cellular homeostasis during subsequent bouts of similar physical activity (59-62).

2.2.2 Parameters and Principles of Exercise Training

Exercise training and adaptation are affected by several key parameters and principles. Training is defined by parameters, such as mode, frequency, duration, and intensity; whereas adaptation is affected by the principles of overload, specificity, and reversibility. The exercise mode (e.g., running, swimming, lifting) will determine the adaptations that occur in SkM, such that subsequent bouts of similar physical activity can be accomplished more easily (59-62). Immediately following an acute exercise bout, SkM responds by modulating adaptive gene transcription and translation for ~3-12 hours. Typically after ~24 hours, conditions return to baseline (39, 60). Hence, performing exercise sessions frequently (3-5 sessions per week) will enhance SkM adaptations (46, 56, 60). The duration of the exercise bout also affects adaptation; typically, the longer the exercise bout lasts, the more potent the activation of gene transcription. However, the duration that can be sustained during an exercise bout will depend on the intensity of the work being performed (39). Intensity is often quantified relative to an individual's maximal volitional capacity (e.g., maximal oxygen consumption rate ($\text{VO}_{2\text{max}}$) or 1-Repetition Maximum (1RM)).

The combination of exercise intensity and duration determines the magnitude of the exercise stimulus, which must achieve a critical threshold to overload SkM and drive adaptive signaling (39, 56, 63). A common training practice to achieve overload is to increase workload by 10% each week (63), but exercise adaptive responsiveness varies considerably between individuals (64). Exercise adaptation does not occur unless the muscle is overloaded, so muscles that are not stressed during an exercise program will not be driven to adapt (i.e., SkM adaptation is limited to the primary working muscles of a given exercise) (14). Further, exercise adaptations are specific to their type of training. Endurance and resistance exercise impose different stresses

on muscles, and therefore promote different SkM adaptations mediated by different molecular signaling events and gene transcription/translation. Finally, SkM exercise adaptations are reversible: the newly synthesized proteins are eventually degraded and conditions will return to their original steady state if training ceases (39, 60).

2.2.3 Endurance Exercise

Endurance, or aerobic, exercise involves rhythmic, high-repetition, low-powered SkM contraction over long durations (minutes to hours). Endurance training aims to improve the individual's ability to resist fatigue and thereby sustain tasks that sub-maximally stress the cardiorespiratory system (e.g., long-distance running, swimming, or cycling). Typically, SkM starts to noticeably adapt to endurance training after weeks or months (39, 40), but the rate and magnitude of adaptation depend on training parameters (frequency, duration, intensity) and genetic predisposition (40, 65, 66). At the cellular level, acute and chronic aerobic training modulate the expression of key genes (metabolic and structural), which determine a myofiber's ability to resist the underlying molecular causes of fatigue.

2.2.3.1 Skeletal Muscle Fatigue

SkM fatigue can be defined as the failure to sustain a target level of force production while performing constant or intermittent contractions (67). Generally, SkM fatigue is associated with metabolic markers of ATP demand. Such markers include 1) increased sarcoplasmic concentrations of H^+ (decreased pH), adenosine monophosphate (AMP), ADP, P_i , NAD^+ , and lactate (40, 46), concomitantly with 2) decreased sarcoplasmic concentrations of ATP, phosphocreatine, NADH, O_2 , and muscle glycogen (40). Many of these molecular markers

allosterically influence the activity of key proteins/enzymes involved in metabolism, contraction, and adaptive signaling.

For example, AMP-activated kinase (AMPK), a potent metabolic regulator, is allosterically activated as the ratios of AMP/ATP and creatine/phosphocreatine rise; AMPK is allosterically inhibited when these ratios fall (40, 68, 69). Acute AMPK activation (i.e., during an acute aerobic exercise bout) serves as a signal for the cell to conserve ATP, hence AMPK acts to restore ATP balance by activating cellular glucose and fatty acid transport and oxidation (70, 71), and by simultaneously suppressing synthesis of glycogen and protein (40, 68, 72). These actions attenuate acute perturbations of cellular ATP status during exercise. Beyond acute effects, AMPK's signaling activities during exercise can also elicit longer-lasting, adaptive responses in SkM by modulating key transcription factors (e.g., peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α)), which modulate protein synthesis and produce significant phenotypic changes in SkM (e.g., increased mitochondrial biogenesis and angiogenesis) (40, 69, 73). However, AMPK is just one of many sensors that respond to muscle contractile activity to maintain cellular homeostasis and activate adaptive processes.

2.2.3.2 From Contraction to Transcription

When SkM is overloaded, the combination of mechanical strains, ATP and Ca²⁺ flux, O₂ deprivation, changing oxidation-reduction (redox) conditions (i.e., NAD⁺/NADH ratio), etc. activate intracellular sensors, which trigger intracellular signaling cascades that modulate gene transcription/translation processes and drive adaptation (40). Thus, significant contractile activity is coupled with molecular, physiological, and functional adaptations in acute and chronic exercise settings. In addition to AMPK, other well-known fatigue sensors include: calmodulin (CaM) and associated kinases (CaMKII and CaMKIV) and phosphatases (calcineurin), p38

mitogen activated protein kinase (p38 MAPK), hypoxia inducing factors (HIF), and sirtuins (SIRT) (40, 74, 75). These sensors are activated by and respond to sarcoplasmic Ca^{2+} transients (74), free radicals (39), low O_2 tension (75, 76), and increased NAD^+/NADH ratio (73, 75), respectively. All of these sensors influence the transcriptional activity of PGC-1 α , which mediates many aerobic phenotype adaptations as a co-activator with other transcription factors, such as the peroxisome proliferator-activated receptor (PPAR) family, nuclear respiratory factors (NRFs), myocyte enhancing factors (MEFs), forkhead box containing protein, O-subclass (FOXOs), etc. (77, 78).

2.2.3.3 Key Endurance Training Adaptations

Key SkM adaptations to endurance exercise include: increased oxidative capacity, increased capillary and mitochondrial density, enhanced fatty acid oxidation and glycogen sparing, and a fast-to-slow fiber type shift (40, 75, 79). At the end of this section, figure 6 illustrates many of the pertinent endurance exercise signals and adaptations that are discussed below (75).

Endurance training improves the oxidative capacity of myofibers by increasing the number of surrounding capillaries, internal myoglobin content, and mitochondrial density of activated fibers (39). Angiogenesis of capillaries is under the control of regulators like HIF1 and vascular endothelial growth factor (VEGF); endurance exercise activates these angiogenic factors through PGC-1 α (77, 80). With high capillarity and myoglobin content, more O_2 can be delivered to mitochondria; and with increased mitochondria, a myofiber can increase its ATP production capacity (39).

SkM mitochondrial biogenesis is under the control of nuclear and mitochondrial genes and transcription factors that respond to exercise, most notably PGC-1 α (77). When PGC-1 α is

activated by exercise sensors (i.e., phosphorylated by AMPK, CaMKs, p38MAPK, and deacetylated by sirtuins), it works with NRFs and other transcription factors in the nucleus to activate aerobic gene transcription programs (78, 81). An important protein product of this transcriptional activity is mitochondrial transcription factor-A (Tfam), which then translocates to the mitochondrial matrix and activates mitochondrial-encoded protein synthesis (77). Other gene transcriptional targets of PGC-1 α (and its co-activators) include ETC, TCA cycle, and β -oxidation enzymes (82). In addition to increased mitochondrial content, recent studies show that endurance exercise also improves mitochondrial quality control by activating mitophagic pathways, which phagocytize old/sub-optimal mitochondria (78). The combination of increasing mitochondrial enzymes and mitochondrial number with endurance training increases myofiber oxidative capacity.

Endurance training also enhances mechanisms associated with fatty acid oxidation (FAO), which produces more ATP per molecule than aerobic glucose oxidation (GO) (i.e., FAO is more metabolically efficient than GO) (14). Training increases expression of fatty acid binding protein, fatty acid translocase, and carnitine palmitoyl transferase 1 (CPT1), all of which help transport free-fatty acids (FFA) into mitochondria for aerobic catabolism (83). This process is also aided by increased capillarity. When FFAs enter the mitochondria, they are immediately catabolized via β -oxidation, which breaks long hydrocarbon chains into acetyl-CoA units for the TCA cycle. By increasing mitochondrial content, SkM increases its overall FAO capacity. Combined, these endurance training changes result in SkM oxidizing relatively more fat during exercise, which helps to produce ATP more efficiently and spare muscle glycogen to delay fatigue (14, 39).

As endurance training increases cellular reliance on FAO, SkM fibers decrease their dependence on anaerobic metabolism during steady-state aerobic exercise (40, 84). Glycolysis and the phosphocreatine system increase sarcoplasmic H^+ concentration, which contributes to SkM fatigue by interfering with cross-bridge cycling; in contrast, oxidative phosphorylation does not contribute to sarcoplasmic H^+ (occurs in the mitochondria). Hence, increased reliance on aerobic metabolism and increased aerobic capacity are advantageous for endurance exercise because each help SkM to resist metabolic acidosis and fatigue (67, 85).

Dramatic metabolic and mitochondrial changes that occur with endurance exercise can shift the phenotype of a given fiber where FG fibers can remodel into FOG fibers (50, 52). These changes partly take place due to increasing mitochondrial density and myoglobin expression (40, 49, 86). However, a more fundamental change can occur in SkM with aerobic training; MHC isoform expression in FG myofibers can shift from fast type IIB/X-dominant expression towards slower type IIA and type I MHC (49, 87). Slower MHC isoforms (type IIA and I) are less powerful but use ATP more efficiently than fast fibers for contraction; hence the slow fiber type shift is adaptive for low-powered, aerobic exercise (14, 40). Not surprisingly, PGC-1 α and related transcription factors (e.g., nuclear factor of activated T-cells (NFAT), nuclear respiratory factors 1/2, MEF2, etc.) play a role in SkM remodeling towards the aerobic phenotype (shown in rodents) (40). Overall, the endurance training-induced changes discussed above help SkM to resist fatigue, and to increase O_2 delivery and metabolic efficiency.

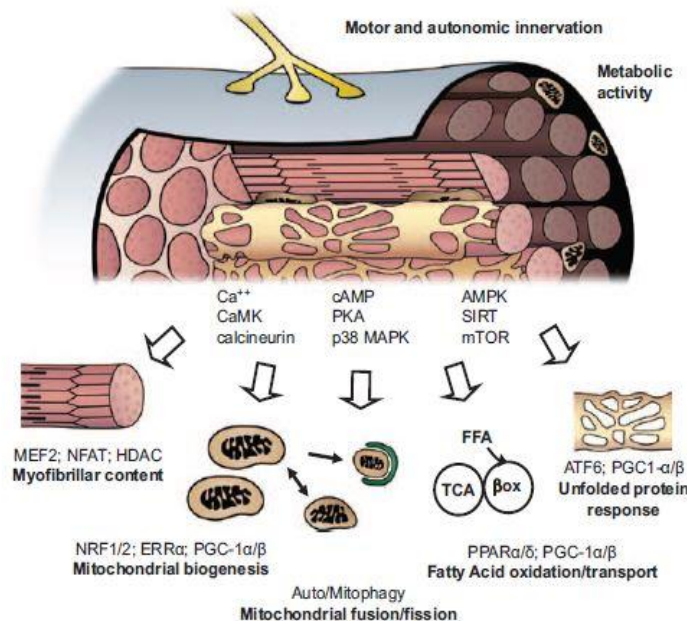


Figure 6. Endurance Exercise Signaling Pathways and Adaptations.

Nervous stimulation of SkM during exercise activates SkM and its adaptive signal transduction pathways. Important adaptive pathways, such as calcium-sensitive signaling (CaMK and calcineurin), cAMP-sensitive signaling (protein kinase A (PKA)), and AMPK signaling promote gene expression for an aerobic phenotype by altering myofibrillar content (towards slow MHC), enhancing mitochondrial biogenesis and quality control (autophagy/mitophagy), and increasing fat metabolism. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: Rowe GC, Sadfar A, and Zolt A, Running Forward: New Frontiers in Endurance Exercise Biology, 2014.

2.2.4 Resistance Training

Resistance, or strength, training involves high-intensity, short-duration muscular contractions against an external load (e.g., weight-lifting). While resistance training comes in many different forms, these can generally be divided into two broad categories: high-load, low-repetition, and low-load, high-repetition. The former aims to maximize muscular strength and power, while the latter aims to increase muscular strength and endurance. Muscular strength can be defined as a muscle's (or muscle group's) maximal force response upon activation, while power can be defined as the product of contractile strength and speed (i.e., the maximal rate at which work can be accomplished).

Over 100 years ago, Benedetto Morpurgo reported that muscular strength is directly correlated with muscular size (39). Most researchers agree that SkM hypertrophy following exercise is primarily the result of increased myofiber size (i.e., myofiber hypertrophy), rather than increased myofiber number (i.e., myofiber hyperplasia) (88). Increases in an exercised muscle's size are primarily mediated by net gains in SkM contractile protein and, partly (but not necessarily), by incorporating the nuclear and cytoplasmic contents of satellite cells into existing myofibers (89). SkM achieves net gains in contractile protein content with resistance training when rates of protein synthesis exceed rates of protein degradation; the newly incorporated myonuclei (from satellite cells) help to carry out protein synthesis in their local cellular domain (89).

Four hours after a single training session, protein synthesis rate increases by ~50%, and after 24 hours protein synthesis rate increases by ~100% (90). However, 36 hours post-exercise, SkM protein synthesis and degradation returns to baseline levels (39, 90). Hence, accumulation of contractile protein is a slow process that requires frequent stimulation; noticeable changes in SkM size typically require weeks to months of training (39). In recent decades researchers from a variety of fields have elucidated many, but not all, of the mechanistic details linking resistance training to increased muscular size and strength.

2.2.4.1 Mechanical Stress and Skeletal Muscle Signaling

The full extent of the mechanisms linking loaded muscle contractile activity with adaptive changes in muscle size and strength are still unclear. From SkM overloading and unloading studies (i.e., synergistic ablation and hindlimb suspension, respectively), researchers have discovered that SkM must chronically bear external load to increase size and strength (91-93). It is also known that adaptive gains in SkM size and strength are mediated by the activation

of protein synthesis pathways (and depression of protein degradation pathways) that promote net gains in SkM contractile protein (91-93).

Mechanisms involving mechanosensors such as increased concentrations of phosphatidic acid (91, 94-96), focal adhesion kinases (40, 97, 98), Ca^{2+} sensing (99), among others have been proposed, but these mechanisms are still hypothetical. Despite the different possible mechanotransduction mechanisms, a common theme that these mediators share is the activation of adaptive protein synthesis, which can be dependent or independent of mammalian target of rapamycin (mTOR) signaling (91, 94, 96, 98). The regulation of protein synthesis/degradation pathways, such as mTOR signaling, is very complicated due to the integration of many different anabolic and catabolic signals, which fluctuate as cellular circumstances change (e.g., transitioning from rest to exercise).

2.2.4.2 Mammalian Target of Rapamycin Complexes

In 1999, mTOR was recognized as a key modulator of adaptive SkM protein synthesis following resistance exercise (2). In SkM, mTOR is a part of two major protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which differ both in their protein constituents and cellular activities (88, 100).

The two mTOR complexes differ in their sensitivity to rapamycin, which is a drug that inhibits cellular growth even in the presence of anabolic stimuli (e.g., amino acids or resistance training) (88, 101, 102). mTORC1 is sensitive-to/inhibited-by rapamycin and directly regulates myofiber size by modulating contractile protein synthesis (88, 102). Further, mTORC1 is activated by many different cellular signals and events related to exercise, such as mechanical stretch (91, 94, 96, 98), cellular nutrient status (88, 103, 104), and growth factors (e.g., insulin-like growth factor 1 (IGF1)) (88, 100). So far, mTORC2 has only been shown to respond to

growth factors like insulin and IGF1 (Ming 2012) and, even though mTORC2 regulates the activity of mTORC1, it is not the focus of most exercise studies since it has not been shown to directly respond to mechanical stretch (88, 94, 105, 106).

2.2.4.3 Mammalian Target of Rapamycin Complex 1 Hypertrophic Signaling

The most completely described activation pathway of mTORC1 is the insulin/IGF1 pathway, which not only affects protein synthesis but also regulates proteolysis, glucose regulation (uptake and glycogenolysis), and cell growth in SkM (60). SkM contraction initiates a signaling cascade that results in the paracrine/autocrine secretion of IGF1 (60).

When mTORC1 is activated by IGF1-Akt signaling (and various other signals), it promotes mRNA translation for protein synthesis by phosphorylating the eukaryotic initiation factor 4E binding protein (4E-BP1) and the ribosomal p70 S6 kinase (p70^{S6K}), both of which help to enhance promote protein synthesis initiation and elongation (107, 108). Enhanced initiation and elongation increase protein synthesis efficiency, which can contribute to myofiber hypertrophy (60). However, muscle hypertrophy following exercise is notoriously transient (muscle loss can occur within days of unloading (109)).

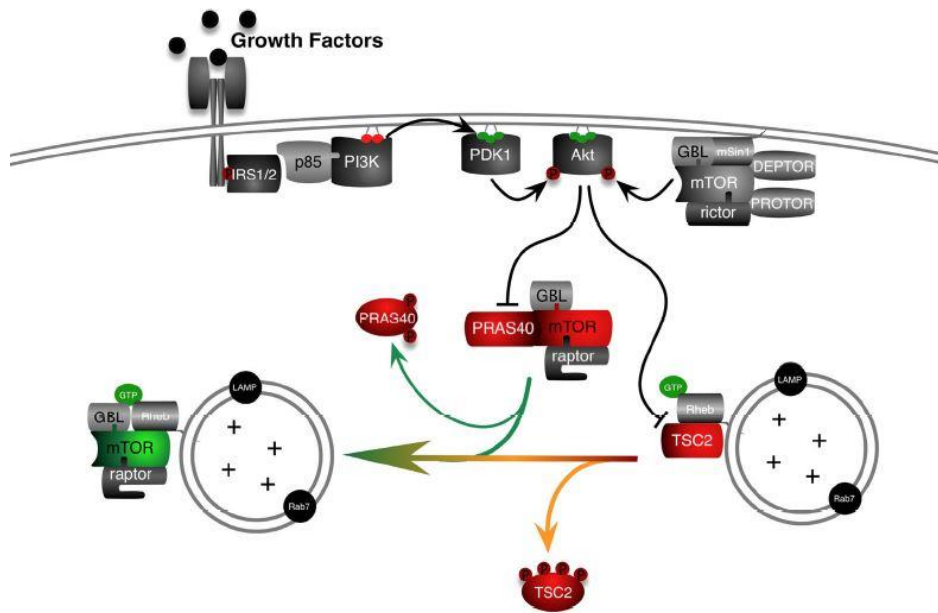


Figure 7. IGF1-PI3K-Akt Axis Activates mTORC1 for Protein Synthesis.

Growth factors and other signals activate key anabolic signaling hubs, such as the IRS1/2-PI3K-Akt axis, which promote mTORC1 activation and relieve mTORC1 inhibition by removing PRAS40 from mTORC1 and TSC2 from the lysosome; these events promote full mTORC1 activation, which enhances protein synthesis. Image reprinted with kind permission from Springer Science and Business Media: Springer and Calcified Tissue International, 2014, The Molecular Basis for Load-Induced Skeletal Muscle Hypertrophy, Marcotte GR, West DWD, and Baar K, Figure 2.

2.2.4.4 Exercise and Protein Turnover

The shifting balance of protein synthesis and degradation determines changes in myofiber size. For muscle accretion to occur, protein synthesis must exceed protein degradation. Fittingly, protein degradation pathways are down-regulated following resistance exercise. The two main proteolytic systems involved in SkM remodeling following exercise are the calcium-dependent calpains and the ubiquitin-proteasome pathway; autophagic lysosomes and caspases are also involved in protein degradation, but to a lesser degree following exercise (36, 60, 110).

Generally, aerobic exercise activates catabolic signaling mechanisms (e.g., Ca^{2+} , AMPK and unc-51-like kinase 1 (ULK1)), which promote protein degradation by upregulating calpains

and ubiquitin ligases, and also inhibit myofibrillar protein synthesis pathways (36, 110-112). Interestingly, increased PGC-1 α activity, in addition to promoting mitochondrial protein synthesis, may prevent excess degradation to spare overall muscle protein by down-regulating FOXOs (36, 113, 114).

In contrast, resistance exercise activates anabolic mechanisms (e.g., IGF1-Akt and mTORC1 signaling), which inhibit FOXOs expression/activity and thereby decreases myofibrillar protein degradation, while simultaneously activating myofibrillar protein synthesis pathways (88). Consequently, protein balance, and exercise adaptations in general, are a function of cross-talk between intramuscular signaling cascades that are activated in response to exercise training and depend on the type of training (36, 88).

2.2.4.5 Satellite Cell Activation and Muscle Hypertrophy

Beyond the regulation of protein synthesis and degradation, adaptive changes in SkM size can be aided by the activation and incorporation of satellite cells into existing myofibers (89). In studies of muscle unloading/reloading, myonuclear numbers did not change despite significant muscle hypertrophy (115, 116). In contrast, more extreme hypertrophic models, like synergistic ablation and eccentric contraction, activate satellite cells and promote their incorporation into myofibers (117, 118). However, even in synergistic ablation hypertrophy models, satellite cell activation has been shown to be dispensable: results from McCarthy et al., showed that even with 90% satellite cell inhibition (mediated by a Pax7-inducible transgene of diphtheria A toxin), synergistically ablated muscles undergo hypertrophy just as much as synergistically ablated muscles from control mice (119). These data indicate that while satellite cells may aid in SkM hypertrophy, their activation is non-essential for hypertrophy.

2.2.4.6 Increasing Muscular Strength with Resistance Training

The initial adaptive responses to resistance exercise involve neural adaptations that increase motor neuron activation during loaded contractions (14, 120). These neural adaptations allow for greater motor unit recruitment, improved motor unit synchronization, and blunted neural inhibition; these effects are responsible for the initial strength gains that occur with training (14, 89, 121). With continued training, myofiber hypertrophy, mediated by increased content of contractile proteins, plays a much larger role in facilitating strength gains than enhanced neural activation (39, 120).

Notably, only the fibers that are sufficiently activated to a certain threshold during loaded exercise will respond by increasing fiber size, whereas insufficiently activated fibers will not undergo hypertrophy. Consequently, myofibers can hypertrophy and increase strength following both endurance and resistance exercise, but endurance exercise utilizes low-intensity contractions, which lead to small size/strength gains, while resistance exercise utilizes high-intensity contractions that promote large size/strength gains (14).

Resistance training can also be performed using lower loads with increased repetitions. This type of training results in lesser strength gains than high-load resistance training, but it enhances muscle endurance, although to a lesser degree than endurance training (39, 122).

2.2.5 Concurrent Training

Concurrent exercise can be defined as conducting both endurance and strength training protocols in the same muscle groups, within the same exercise session, to promote both endurance and strength training adaptations (123). Studies investigating the adaptive effects of concurrent training have demonstrated mixed results, partly because of the heterogeneity in

exercise programs across studies. Several key features of concurrent training studies are 1) whether the endurance and resistance exercise sessions occurred on the same day or alternating days, 2) whether the endurance and resistance exercises worked the same primary muscle groups, and 3) the order of endurance and resistance exercise during the concurrent training sessions.

When focusing on concurrent training as defined above, the literature suggests that the most effective way to drive endurance or strength adaptations is to exclusively train in their respective modes (123). Additionally, most of the literature suggests that concurrent training primarily attenuates strength gains, rather than endurance gains (124-126). However, concurrent training has been shown to inhibit both strength and endurance gains. For example, Glowacki et al. conducted a study where untrained men underwent strength, endurance, or same-day concurrent training for 12 weeks (127). The endurance-only group increased their $\text{VO}_{2\text{max}}$ significantly more than the concurrent training group. Meanwhile, the strength-only group experienced significantly greater increases in peak torque production in knee flexion and extension than the concurrent group (127). However, when endurance and strength exercise sessions are performed on alternating days, alternating-concurrent training yields similar endurance and strength gains, compared to endurance-only and strength-only programs, respectively (128). These results suggest that the acute signaling mechanisms associated endurance training inhibit resistance training signaling, and possibly vice-versa.

2.2.5.1 Concurrent Training and Exercise Signaling

As discussed in sections 2.B.3 and 2.B.4, AMPK activation and mTORC1 activation play large roles in SkM adaptive signaling. It has been hypothesized that these signaling pathways,

and others (e.g., calmodulin signaling, HIFs, sirtuins, etc.), interact with each other during same-day concurrent training, which limits potential strength gains (60, 129).

AMPK and related endurance exercise signals (e.g., CaMK) increase eEF2K activity, which inhibits protein translation (130, 131). Additionally, AMPK phosphorylates TSC2 to inhibit mTORC1 activity (60, 72). AMPK also regulates protein degradation by activating ULK1 and activating FOXOs, both of which promote proteolytic pathways (107, 132-134). Therefore, activation of AMPK during endurance exercise inhibits anabolic signaling and promotes negative protein balance. However, the activation of PGC-1 α with endurance exercise may help to curb muscle atrophy by inhibiting protein degradation, without affecting protein synthesis rates (36, 107, 135, 136).

Resistance exercise, on the other hand, promotes myofiber hypertrophy by activating anabolic signaling, such as the IGF1-Akt axis and mTORC1. When Akt is activated during resistance exercise, it phosphorylates FOXOs (particularly FOXO1, 3, and 4) to help shuttle them out of the nucleus, which inhibits their transcriptional activities (137-139). Thus, Akt activation during resistance exercise inhibits protein degradation pathways associated with endurance exercise (137). Akt also phosphorylates TSC2 to promote mTORC1 activity, which increases protein synthesis following resistance exercise (137, 140, 141).

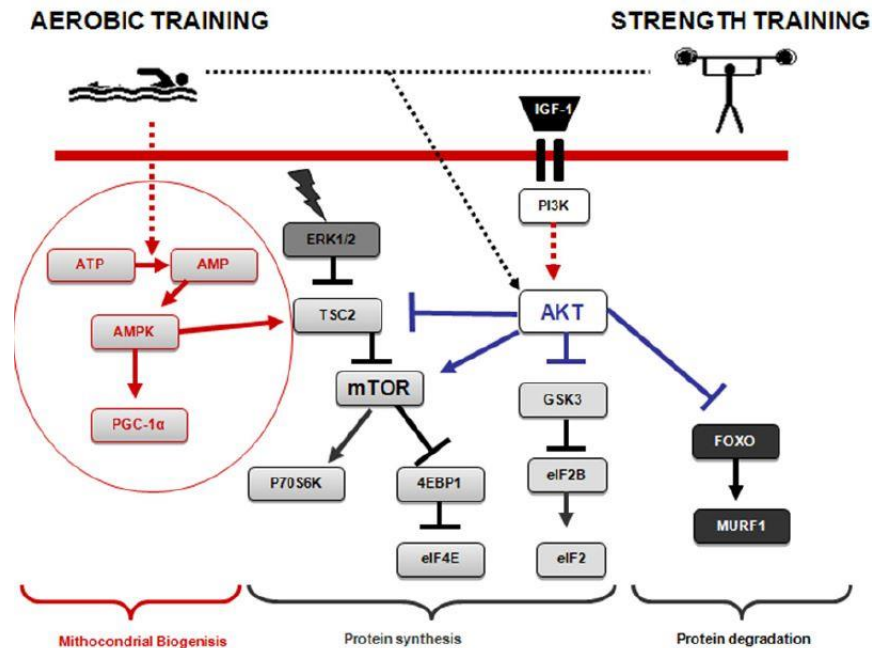


Figure 8. Interaction of Endurance and Resistance Exercise Signals.

Adaptive signaling pathways for aerobic and strength training signals interact with, and mutually inhibit, each other. Increased AMPK activity with aerobic training promotes transcriptional activity of PGC-1 α for aerobic adaptations (e.g., mitochondrial biogenesis). Increased IGF1-Akt-mTOR signaling promotes myofibrillar protein synthesis and inhibits protein degradation for net gains in SkM mass. AMPK and Akt have opposing effects on TSC2, which regulates SkM protein synthesis. Hence, concurrently training SkM for aerobic and strength gains may yield sub-optimal results. This graphic was adapted from original work by Tiago Fernandes, Úrsula P.R. Soci, Stéphan F.S. Melo, et al. (2012). Signaling Pathways that Mediate Skeletal Muscle Hypertrophy: Effects of Exercise Training, Skeletal Muscle - From Myogenesis to Clinical Relations, Dr. Julianna Cseri (Ed.), ISBN: 978-953-51-0712-5, InTech, DOI: 10.5772/51087. Available from: <http://www.intechopen.com/books/skeletal-muscle-from-myogenesis-to-clinical-relations/signaling-pathways-that-mediate-skeletal-muscle-hypertrophy-effects-of-exercise-training>

Hence, resistance training Akt-activation can be considered an anabolic signal, which is inhibited by catabolic signaling associated with endurance training. The phenomenon of adaptive signaling interference may account for sub-optimal strength and power gains seen in concurrent training, compared to resistance-only training (60, 129). Figure 10 illustrates how endurance training signaling antagonizes resistance training signaling by activating TSC2 (137). This thesis study sought to investigate the effects of resistance wheel running, which may promote SkM

adaptations similar to those seen in concurrent training (i.e., increased SkM strength and endurance).

Section 2.3: Rodent Models

Rodent models, specifically mice and rats, have played a crucial role in understanding the molecular mechanisms of exercise adaptation, particularly due to their homology to humans (142, 143) and widespread availability of genetically modified strains (i.e., targeted gene knockouts/overexpression) (75, 144, 145). Rodents are used to model both endurance and resistance exercise, and are used for SkM hypertrophy models. Commonly used endurance exercise models include unloaded wheel running (145, 146), treadmill running (144, 146), and swimming. Commonly used resistance exercise models include climbing/hanging (147, 148), while common hypertrophic models include synergistic ablation (4, 5), myostatin suppression (9), limb unloading/reloading (149, 150), and high-frequency electrical stimulation (151, 152). Resistance wheel running may serve as a model for simultaneous endurance and resistance training, which promotes SkM adaptations similar to those seen in concurrent training (10, 11, 153). The sections below (2.C.1-2.C.4) will summarize important findings, regarding SkM exercise adaptation, from the literature of rodent exercise studies that utilized endurance, resistance, and RWR training modes. These sections will mostly emphasize the results from studies that assessed SkM adaptations to exercise in wild-type mice and rats (when data are available), rather than genetically-modified animals, because this thesis work was performed using wild-type mice (C57BL/10SnJ).

2.3.1 Rodent Endurance Exercise Models

Many endurance exercise studies have been performed using mice and rats. These studies typically use either voluntary free (unloaded) wheel running (FWR) or forced treadmill running. Studies that use FWR allow rodents to run on an unloaded running wheel *ad libitum*, whereas treadmill studies control the speed, duration, and incline of the running exercise performed by rodents. Although study designs using FWR and treadmills vary in their details (e.g., choice of animal, training duration, intensity, frequency, etc.), both of these exercise modes have been shown to produce classic endurance training adaptations in rodent skeletal muscles. The following sections will focus on FWR studies because the present thesis work was performed using running wheels.

2.3.1.1 Running Adaptations in Mice

Mice are night-time foragers and voluntarily exhibit endurance exercise behaviors (154). Remarkably, when running wheels were placed in nature, wild field mice used the wheels, even in the absence of food rewards (155). In laboratory settings, mice provided with running wheels will voluntarily run, sometimes up to 20km during the night. Hence, endurance exercise is frequently modeled in mice using FWR. The following paragraphs describe findings from a several FWR studies in mice that investigated SkM adaptation. Important findings are summarized in table 3.

Generally, FWR studies in mice show that SkM responds to FWR by activating gene programs that promote metabolic and structural changes, which enhance SkM fatigue resistance and aerobic capacity. Davidson et al. (2006) demonstrated that 16 weeks of voluntary FWR (~15-30km/week on average) in young, female C57BL/6 mice (starting at 4-6 weeks old) increased CS activity in SOL (15.31 ± 0.71 vs. 12.98 ± 0.75 mol/kg protein/hr), red-GAS (13.98

± 1.08 vs. 11.13 ± 0.68 mol/kg protein/hr), and plantaris (PLN) muscles (12.09 ± 0.36 vs. 10.42 ± 0.29 mol/kg protein/hr), relative to sedentary (SED) controls (156). Additionally, SDH activity (7.57 ± 0.38 mol/kg protein/hr vs. 4.69 ± 0.61 mol/kg/hr) and phosphofructokinase (PFK) activity (17.64 ± 1.49 mol/kg protein/hr vs. 9.42 ± 0.69 mol/kg protein/hr) were higher in white-GAS muscle of runners, relative to SED. These differences in oxidative and glycolytic enzyme capacities coincided with nearly doubled maximal treadmill-running capacity (5 min warm-up, then 0.46m/s to failure) in runners vs. non-runners (104 ± 6.98 min vs. 56.2 ± 1.97 min, respectively). Furthermore, runners had ~10% higher VO_{2max} than non-runners (81.27 ± 2.70 mL/kg/min vs. 74.44 ± 1.76 mL/kg/min, respectively) (156). These results suggest that long-term FWR can promote adaptive changes in mice that increase SkM oxidative capacity and whole-body aerobic capacity.

Similarly, Tanner and coworkers (2013) reported that 3-5 month old male and female mice that underwent 21 days of voluntary FWR increased cytochrome C oxidase (COXIV; ~175% of control) enzyme activity and ETC protein content, specifically complexes I (+150%), II (+150%), IV (~150%), and V (~160%), in GAS muscle (157), which indicates that FWR increases expression of mitochondrial genes that enhance oxidative capacity in SkM. However, CS activity was only mildly elevated in GAS of runners compared to control (~65 vs. ~62 μ mol/g protein/min), despite the large volume of wheel running (ranging from ~4 to 10km per day) (157). Surprisingly, PGC-1 α protein was unchanged, relative to control (157), but some studies using knockout animals have shown that PGC-1 α activation is not completely necessary for SkM endurance training adaptations (77, 158, 159).

In addition to Tanner et al., the results of Ikeda et al. (2006) showed that female mice that voluntarily ran on unloaded wheels for up to 8 weeks (distance per day range: 6.0 ± 0.8 km to

12.2 ± 1.0km) increased malate dehydrogenase, cytochrome C, COXIV, and ATP-synthase β activities in PLN muscles, relative to sedentary controls (160). Tibialis anterior muscle similarly responded to training with increased cytochrome C, COXIV, and ATP-synthase β activities (not malate dehydrogenase), after at least 4 weeks of FWR (160). Changes in PGC-1 α protein were seen after 4 weeks in PLN muscle of runners (~22% higher than control) (160). These studies strengthen the notion that FWR promotes endurance training adaptations in SkM, such as increased expression of oxidative/mitochondrial genes.

Matsakas et al. (2010) gave 4-week old male C57BL/6 mice (and age-matched myostatin-null mice) access to unloaded running wheels for 5 weeks and ran a battery of tests to assess SkM exercise adaptation (161). Of note, only ~1-2km/day of running on average increased the percentage of type IIA fibers in TA muscle compared to sedentary controls (5.4 ± 1.0% vs. 3.0 ± 0.3%, respectively). Further, in both TA and PLN muscle, runners had lower type IIB fiber percentage relative to control (TA= 56.6 ± 1.4% vs. 67.5 ± 0.06%, respectively; PLN= 30.0 ± 2.8% vs. 41.4 ± 4.1%, respectively), with increased percentage of type IIB/X fibers (TA= 4.1 ± 0.9% vs. 1.3 ± 0.5%, respectively; PLN= 2.1 ± 0.6% vs. 0.5 ± 0.4%, respectively) and type IIX fibers (TA= 30.9 ± 1.8% vs. 27.7 ± 0.1%, respectively; PLN= 31.7 ± 1.2% vs. 25.9 ± 5.1%, respectively) (161), indicating that a shift towards slower fibers occurred in runners. These results suggest that a relatively small, but consistent FWR stimulus promotes slow myofiber shift in wild-type mice (161).

In a follow-up study, Matsakas et al. (2012) showed that similar mice performing FWR under similar conditions had more type IIA fibers than sedentary controls (7.7 ± 0.3 vs. 3.6 ± 1.1%, respectively) in extensor-digitorum longus (EDL) (53), and these results coincided with

higher mRNA expression of VEGF-b (~150% of control) in EDL (53). Taken together, these data suggest that FWR can promote aerobic adaptations in SkM.

Beyond the studies reviewed above, others have reported similar effects of FWR in SkM, such as increased mitochondrial gene expression (162, 163) and oxidative capacity (162, 164), metabolic adaptations (165, 166), slow fiber-type shift (52, 167, 168) and increased capillarity (167). Combined, these results suggest that voluntary FWR in mice can effectively model endurance exercise training.

Table 3. Brief Summary of Murine Skeletal Muscle Adaptations to Free Wheel Running			
First Author (Year)	Running Behavior	Aerobic Adaptation	Data (Runners vs. Control)
Davidson (2006)	16 wk (~15-30km/wk)	↑ Oxidative and glycolytic enzyme activities, and ↑ aerobic capacity	↑ CS in SOL (~15.3 vs. ~13.0 mol/kg pro/hr) ↑ CS in red-GAS (~14.0 vs. ~11.1 mol/kg pro/hr) ↑ CS in PLN (~14.0 vs. ~11.1 mol/kg pro/hr) ↑ SDH in white-GAS (~7.6 vs. ~4.7 mol/kg pro/hr) ↑ PFK in white-GAS (~17.6 vs. ~9.4 mol/kg pro/hr) ↑ Run-time to exhaustion (~104.0 vs. ~56.2 min) ↑ VO _{2max} (~81.3 vs. ~74.4 mL/kg/min)
Tanner (2013)	3 wk (~4-10km/d)	↑ Mitochondrial proteins	↑ COXIV protein in GAS (~175% of control) ↑ Complex I protein in GAS (~150% of control) ↑ Complex II protein in GAS (~150% of control) ↑ Complex IV protein in GAS (~150% of control) ↑ Complex V protein in GAS (~160% of control)
Matsakas (2010)	5 wk (~1-2km/d)	Slow-fiber type shift	↑ Type IIA fibers in TA (~5.4 vs. ~3.0%) ↑ Type IIX fibers in TA (~30.9 vs. ~27.7%) ↑ Type IIX fibers in PLN (~31.7 vs. ~25.9%) ↑ Type IIX/B fibers in TA (~4.1 vs. ~1.3%) ↑ Type IIX/B fibers in PLN (~2.1 vs. ~0.5%) ↓ Type IIB fibers in TA (~56.6 vs. ~67.5%) ↓ Type IIB fibers in PLN (~30.0 vs. ~41.4%)

2.3.2 Rodent Resistance Exercise and Hypertrophic Models

As noted above, many studies of endurance exercise have been performed using rodents, but fewer studies have investigated the effects of resistance exercise in rodents. This may be partly due to the difficulty of motivating mice and rats to perform resistance training; however, several studies using rodent resistance exercise models have reported SkM adaptations similar to human resistance training. In addition to voluntary models, some researchers have studied rodent hypertrophic signaling using involuntary models that involve externally-imposed stimuli. Several commonly used involuntary hypertrophy models include high-frequency electrical stimulation, and synergistic ablation. Table 4 the end of section 2.C.2.3 summarizes studies using these models.

2.3.2.1 Voluntary Resistance Training Paradigms

In 1988 Henrik Klitgaard reported results of a 36-week resistance training study that he performed using 19-month old male Wistar rats (169). Klitgaard used a device that progressively trained rats to perform plantar flexion (in a squat-like maneuver) to lift a platform for food rewards. By the end of the study, the rats were lifting 85% more weight for each reward, which resulted in greater SOL mass ($234 \pm 26\text{mg}$ vs. $174 \pm 36\text{mg}$) and PLN mass ($434 \pm 60\text{mg}$ vs. $351 \pm 43\text{mg}$), as well as SOL tetanic strength ($2.47 \pm 0.26\text{N}$ vs. $1.50 \pm 0.18\text{N}$) and PLN tetanic strength ($5.19 \pm 1.21\text{N}$ vs. $3.79 \pm 0.36\text{N}$), relative to untrained controls (169). Similarly, Rader et al. (2014) studied the effects of high-load (700g; ~200% rat body mass (BM)) and low-load (70g; ~20% rat BM) squat-like training in male Sprague-Dawley rats (starting at 3-4 months old) (170). Daily training sessions consisted of 100 lifts at their respective loads. After 1 month of training, high-load rats increased their peak reactive force production during lifting sessions *in-*

vivo, relative to baseline and low-load rats. Surprisingly, there were no differences in SkM masses (SOL, TA, GAS, and PLN) of low and high-load rats, compared to untrained controls. Further, high-load rats had smaller and more numerous SOL and TA myofibers than untrained controls (170). However, in another study, the same research group showed that rats that underwent 2 months of a very similar training regimen (just 4 weeks longer than Rader et al.) increased their normalized GAS mass ($\sim 5.5\text{mg/g BM}$ vs. $\sim 5\text{mg/g BM}$) and SOL mass ($\sim 4.3\text{mg/g BM}$ vs. $\sim 4\text{mg/g BM}$), relative to untrained controls (171). These results seem to suggest that strength gains achieved by rats with resistance training can precede hypertrophy, which has been also been shown in humans (120, 172).

Using a different kind of hypertrophy model, Itoh et al. (2014) studied the SkM effects of 2-week tail suspension (promotes hindlimb atrophy) followed by operant conditioning (shock therapy) that encouraged 10-week old male ICR mice to rear up on their hind-legs 50 times per day (performed in 2 sets of 25) (173). After 7 days of training, SOL muscle CSA increased (Trained: $\sim 1.1\text{mm}^2$ vs. Control: $\sim 0.9\text{mm}^2$) and myofiber CSA increased (Trained $\sim 1800\mu\text{m}^2$ vs. Control: $\sim 1300\mu\text{m}^2$) in the stand-trained group, relative to untrained controls (173). Myonuclear number was higher in stand-trained mice compared to untrained (~ 1.0 vs. ~ 0.6 nuclei per fiber, respectively). Further, the fraction of 5-ethynyl-2'-deoxyuridine (EdU)-positive nuclei (a probe for active DNA synthesis) was higher in the stand-trained mice after 48 hours, which suggests that acute satellite cell activation and proliferation may have contributed to myofiber hypertrophy following training (173).

In addition to squatting and rearing, other researchers have modeled resistance exercise in rodents by climbing and hanging. Matheny and co-workers (2009) performed a 16-week voluntary resistance training study in 12-13-month-old male mice, where the exercised group

climbed an 85° inclined ladder (148). The mice were trained to climb with progressively increasing external weights, beginning with 50% of BM, followed by 75%, 90%, and 100% of BM. If the mouse could continue to climb the ladder with 100% BM of external weight, then 3g weights were progressively added to failure (148). Over the course of the study, the mice increased maximal climbing weight by roughly 100% (from ~125% BM to ~250% BM). Relative to control, trained mice expressed higher IGF1 mRNA in GAS (26% higher) and increased GAS mass (~0.37% BM vs. ~0.31% BM) (148). These data tentatively suggest that climbing activates SkM anabolic signaling and hypertrophy in a manner similar to resistance exercise.

To study the effects of isometric grip-strength training, Krüger and colleagues (2013) assigned male C57BL/6N mice to one of three groups for a 5-10-week study: strength train, endurance train, or control (147). The strength training involved vertical hanging from a wire grid (3 sets X 3 minutes; 5 days per week), while the endurance training mice performed relatively low-intensity treadmill exercise (0.05m/s for 30 min; 5 days per week) (147). As expected, after 5 and 10 weeks, only mice in the strength-train group increased their maximal vertical hanging time (from ~10 to ~50 min; ~500%), whereas only endurance-trained mice increased VO_{2max} (from ~45 to ~55mL O₂/kg/min; ~22%); these changes were significant relative to group baseline and relative to all other groups. The endurance trained group decreased type II: type I fiber ratio (~1.2:1 vs. ~2.0:1 type II: type I) and increased type I fiber diameter (~70 vs. ~65μm) in medial-GAS compared to control. The strength trained group increased type II fiber diameter (147). However, strength-trained mice increased SDH mRNA expression in medial-QUAD (~160%), SOL (~400%), and medial-GAS muscles (~150%), but only increased PFK expression in medial-QUAD (~300%) compared to control (147). These results suggest that

hanging exercise in mice can increase muscle endurance and glycolytic myofiber size during demanding isometric exercise. However, these researchers did not report fiber type changes in the strength-trained group. Unfortunately, these researchers did not assess functional measures of muscle strength or power, which limits similarities between this rodent isometric hanging model and resistance exercise (147).

To summarize, studies using rodent models of voluntary resistance training are limited. Generally, these studies suggest that SkM adaptations to voluntary resistance training in rodents, such as increased muscle size and strength, are similar to human resistance training adaptations. However, a major limitation to these kinds of training studies is variability in animal exercise volition, which decreases the standardization of conditions. Thus, it is still unclear whether these voluntary resistance exercise models in rodents can truly mimic human strength training. Some researchers have chosen to work around this problem by designing experimental models that are minimally affected by animal exercise volition.

2.3.2.2 Involuntary Rodent Resistance Training: High Frequency Electrical Stimulation

Several common models of involuntary SkM hypertrophy/resistance training in rodents are: high-frequency electrical stimulation (HFES) and synergistic ablation. Electrical stimulation involves a procedure that anesthetizes the rodent prior to high-frequency muscle stimulation by needle electrodes. HFES directly activates myofibers in target muscles via their intramuscular nerves, unlike myofiber activation *in-vivo* where motor units are progressively recruited (1, 174). By manipulating the stimulation parameters (e.g., stimulation frequency, current, pulse and train durations, etc.), researchers can more carefully control muscular contractile conditions. Traditionally, HFES studies in rodents have focused on activating plantar flexion in the lower leg muscles either by sciatic or tibial nerve stimulation.

Among the first to utilize HFES in exercise research, Wong and Booth (1988) had adult female Sprague-Dawley rats perform one-legged, sub-maximal, concentric weight training with their plantar flexor muscles for 16-weeks via HFES (1). The rats began lifting 600-800g (4 sets of 6 reps; 3 days rest between lifting bouts) and progressively increased by 100g increments. Trained animals increased GAS mass in trained legs ($1.97 \pm 0.07\text{g}$ vs. $1.65 \pm 0.08\text{g}$) and GAS total protein content ($375 \pm 35\text{mg}$ vs. $321 \pm 31\text{mg}$), relative to the untrained contralateral leg (1). Further, by the end of the training, the rats were able to perform 66% more work on average per bout than at baseline (final maximum loads of 600-1100g vs. initial maximum loads of 600-800g). Results from their study suggest that the HFES-induced weight training promoted adaptive SkM hypertrophy (1).

More recent studies have expanded on Wong and Booth's efforts by investigating the acute signaling effects of lower-limb HFES. Upon HFES activation, both the plantar flexors (SOL, PLN, GAS) and the dorsiflexors (TA and EDL) contract, but the powerful concentric contraction of the plantar flexors overpowers the dorsiflexors and forces the TA and EDL to eccentrically contract, so Baar and Esser (1999) decided to study the effects of HFES in dorsiflexors muscles (2). In adult female Wistar rats, Baar and Esser (1999) showed that 6 weeks of HFES training (60 tetanic contractions per bout; 2 bouts per week) increased p70^{S6K} phosphorylation up to 36 hours post-stimulation in TA muscles ($+204.1 \pm 28.3\%$) and EDL muscles ($+135.7 \pm 20.7\%$), compared to unstimulated contralateral control legs. Further, compared to contralateral control, TA muscles from stimulated legs were $14.4 \pm 3.15\%$ larger and EDL muscles were $+13.9 \pm 3.01\%$ larger. The activation of p70^{S6K} was positively correlated with muscle mass changes ($R = 0.998$; $p < 0.05$) (2). As p70^{S6K} is a direct target of mTORC1, these results suggest that HFES activates anabolic signaling associated with resistance exercise.

To investigate mTORC1 activation more closely, Frey et al. (2014) subjected 8-10 week old C57BL/6 mice to acute HFES (60 tetanic plantar flexions), which triggered eccentric contractions in dorsiflexor muscles, and soon-after measured effects on mTORC1 signaling (40 minutes post-stimulation) (3). Their results showed that HFES-induced eccentric contraction of the TA increased p70^{S6K} phosphorylation (3). Combined, these studies of acute mTORC1 signaling show that HFES activates hypertrophic signaling.

2.3.2.3 Involuntary Rodent Resistance Training: Synergistic Ablation

A more commonly used model than HFES is synergistic ablation, which involves the surgical removal of synergist muscles to overload remaining muscles *in-vivo* (e.g., removing GAS and SOL muscles to overload the PLN). This resistance training model has been used in rodents to overload SkM and drive adaptive hypertrophy.

Linderman et al. (1996) showed that 4 weeks after bilateral removal of both the GAS and PLN muscles, the SOL muscles were overloaded enough to drive hypertrophy (overloaded SOL= 138 ±6mg; control SOL= 120 ±3mg) (175). At the signaling level, Hamilton and colleagues (2010) showed that ten days after GAS muscles were ablated in rats, higher levels of p70^{S6K} Thr389 phosphorylation in SOL (~2.75 phospho/total vs. ~0.75 phospho/total) and PLN muscles (~1.05 phospho/total vs. ~0.50 phospho/total) coincided with increased SOL mass (~0.55mg/g BM vs. ~0.45mg/g BM) and PLN mass (~1.05mg/g BM vs. 0.70mg/g BM) relative to controls (5). Additionally, Chalé-Rush (2009) showed that 4 weeks of synergistic ablation increased SOL and PLN muscle mass, along with increased mTORC1 and ribosomal S6 phosphorylation, but surprisingly p70^{S6K} phosphorylation was not different than control (176).

When Phelan and Gonyea (1997) overloaded SOL muscles in rats for 4 weeks by removing the GAS, PLN, and flexor digitorum profundus, the overloaded SOL muscles robustly

increased in mass (~40% compared to contralateral control leg); whereas SOL overload hypertrophy was blunted in legs that were exposed to satellite-cell inhibiting radiation (3000 rads; does not inhibit typical myonuclear activities) (4). Further, this study showed that 4 weeks after plantar flexor ablation, irradiated SOL muscles exhibited a smaller percentage of 5-bromo-2'-deoxyuridine (BrdU)-positive nuclei ($8.4 \pm 4.4\%$), relative to non-irradiated controls ($17.1 \pm 3.9\%$) (4). Similarly, Rosenblatt et al. (1992) showed that 4 weeks of overloading the EDL muscle (by removing the TA) increased EDL muscle mass ($13.1 \pm 0.6\text{mg}$ vs. $10.8 \pm 0.3\text{mg}$) and increased type IIB fiber CSA ($2258 \pm 19 \mu\text{m}^2$ vs. $1903 \pm 17 \mu\text{m}^2$); but muscle mass gains were prevented by irradiation (2500 rads) (37). These data are supported by others (177, 178); together, they indicate that satellite cell activation can contribute to SkM overload-induced hypertrophy, similar to resistance training. Taken together, data from the studies discussed above suggest that SkM overload by synergistic ablation can model SkM hypertrophic responses similar to those elicited by resistance training.

Of note, another study by Kandarian and others (1992) showed that bilateral removal of the smaller plantar flexor muscles (SOL and/or PLN), leaving the GAS intact, had no hypertrophic or metabolic effects on remaining muscles after 40 days (179). Results from this study imply that SkM adaptive hypertrophy to synergist ablation only occurs when SkMs are overloaded to a critical threshold, which was not achieved when the larger GAS muscle contributed to weight bearing. This highlights the importance of functional overload when trying to drive SkM adaptation.

Table 4. Summary of Rodent Adaptations to Resistance Training/Hypertrophy Models			
First Author (Year)	Exercise Mode	Voluntary?	Adaptation (Magnitude)
Klitgaard (1988)	Progressive Squat	Yes	↑ Workload by 85% after 36 weeks ↑ SOL mass vs. control (~234mg vs. ~174mg) ↑ PLN mass vs. control (~434mg vs. ~351mg) ↑ SOL strength vs. control (~2.47N vs. ~1.5N) ↑ PLN strength vs. control (~2.47N vs. ~1.5N)
Matheny (2009)	Ladder Climbing	Yes	↑ Workload by ~100% after 16 weeks ↑ GAS mass vs. control (~0.37% BM vs. ~0.31% BM) ↑ GAS IGF1 mRNA vs. control (26%)
Krüger (2013)	Hanging	Yes	↑ Hanging time ~500% after 10 weeks ↑ Hanging time vs. control (~50 min vs. ~20 min) ↑ Type II fiber diameter vs. control (~125µm vs. ~85 µm)
Itoh (2014)	Rearing	Yes	↑ SOL CSA vs. control (~1800µm ² vs. ~1300µm ²) ↑ Myonuclei per fiber vs. control (~0.9 vs. 0.6 per fiber) ↑ Fraction of EdU-positive myonuclei within first 48 hr
Wong (1988)	HFES With Progressive Resistance	No	↑ Workload by ~66% after 16 weeks ↑ GAS mass vs. control leg (~1.94g vs. ~1.65g) ↑ GAS protein vs. control leg (~375mg vs. ~321mg)
Baar (1999)	HFES-induced Lengthening	No	↑ TA (~14.4%) and EDL (~13.9%) mass vs. control leg 3 hr post-HFES ↑ TA p70 ^{S6K} activity (~363.2%) 3 hr post-HFES ↑ EDL p70 ^{S6K} activity (~353.4%) 36 hr post-HFES ↑ TA p70 ^{S6K} activity (~204.1%) 36 hr post-HFES ↑ EDL p70 ^{S6K} activity (~135.7%)
Hamilton (2010)	Synergistic Ablation	No	↑ (p)p70 ^{S6K} in SOL vs. control (~2.75 vs. ~0.75) ↑ (p)p70 ^{S6K} in PLN vs. control (~1.05 vs. ~0.50) ↑ SOL mass/BM vs. control (~0.55 vs. ~0.45mg/g) ↑ PLN mass/BM vs. control (~1.05 vs. ~0.70mg/g)
Phelan (1997)	Synergistic Ablation	No	↑ SOL mass vs. control (~40%) Radiation blocked SOL muscle hypertrophy vs. control Radiation ↓ Budr ⁺ -nuclei vs. control (~8.4% vs. ~17.1%)

2.3.3 Resistance Wheel Running

As illustrated in table 4 above, resistance training models in rodents have shown some promise in their ability to drive adaptive SkM hypertrophy. However, the voluntary models of resistance training discussed (weight lifting, climbing, hanging) require significant researcher time since the animals must be constantly monitored during their workouts. The involuntary models discussed (HFES and synergistic ablation) also require similar researcher burden and

attention, and these models push muscles to adapt in ways that are virtually irrelevant to humans. Forced exercise methods, such as HFES, synergistic ablation or forced treadmill running, are also thought to stress animals more than voluntary exercise paradigms like wheel running, which rodents willingly perform (180, 181).

Knowing that rodents voluntarily exercise when given free access to a running wheel, some researchers have attempted to model resistance training in rodents by applying external resistance (force) to running wheel axles; this increases the work (wheel resistance force X distance) performed by running animals. Several studies have investigated effects of resistance wheel running (RWR) on rodent SkM (10-12, 153, 182-186). Table 5 summarizes these studies at the end of section 2.C.3.1.

2.3.3.1 Review of Resistance Wheel Running Literature

In 1998, Ishihara and colleagues were the first group to publish on the effects of RWR in rodents (10). They developed an automated resistance running wheel system designed for rat use. In this study, young male Sprague-Dawley rats were given free access (during 12hr dark cycle) to a resistance wheel for 8 weeks (10). During the 8 weeks, wheel resistance was progressively increased from no-added resistance (4.5g of inherent resistance; ~1.5% BM) up to 220g (~73% BM) by week 8. The resistance runners increased their daily external work (adjusted to BM) throughout the study from ~250 N*m/kg to ~12000 N*m/kg, which greatly exceeded the free wheel runners (from ~250 N*m/kg to ~400 N*m/kg), but there was marked heterogeneity in work output among resistance runners. Clear sub-groups of work performance emerged among the resistance runners by the end of the study: low (n= 2; ~2500 N*m/kg/day), medium (n= 6; ~7500 N*m/kg/day), and high resistance runners (n= 2; ~12000 N*m/kg/day). Even with large work variability among resistance runners, Ishihara et al. (1998) reported increased PLN mass in

resistance runners as a whole ($374 \pm 8\text{mg}$), relative to sedentary controls and free-wheel runners ($313 \pm 9\text{mg}$ and $319 \pm 5\text{mg}$, respectively). Additionally, resistance runners increased CSA of SO, FOG, and FG fibers in PLN, relative to sedentary (SED) controls. However, neither resistance runners nor free-wheel runners experienced changes in PLN fiber type percentages (SO, FOG, or FG fibers), relative to SED rats (10). Taken together, these data suggest that RWR can increase muscle mass and myofiber size, similar to resistance training.

Konhilas et al. (2005) tested the effects of 7 weeks of progressive RWR training in 12-week-old male C57BL/6 mice (153). They altered wheel resistance in their RWR system by manually turning a screw to adjust the tension on a fishing line that was wrapped around the axle of a running wheel (153). Throughout the study, wheel loads progressed for the resistance runners from inherent wheel resistance ($\sim 2\text{g}$) up to 12g resistance; or from $\sim 7\%$ BM to $\sim 41\%$ BM. By week 3, runners randomized into the low-resistance group maintained 5g for the duration of the study, while high-resistance runners progressed up to 12g . As load exceeded 5g , distance run by the high-resistance runners diminished (from $\sim 3.5\text{km/day}$ to $\sim 0.05\text{km/day}$), while the other groups remained consistent (no-resistance $\sim 2.5\text{-}3.5\text{km/day}$; low-resistance $\sim 4\text{-}5\text{km/day}$). By week 4, external work peaked for low (still at 5g ; $8126 \pm 1509\text{N}\cdot\text{m/kg}$) and high-resistance runners (at 7g ; $8171 \pm 1285\text{N}\cdot\text{m/kg}$), but external work for the high resistance runners dropped sharply when the wheel load exceeded 7g (from $6193 \pm 1429\text{N}\cdot\text{m/kg}$ at 9g , to $2803 \pm 787\text{N}\cdot\text{m/kg}$ at 12g). When comparing running groups at the muscular level, Konhilas et al. reported increased SOL mass in resistance runners only ($\sim 20\%$), relative to sedentary controls, but no changes in PLN, GAS, or TA mass. However, the resistance runners increased the size of MHC IIA ($\sim 20\text{-}30\%$) and IIX/D ($\sim 5\text{-}10\%$) isoform-dominant fibers in whole-calf muscle sections, relative to sedentary controls. Surprisingly, type IIB fiber size increased in low-

resistance (~15%) but decreased in high-resistance runners (~5%), relative to sedentary controls (153). The authors suggested that future RWR studies should investigate fiber type conversions. Konhilas et al. (2005) also reported changes in cardiac hypertrophic signaling primarily in free-wheel runners, which coincided with their heart mass data (153).

Call and colleagues (2010) reported the SkM effects of RWR in 4-week-old male *mdx* mice (11). The dystrophin-deficient *mdx* mouse is a widely used mouse model of Duchenne Muscular Dystrophy. Call et al. (2010) gave *mdx* mice free access to running wheels for 12 weeks and progressively increased the resistance from inherent resistance (1g; ~3% BM) up to 7g (~20% BM). Resistance was applied via a threaded aluminum collar that was calibrated using hanging weights. During the 12-week training period, resistance was adjusted individually (+1g) on a weekly basis for resistance runners. Resistance was not increased for the resistance runners if average daily distance dropped by 50% or dropped below 2 km/day. Resistance runners ran significantly less than free-wheel runners after 5 weeks (~2km/day vs. ~6-9km/day, respectively), but after 2 weeks resistance runners performed more external work than free wheel runners (~4000-9000 N*m/kg vs. ~2000-4000 N*m/kg, respectively). Every 4 weeks, Call et al. (2010) performed measures of ankle dorsiflexion torque *in-vivo*, but there were no differences between groups over time (this result could be explained by the limited role played by dorsiflexors in wheel running). After 12 weeks, grip strength tests revealed that free-wheel runners (3.7 ± 0.2 g/g BM) and resistance runners (3.3 ± 0.1 g/g BM) were stronger than sedentary (2.8 ± 0.1 g/g BM), but no different from one another. After sacrifice, triceps mass of resistance runners (normalized to BM) was slightly greater than sedentary *mdx* mice (~10%), but SOL, GAS, TA, EDL, heart were no different. However, SOL muscle tetanic strength *in-vitro* was ~26% greater in resistance and free-wheel runners than sedentary. Further, although Call et

al. (2010) reported differences in work performance and SOL muscle strength, they were unable to detect any major differences in SOL cytoskeletal proteins (talin, integrin, and β -dystroglycan) except a minor increase in vinculin expression in free-wheel runners (~10%) relative to control (11). These results, similar to Konhilas et al., are inconclusive and warrant further inquiry into RWR's effects on SkM.

Soffe et al. (2015) recently published results from a 10 week study of voluntary RWR in young (15 weeks old) male C57BL/6J mice (185). The mice were subjected either to progressive low resistance (LR; up to 4g) or high resistance (HR; up to 6g) wheel running. Running performance declined over time for both groups as wheel load increased (LR: from ~7km/day during week 1 to ~1.6km/day during week 10; HR: from ~7km/day to ~0.9km/day). The low- and high-resistance groups both increased QUAD (by ~12.5%) and SOL mass (by ~50%), relative to sedentary controls. However, SOL fiber CSA was unaffected by wheel running (185).

Several other studies of RWR have been published using rats (12, 182-184). Legerlotz et al. (2008) had young (4 week old), male Sprague-Dawley rats run against progressively increasing loads for 45 days. Unlike the studies mentioned above, Legerlotz et al. increased wheel resistance very quickly during the study (0% BM days 1-8, ~83% BM days 8-12, ~85% BM days 12-16, ~118% BM days 16-20, up to 176% BM during days 40-45). As expected, these researchers found that very high wheel loads decreased the distance and time of wheel bouts, compared to free wheel runners. Also, the RWR group performed bouts at higher velocity than the FWR group, which suggests that their activity may have been more similar to sprint training than endurance training (182). However, Legerlotz et al. (2008) reported no differences in absolute muscle masses (GAS, SOL, PLN, TA, vastus lateralis (VL), extensor carpi radialis longus and brevis) among any of the groups. Yet, the RWR group had larger muscle CSA,

compared to control, in PLN ($\sim 2600\mu\text{m}^2$ vs. $\sim 2400\mu\text{m}^2$, respectively; $+7.8\%$ ($p < 0.001$)) and SOL ($\sim 3100\mu\text{m}^2$ vs. $\sim 3000\mu\text{m}^2$, respectively ($p < 0.05$)) (182). Again, seemingly conflicting results (i.e., increased muscle CSA with no differences in muscle mass) highlight the need for further inquiry of RWR's effects on SkM.

In 2012, the same research group published a similarly-designed study that investigated the response of SkM satellite cells to progressive RWR in 4-week-old, male Sprague-Dawley rats (12). However, in their 2012 study, rats were sacrificed after 1, 3, and 6 weeks of RWR to obtain time-course results (12). Similar to the Legerlotz et al. (2008) study, Smith and Merry (2012) reported no differences in GAS, VL, or TA mass (relative to BM) after 6 weeks of RWR, relative to sedentary control (12). SOL mass (relative to BM) was greater in runners (FWR and RWR), compared to control. SOL and VL fiber CSA increased in the RWR group, compared to control, while GAS fiber CSA was unchanged. In the RWR group, larger fiber size in SOL and VL coincided with a higher numbers of satellite cells per myofiber and active myofiber nuclei, suggesting that satellite cell proliferation and incorporation contributed to myofiber hypertrophy (12). These data suggest that RWR may have resistance-like training effects on SkM.

Other studies of RWR have focused on its neurotrophic effects in the hippocampus (183, 184), but also reported some effects on SkM as a secondary outcome. For example, Lee et al. (2012) reported that both 4 weeks of FWR and progressive RWR (from no-added to $\sim 30\%$ BM) increased SOL and PLN muscle mass and CS activity in male Wistar rats, relative to control (184), which suggests that RWR against relatively high loads also induces endurance-like training effects in SkM. However, when the same research group performed a repeat study under the same conditions, there were no differences in muscle mass among groups and only the RWR group exhibited higher CS activity in PLN muscle, relative to control (183). In both studies

RWR rats ran less than the FWR groups, but performed much more work. An interesting feature of these studies was the heterogeneous running performance among animals the RWR groups (i.e., high standard error) (183, 184).

Finally, Suijo et al. (2013) performed a study that assessed RWR's effects, both on hippocampal neurotrophic signaling and SOL muscle hypertrophic signaling in mice (186). These researchers put 8-week old male C57BL/6 mice on a progressive RWR program for 2-weeks (wheel loads increased from 1.00 to 20.5g; ~4.3 to 88% BM; +1.5g per day) (186). Remarkably, even at very high loads (20.5g; ~88% BM), running performance (distance per day) did not dramatically decrease over the 14 days of RWR training (Day 1 ~ 7.5km/day vs. Day 14 ~6.0km/day, on average); further, there were no significant differences in distance accomplished per day between loaded and unloaded wheel runners (186). The FWR ($9.64 \pm 0.38\text{mg}$) and RWR ($9.84 \pm 0.11\text{mg}$) groups had similarly greater SOL mass than sedentary controls ($8.53 \pm 0.14\text{mg}$). Despite similar running distance and SOL mass, the RWR group increased mTORC1 and p70^{S6K} protein phosphorylation in SOL, relative to control, while the FWR group did not. These data indicate that RWR promotes hypertrophic signaling more potently in prime movers than FWR (186). However, SOL mass was no different between running groups (186), which highlights that, while RWR may promote resistance-like training effects, RWR may promote adaptive signaling interference similar to concurrent training.

Table 5. Brief Summary of Resistance Wheel Running's Effects on Skeletal Muscle			
First Author (Year)	Duration	Load Range (species)	Skeletal Muscle Effects (Magnitude)
Ishihara (1998)	8 weeks	~1.5→~73% BM (rats)	↑ PLN mass vs. control (~374mg vs. ~313mg) ↑ PLN SO fiber CSA vs. control (~3247 vs. ~2812 μm^2) ↑ PLN FOG fiber CSA vs. control (~3918 vs. ~3142 μm^2) ↑ PLN FG fiber CSA vs. control (~4117 vs. ~3431 μm^2)

Konhilas (2005)	7 weeks	~7.0→~41% BM (mice)	↑ SOL mass vs. control (~20%) ↔ PLN, GAS, or TA mass vs. control ↑ Size of type IIA fibers in calf vs. control (~20-30%) ↑ Size of type IID/X fibers in calf vs. control (~5-10%)
Legerlotz (2008)	45 days	~0.0→~176% BM (rats)	↔ GAS PLN, SOL, or TA mass vs. control ↑ PLN fiber CSA vs. control and FWR (7.6% and 6.0%) ↑ SOL fiber CSA vs. control (~5%)
Call (2010)	12 weeks	~3.0→~20% BM (mice)	↑ Triceps mass/BM vs. control (~10%) ↔ GAS, SOL, TA, EDL mass/BM vs. control ↑ SOL tetanic force vs. control (~26%)
Smith (2012)	6 weeks	~0.0→~176% BM (rats)	↑ SOL mass/BM vs. control (0.50 vs. 0.40mg/g BM) ↔ GAS, TA, or VL mass/BM vs. control ↑ SOL fiber CSA vs. control (~2900 vs. 2300 μm^2) ↑ SCs per fiber in SOL, GAS, and VL (~25-100%) ↑ Myonuclei per fiber in SOL, GAS, and VL (~10-15%)
Suijo (2013)	2 weeks	~4.3→~88% BM (rats)	↑ SOL mass vs. control (~9.84mg vs. ~8.53mg) ↑ SOL (p)mTORC Ser2448 vs. control (~81%) ↑ SOL (p)p70 ^{SGK} Thr389 vs. control (~60%)
Soffe (2015)	10 weeks	~0.0→~20% BM (mice)	↑ SOL mass vs. control (~50%) ↑ QUAD mass vs. control (~12.5%) ↔ SOL fiber CSA vs. control

As reviewed above and summarized in table 5, RWR is a relatively understudied exercise model in rodents, which may promote resistance-like training effects. There was considerable variability in study designs, specifically in wheel resistances achieved by mice, but most of the studies of RWR were conducted on relatively short-term timescales (2-12 weeks) and their RWR systems were not equipped to easily apply resistance as a function of body mass. Only one study in mice investigated the effects of RWR on SkM endurance training adaptations. None of these studies looked at RWR's effects on plantar flexor function, despite this muscle group's integral role in locomotion. To address these deficiencies, we sought to develop an improved RWR system, and to investigate the long-term effects of RWR on endurance training adaptations and plantar flexor contractile function *in-vivo*.

Section 2.4: Restatement of the Problem

Mice are commonly used for endurance exercise studies, but fewer mouse models of voluntary resistance exercise are available. Previous studies have shown that resistance wheel running may promote resistance-like training adaptations in mice of the C57 genetic background (11, 185, 186). However, existing RWR systems either crudely apply wheel resistance using weights, or are very expensive (e.g., ~\$1500 per wheel). Additionally, few studies have assessed the SkM endurance training effects of RWR, and no studies have assessed RWR's effects on prime mover muscle function over time. The primary purposes of this study were 1) to develop a computer-controlled RWR system that could apply resistance as a function of body mass, 2) to determine how RWR alters various physiological parameters of male C57BL10/SnJ mice over time (e.g., plantar flexor function and body composition), 3) to determine how RWR alters various physiological parameters in isolated tissues (e.g., muscle mass, CSA, myosin heavy chain expression, central nucleation, plasma creatine kinase activity, etc.), and 4) to determine if RWR promotes functional and physiological adaptations similar to both endurance and resistance training.

Chapter 3: Methods

Section 3.1: Study Design

3.1.1 Animals

Forty male C57BL/10SnJ mice were purchased from Jackson Labs. The C57BL/10SnJ strain was used because the C57BL/10 mouse serves as the genetic background for the dystrophic *mdx* mouse, which we intended to use in future studies of resistance wheel running. Mice were delivered in 2 cohorts of 20 at age 3 weeks; cohort 1 arrived two weeks prior to cohort 2. Mice were individually housed for the duration of the study (20 weeks total for cohort 1; 23 weeks total for cohort 2), had equal light/dark cycles (starting at 7:00AM and 7:00PM, respectively), and consumed standard mouse chow and water *ad libitum*. We intended to only use the best 32 runners (based on a 3-day screening process), but only 30 of those mice survived to the end of the study (16 mice in cohort 1; 14 mice in cohort 2). The remaining mice were used for other study purposes (e.g., troubleshooting methods). Food consumption and body mass were recorded every 7-14 days.

3.1.2 Resistance Running Wheels

To facilitate RWR, resistance was applied to the axle of running wheels (11.5cm diameter; Mini Silent Spinner; Super Pet, Inc.). To apply resistance, a stepper motor turned a threaded rod, which compressed a spring that acted on an actuator arm. Two plastic wheels on the actuator arm interfaced with a rubberized portion of the running wheel axle (Figure 9). A computer program (Running Wheel Analyzer; VT Metabolic Phenotyping Core), which was connected to a magnetic wheel counter and to the stepper motor via microcontroller (Arduino Uno; Arduino, Turin, Italy), recorded distance run (m) and modulated wheel resistance (in grams

(g)) as a percentage of animal body mass (% BM). Distance data were recorded in 10 second intervals. Running wheels had ~0.2g of inherent (no-added) resistance. Wheel work in joules (J) was calculated as a function of distance run (m) and wheel resistance (g).

$$Work (J) = \frac{Distance (m) * Resistance (g)}{101.94 \frac{g}{N}}$$

To quantify wheel resistance and to check wheel calibrations, the wheels were temporarily removed from the axle and were replaced with a light metal bar (11.5cm long); masses were hung from the end of the metal bar until the gravitational force acting on the masses overcame the axle's inertia (Appendix C).

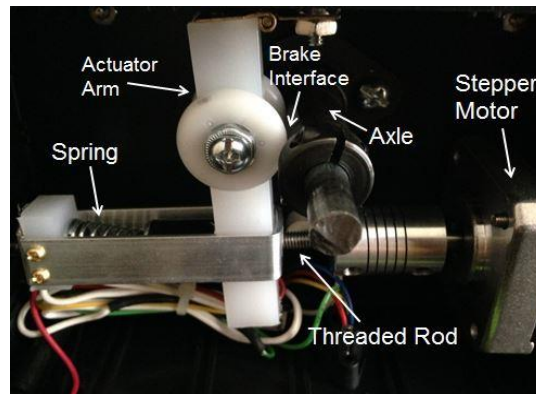


Figure 9. Resistance Running Wheels.

Resistance was applied to running wheels by a computer-controlled stepper motor that turned a threaded rod, which compressed a spring. The spring was contiguous with an actuator arm, which applied frictional (braking) force to a rubberized portion of the running wheel axle.

3.1.3 General Timeline, Screening, and Baseline Measures

This study consisted of four major phases: screening (3 days), pre-training (8 weeks), static training (5 weeks), and dynamic training (lasted 7 weeks for cohort 1; lasted 10 weeks for

cohort 2) (Figure 10). During the first week of the study, all mice were screened for running status for 3 days (wheels had no-added resistance) and baseline measures of hindlimb plantar flexor torque (PFT), body composition, and treadmill running fitness were obtained (all described in section 3B). Detailed descriptions of each training phase are provided in section 3.A.4.

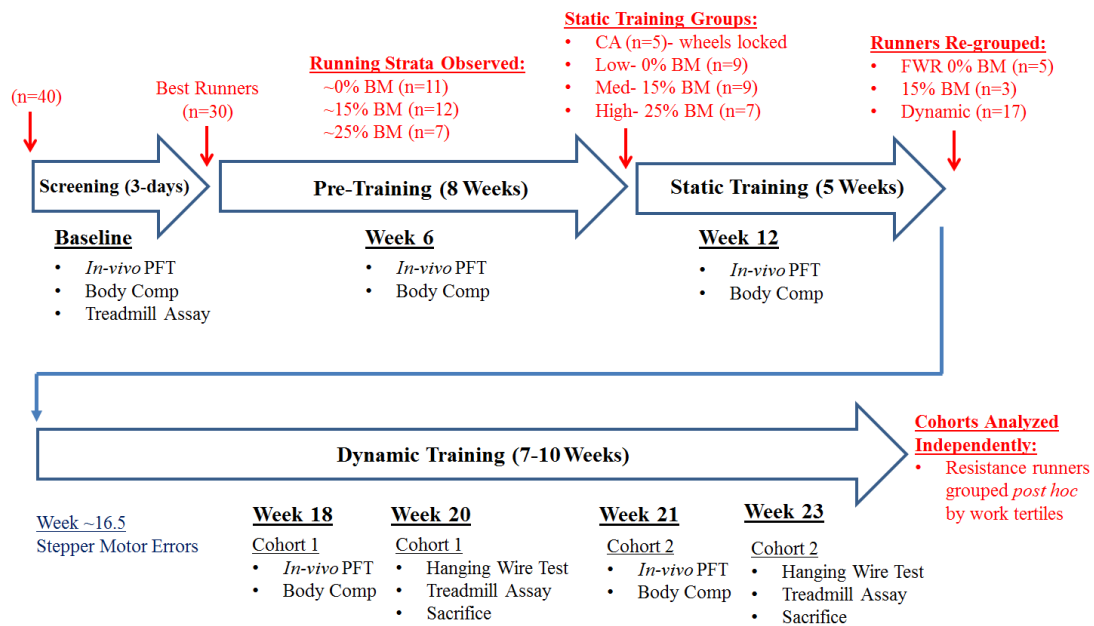


Figure 10. Schematic of Study Timeline and Training Phases.

Training Phases: First, all mice (n= 40) underwent 3 days of screening for running status. The top 30 runners were pre-trained to run against progressively increasing resistance ($\pm 2\text{-}3\%$ body mass (BM) every ~ 3 days). At the end of pre-training, 5 mice were selected as cage-activity controls (CA; n= 5) and their wheels were locked for the remainder of the study. During the next 5 weeks (static training), the remaining 25 mice ran against a constant load of 0% BM (n= 9), 15% BM (n= 9), or 25% BM (n= 7), based on their performance during pre-training. After static training, mice either continued running against 0% BM (FWR 0% BM; n= 5), 15% BM (n= 3), or began dynamic training (n= 17), which involved dynamic adjustments to wheel resistance, in real-time. After ~ 16.5 weeks, we discovered a problem with the stepper motor resistance mechanism, which was resolved after two weeks of troubleshooting (~ 18.5 weeks). Mice were sacrificed at the end of the dynamic training phase (7 weeks for cohort 1 (20 weeks total), and 10 weeks for cohort 2 (23 weeks total)). Final sub-groups for resistance runners (included both static and dynamic resistance runners) were formed *post hoc* for each cohort based on work performed during dynamic training, after the stepper motors were fixed (~ 18.5 weeks until sacrifice). Final groups for cohort 1 were: CA (n= 3), FWR 0% BM (n= 2), Low-Work (n= 3), Med-Work (n= 4), and High-Work (n= 4). Final groups for cohort 2 were: CA (n= 2), FWR 0% BM (n= 3), Low-Work (n= 3), Med-Work (n= 3), and High-Work (n= 4). Data collected during the dynamic training phase (and after sacrifice) were independently analyzed for each cohort. **Assays:** Measures of plantar flexor torque (PFT) and body composition were obtained at baseline, 6 weeks (during pre-training), 12 weeks (during static training), and two weeks prior to sacrifice during dynamic training (18 weeks for cohort 1; 21 weeks for cohort 2). Two days prior to sacrifice, hanging wire tests were performed. At baseline and one day prior to sacrifice, treadmill assays were performed.

3.1.4 Training Details

3.1.4.1 Pre-training Phase

Following baseline measures, 30 mice were selected for the study (16 from the first cohort; 14 from the second cohort). Only 29 of 30 engaged their wheel during 3-day screening. All 30 mice were given free access to resistance running wheels to learn to run against resistance and to establish groups based on running performance.

Note that we did not use a sedentary control during pre-training for multiple reasons: 1) running wheels affected the cage environment (i.e., available space, enrichment, temperature (+1-5°C, depending on proximity to the stepper motor)), so we wanted sedentary controls to also have a (locked) resistance wheel in their cages; 2) only 32 resistance wheels were available; 3) a power analysis showed that group sizes of at least 6 were necessary for sufficient study power ($\beta = 0.80$; Appendix A), but we did not know *a priori* the mice that would consistently run against resistance. Hence, all mice were pre-trained to run against resistance and sedentary controls were chosen after pre-training.

During the first week of pre-training for cohort 1, wheel resistances were increased by 3% BM daily, but we found that running performance was lower than expected: $143 \pm 86\text{m/day}$ when resistances reached 18% BM ($3.9 \pm 0.1\text{g}$). So we hypothesized that resistances were increased too rapidly, and we decided to reset the wheels to 0% BM and adjust the pre-training protocol.

Instead, we incrementally altered the resistance $\pm 2\text{-}3\%$ BM ($\pm \sim 0.5\text{-}1.0\text{g}$) every ~ 3 days for each mouse, based on running performance. On days when resistances were adjusted, if mice ran $> 1000\text{m/day}$ during previous 24 hours, then wheel resistance was increased. If mice ran

<500m/day, wheel resistance was decreased. Wheel resistance was held constant (relative to BM) if mice ran 500-1000m/day. Each time wheel resistances were adjusted, the stepper motors were recalibrated with a 2g hanging mass. This incremental adjustment protocol was used for the remainder of pre-training for cohort 1 (~6.5 weeks). Mice in cohort 2 immediately began pre-training with the incremental adjustment method, rather than daily resistance increases.

3.1.4.1.1 Running Strata and Selection of CA-control Mice

By the end of pre-training for cohort 1, we noticed three distinct running strata (based on weeks 7 and 8): some mice consistently ran against relatively low resistance (~0-5% BM; $1.2 \pm 0.4\text{g}$; $1447 \pm 449\text{m/day}$; $n=6$), others ran against medium resistance (~12-18% BM; $4.2 \pm 0.3\text{g}$; $1677 \pm 533\text{m/day}$; $n=6$), and a third group ran against higher resistances (~25-28% BM; $6.5 \pm 0.4\text{g}$; $2064 \pm 792\text{m/day}$; $n=4$). These running strata were used to group mice in cohort 1 for static training.

However, during the last week of pre-training a subset of these mice (3 of 16), dramatically reduced their wheel activity (from $2052 \pm 454\text{m/day}$ to $96 \pm 32\text{m/day}$; $p=0.049$), and we were not confident that they would resume training during the next phase, so they were selected for the cage-activity (CA) control group.

We observed similar running patterns for mice in the second cohort: low resistance runners (~0-5% BM; $1.2 \pm 0.4\text{g}$; $4264 \pm 1122\text{m/day}$; $n=5$), medium resistance runners (~12-18% BM; $4.2 \pm 0.3\text{g}$; $2832 \pm 867\text{m/day}$; $n=6$), and high resistance runners (~25-28% BM; $6.8 \pm 0.4\text{g}$; $2968 \pm 1169\text{m/day}$; $n=3$). At the end of cohort 2's pre-training, 2 mice (of 14) were randomly selected for the CA-control group. Running wheels were locked for CA-control mice, and remained locked for the rest of the study; these mice served as sedentary/detrained controls.

3.1.4.2 Static-Resistance Training Phase

After pre-training, running groups trained against static (constant) wheel resistance for 5 weeks, based on the running strata described above. Resistances were set to 0% BM (no-added resistance; $n=9$), 15% BM ($n=9$), or 25% BM ($n=7$).

We sought to minimize variability in work accomplished during static training by setting maximum work limits for runners. The limit was based on the average maximum work conducted by mice in cohort 1 during their final week of pre-training (106.2J), plus one standard deviation (96.7J), which was ~ 200 J. If the control software calculated that a given mouse reached its work limit, the wheel was locked until the start of the next dark phase (7:00PM). Maximum work limits were raised by 10% each week for the duration of the study to model human training-overload regimens (i.e., 200J week 9, 220J week 10, 240J week 11, etc.) (63). However, very few mice ever reached the work limit.

3.1.4.2.1 Switching Groups for Dynamic Training Phase

Three weeks into cohort 1's static training, PFT measures were performed to determine if RWR had affected muscle function, but there were no differences in strength between resistance runners ($1.95 \pm 0.1\text{N}\cdot\text{cm}$; $n=9$) and cage-activity controls ($2.12 \pm 0.2\text{N}\cdot\text{cm}$; $n=3$) ($p=0.396$), so we hypothesized that resistance runners were not performing enough work on the running wheels to promote adaptive strength gains, as seen by others (Call 2010). Additionally, for the majority of mice running against added-resistance (13 of 16, from both cohorts), running distance appeared to decline during the latter half of the static training phase (first half= $1984 \pm 349\text{m/day}$ vs. second half= $1420 \pm 324\text{m/day}$; $p=0.12$). These 13 resistance runners and several of the consistent 0% BM runners ($n=4$ of 9; first half= $3054 \pm 1271\text{m/day}$ vs. second half= $2600 \pm 1383\text{m/day}$; $p=0.817$), were placed on an alternative training program where wheel resistance

was dynamically adjusted in real-time to attempt to maximize their voluntary work output (17 mice total; described in section 3.A.4.3). The remaining resistance runners (3 of 16) continued to perform moderately well throughout static training (first half= $1992 \pm 544\text{m/day}$ vs. second half= $2040 \pm 1036\text{m/day}$; $p= 0.97$), so they were kept on their static resistance program (all 15% BM) for the remainder of the study (Table 6).

Table 6. Re-grouping Mice for the Dynamic Training Phase		
Group	n	Wheel Settings
Cage-Activity Control	5	N/A
FWR 0% BM	5	No-added resistance
Resistance Runners	17	Dynamic
	3	15% BM

3.1.4.3 Dynamic Training Phase

To attempt to maximize work performed on the wheels, Charlie Stylianios (IT Support Technician; HNFE Department; Virginia Tech) modified the running program to enable the stepper motors to modulate wheel resistance in real-time. We called this kind of RWR program “dynamic training”.

After two weeks of development and testing, the following parameters were used for dynamic training: starting at 5% BM, wheel resistance was progressively increased by 2% BM for every 1J performed on the wheel. Providing 5% initial resistance resulted in a rapid increase in wheel load during running bouts (e.g., a 30g mouse would only need to run ~70m to reach its first step goal, then 50m, then 40m, etc.). Following initial wheel activity, if the wheel was not re-engaged within 120 seconds, then resistance was reset to baseline (5% BM). We hypothesized that rapidly increasing wheel resistance might simulate higher-intensity, short-duration exercise bouts similar to interval training. Further, we hypothesized that wheels needed to quickly reset

(to 5% BM) to encourage frequent activity and to prevent excessive wheel load from inhibiting running behavior.

The dynamic training phase for the first cohort lasted 7 weeks, but the second cohort's lasted 10 weeks; difference in dynamic training duration between cohorts was caused by holiday scheduling conflicts among the essential personnel for mouse sacrifices. Consequently, data collected during the dynamic training phase (and after sacrifice) were split by cohort.

3.1.4.4 Summary of Resistance Wheel Problems and Solutions

This section describes issues that were encountered with wheel resistance settings and how they were addressed. A timeline of these events is provided at the end of the section (Figure 11).

3.1.4.4.1 Calibration Checks

Wheels were frequently calibrated throughout the study (at least once per week). Additionally, actual wheel resistances were checked qualitatively and quantitatively, but less frequently than wheels were re-calibrated.

Qualitative checks involved turning the running wheels by hand to 1) confirm that resistance was/was not being applied to the wheels when appropriate, and 2) to confirm that there were noticeable differences between high-load and low-load running wheels. If wheel resistances seemed incorrect during the qualitative checks, a quantitative check was performed.

Quantitative checks involved direct measurement of wheel resistance by hanging masses in 0.1g increments from a light metal bar on the axle; wheel resistance was determined when the gravitational force of the hanging mass exceeded the axle's inertia. If actual wheel resistance

deviated from the intended (programmed) resistance by more than 0.5g during quantitative checks, then the wheel was recalibrated and its individual brake program was reset.

3.1.4.4.2 Stepper Motor Correction

Despite frequent checks and calibrations, the intended (programmed) wheel resistances may have deviated from actual wheel resistances during the study; this issue was discovered during a series of quantitative checks performed ~3.5 weeks into cohort 1's dynamic training phase (~16.5 weeks into the study), and was resolved after 2 weeks of troubleshooting (~18.5 weeks into the study) (Figure 11).

During troubleshooting, we discovered that, even after wheels were calibrated, they quickly lost calibration (within 24 hours) because the computer program commanded the stepper motors to turn too quickly. We fixed the problem when we decreased stepper motor turning speeds by 25%. Deviations in wheel resistance did not affect the wheels of mice running against no-added resistance.

After slowing down the stepper motors, wheel resistances were mostly within 0.5g of target resistance (159 of 179 quantitative checks), and wheels were re-calibrated when deviations exceeded 0.5g. Unfortunately, this indicates that wheel resistances (and work calculations) were uncertain for mice running against added-resistance prior to ~18.5 weeks. However, distance data throughout the study were reliable and wheel resistances were reliable after ~18.5 weeks. On this basis, wheel work comparisons could still be made between resistance running groups from ~18.5 weeks to sacrifice.

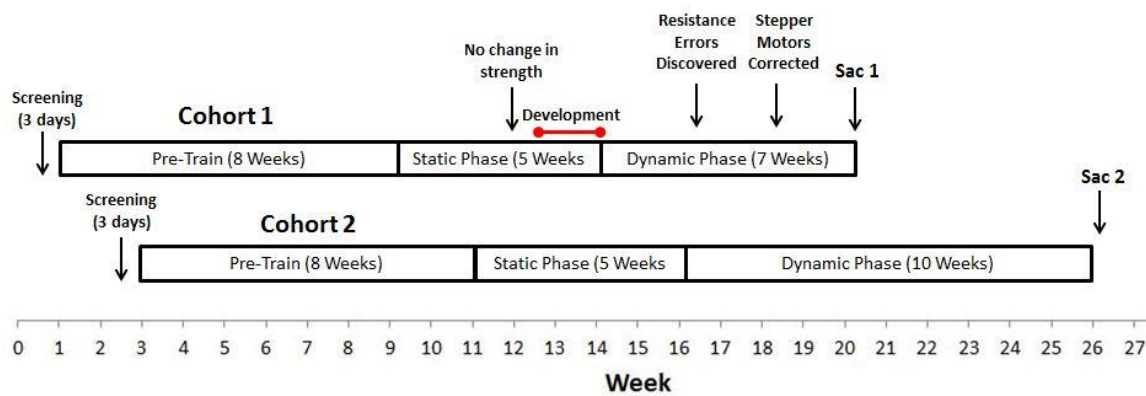


Figure 11. Timeline of Study Problems and Solutions.

After 3 weeks of static training (12 weeks total), *in vivo* PFT measures were obtained for the first cohort, and RWR had no observable effects on plantar flexor function; so we decided to develop (red bar) and implement the dynamic training program. Roughly 3.5 weeks into dynamic training (~16.5 weeks into the study), we discovered errors in wheel resistance during a series of quantitative checks. The errors were resolved in 2 weeks (~18.5 weeks into the study) when stepper motor turning speeds were decreased. At least 12 days of reliable work data were available for *post-hoc* grouping of resistance runners.

3.1.4.5 Establishing Final Groups *Post-Hoc*

Since dynamic training phases were of different lengths for cohorts 1 and 2 (7 weeks and 10 weeks, respectively), we split up analyses of data collected during dynamic training (and after sacrifice) by cohort. Within cohorts, mice running against added-resistance (both dynamic and static) were divided into tertiles *post-hoc*, based on average work accomplished after the stepper motor correction (i.e., from ~18.5 weeks until sacrifice). Using this approach, at least 12 days of reliable work data were available for each resistance runner until sacrifice.

For each cohort, the resistance runner tertiles consisted of a Low-Work sub-group (Cohort 1: $n = 3$; $1.7 \pm 1.0\text{J/day}$; Cohort 2: $n = 3$; $8.1 \pm 1.0\text{J/day}$), a Med-Work sub-group (Cohort 1: $n = 4$; $28.6 \pm 3.1\text{J}$; Cohort 2: $n = 3$; $31 \pm 0.5\text{J/day}$), and a High-Work sub-group (Cohort 1: $n = 4$; $44 \pm 2.5\text{J/day}$; Cohort 2: $n = 3$; $58.5 \pm 5.3\text{J/day}$). Data from the resistance runner sub-groups

were also compared to the CA-control groups (Cohort 1: n= 3; Cohort 2: n= 2) and to the free-wheel running groups (FWR 0% BM; Cohort 1: n= 2; Cohort 2: n= 3) (Table 7).

Table 7. Final Groups Formed for Analyses During/After Dynamic Training			
Group	Sub-Group	Cohort 1 (n)	Cohort 2 (n)
CA	n/a	3	2
FWR 0% BM	n/a	2	3
Resistance Runners	Low-Work	3	3
	Med-Work	4	3
	High-Work	4	3
Total		16	14

Section 3.2: Assays Before Sacrifice

3.2.1 Hindlimb Plantar Flexor Torque Measures

Measures of *in-vivo* hindlimb PFT contractile properties (i.e., torque and temporal data) were obtained at baseline, 6, and 12 weeks for both cohorts; final measures were obtained 2 weeks prior to sacrifice (cohort 1 at 18 weeks; cohort 2 at 20 weeks). Mice were deeply anesthetized with 2-3% Fluriso™ (99.9% isoflurane; VetOne) mixed with O₂ (100%; flow-rate of 1.5L/min; Airgas) in a vaporizer and delivered to a plexiglass induction chamber (model Impac5; VetEquip). Excess isoflurane gas was trapped with VaporGuard™ activated charcoal filters (VetEquip). Depth of anesthesia was tested by foot pinch after 3-5 minutes. Once sufficiently anesthetized, the mouse was removed from the induction chamber, quickly weighed on a portable scale (model PT1200; Sartorius), and transferred to an adjacent preparation table with a nose cone (to continue delivery of isoflurane anesthetic).

At the preparation table, the right hindlimb was shaved with a Wahl trimmer (Wahl) to expose the underlying skin, and remaining hair was removed with Nair depilatory cream (applied

for ~1 minute; Church & Dwight). Excess Nair was removed with a 2x2 gauze soaked with clean water. After shaving, the mouse (with nosecone) was transferred from the preparation area and laid supine on a heated platform (35°C; temperature maintained via heated water pump (model HTP-1500; Adroit Medical Systems) of an 809B *in-situ* mouse stimulation apparatus (Aurora Scientific). The mouse was positioned with its tail and right hindlimb between a U-bracket (3.17cm wide X 2.0cm tall X 0.4cm thick), while its left hindlimb rested outside of the U-bracket (Figure 12).

The mouse's right foot was taped (Transpore™ Surgical Tape (3M)) to the metal footplate of a 300C-LR Dual-Mode servomotor (Aurora Scientific). The foot was positioned parallel to the tibia, and tibia parallel to the platform. The knee was held in place by a plastic/metal plunger (4.0cm long X 0.5cm thick) that pressed the lateral epicondyle against the inside of the U-bracket on the 809B platform (note: the inside face of the U-bracket was modified to have the tip of a 26 gauge needle assist in securing the lateral epicondyle). To immobilize the knee, the plunger was secured with a hex screw and Allen wrench (1.5mm size). The knee was flexed as close to 90° as possible; when measured, knee angles ranged between 110-130° (range of motion was limited by size of the U-bracket; further described in Appendix B) (Figure 12). After securing the knee, the mouse's tail and ventral mid-section were taped from one side of the platform to the other. The nosecone was also secured to the platform with tape.

Servomotor position was controlled by Dynamic Muscle Control software version 5.41 (Aurora Scientific). Two sterilized platinum needle electrodes (model E2-12; 0.3mm diameter; Grass Technologies) were inserted between the U-bracket and the skin of the knee, superior and inferior to the patella (~2mm apart) to activate the plantar flexor muscles (GAS, SOL, and PLN)

via the tibial nerve. All plantar flexor stimulations were delivered as square-wave pulses (35mA; 0.2ms pulse width; current optimization described in Appendix B).

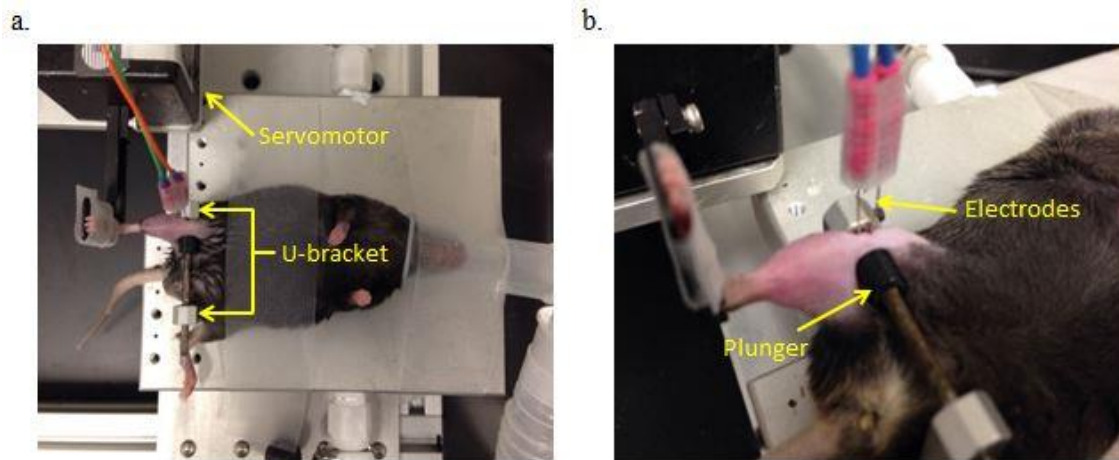


Figure 12. Setup of *In-vivo* Hindlimb Plantar Flexor Torque Assay.

a) This is an overhead view of a mouse positioned for *in-vivo* plantar flexor torque measures on the 809B stimulation apparatus (Aurora Scientific). The mouse's right hindlimb and tail are between the U-bracket, and the right foot is secured with tape to the foot-pedal (attached to the servomotor). **b)** Lateral view of the right hindlimb shows the knee flexed in the neutral position and held in place with the plunger. Needle electrodes were inserted between the U-bracket and the skin, superior and inferior to the patella (~2mm apart) to activate the tibial nerve.

The footplate was fixed for an initial series of isometric stimulations (30 seconds rest between stimulations): first a tetanic contraction (150Hz pulse frequency) and then two twitches (1Hz).

After 1 minute rest, plantar flexor contractile properties were obtained while the ankle was rhythmically moved in a sinusoidal arc about the neutral position by the stepper motor (± 0.0743 cm amplitude; two consecutive sine waves completed; 0.143s per cycle). Protocols were based on work previously described (187). During the first sine wave, the plantar flexors produced passive torque. At the start of the second sine wave, the plantar flexors were stimulated

(1, 20, or 40Hz; ~30 seconds between each). Torque and displacement data from the sine wave stimulations were analyzed for passive and stimulated work-loops, but excluded from results because of technical errors (Appendix B).

After 2 minutes of rest, the plantar flexors were serially stimulated isometrically at increasing frequencies (1, 10, 30, 50, 65 80, 100, 120, 150, 180Hz; 30 seconds rest between each stimulation). Fused tetanus usually occurred at 150-180Hz. These data were analyzed as plantar flexor torque-frequency curves.

After assessing torque-frequency, mice were given 5 minutes rest before undergoing an isometric plantar flexor fatigue protocol: the leg was stimulated at 100Hz every 5 seconds for 5 minutes (60 stimulations total).

At the end of the fatigue protocol, the isoflurane was set to 0%, the nose cone was removed, the leg was aseptically cleaned with 10% povidone-iodine solution (CVS Pharmacy, Inc.), and then the mouse was placed in the induction chamber to recover from anesthesia (~2-5 minutes in 100% O₂; 1.5L/min). Hindlimb PFT data were analyzed using Dynamic Muscle Analysis (DMA) software versions 3.2 and 5.211 (Aurora Scientific).

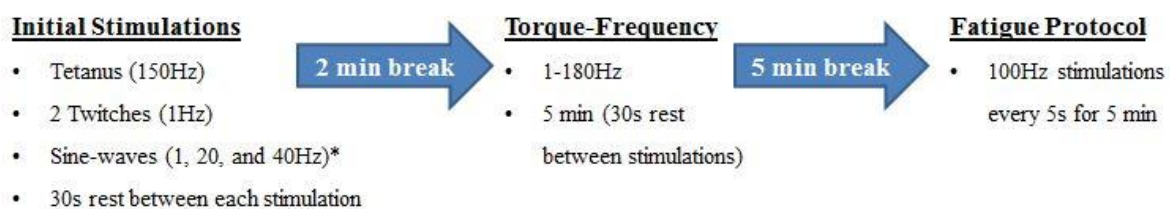


Figure 13. Schematic of Stimulations Performed during Plantar Flexor Torque Protocols.

During the initial stimulations plantar flexors were underwent a tetanus (150Hz), two twitches (1Hz), and three sine-wave contractions (at 1, 20, and 40Hz). After a 2 minute break, plantar flexors underwent torque-frequency (i.e., serial stimulations increasing frequencies; 1, 10, 30, 50, 80, 100, 120, 150, 180Hz; 30 seconds rest between each). Then after a 5 minute break, plantar flexors were finally stimulated to fatigue (100Hz stimulations every 5 seconds for 5 minutes). * The sine wave stimulations were not used in data analyses because of technical errors (Appendix B).

3.2.2 Body Composition

Immediately after all *in-vivo* PFT measures, mouse body composition was determined using a Bruker Minispec LF90-II time-domain nuclear magnetic resonance system (Bruker Corporation). Prior to each session, the Minispec was calibrated according to the manufacturer's instructions. Then mice were placed in the Minispec, which measured spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) of hydrogen atoms (6.2MHz proton frequency; 30 sets of 1ms Carr-Purcell-Meiboom-Gill echo trains) to quantify the relative proportions of fat, lean, and fluid tissues.

3.2.3 Hanging Wire Tests

To assess skeletal muscle endurance at the end of the study (2 days prior to sacrifice), mice underwent hanging wire tests similar to those previously described (188). Before the test, mice were weighed on a portable scale (model PT1200; Sartorius). Briefly, the investigator grasped the mouse by its tail and placed onto an elevated hanging wire (55cm long X 2mm thick suspended horizontally between two posts 35cm above a thick layer of bedding). Once the mouse secured itself on the wire with all four paws, the timer was started and the tail was released (Figure 14). Then the mouse held onto the wire for as long as possible. When the mouse fell, the timer was stopped. Using BM (g) and hang time data (s), hanging impulse (g*s) was calculated to account for hang time differences due to differences in BM (188).

$$Impulse (g * s) = BM (g) * Hang Time (s)$$

Each mouse was given 3 attempts to perform the hanging wire test with 30 seconds of rest between bouts; the best attempt by each mouse was used for analysis. However, not all of

the mice were compliant during all of their tests; some would hang from their hindlimbs, spot a place to land, and then intentionally release from the wire. One mouse did not comply with any of its tests. Non-compliant tests were disregarded in subsequent analyses.

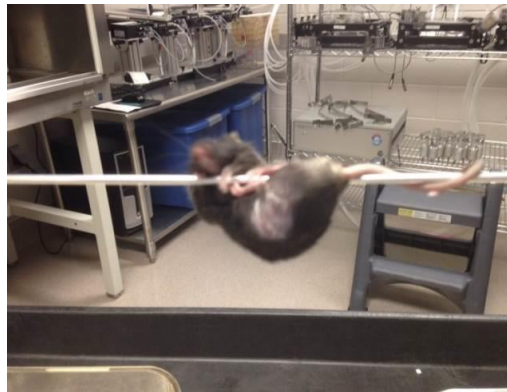


Figure 14. Hanging Wire Test.

Mice were placed onto a horizontal hanging wire (55cm long X 2mm thick), which was suspended 35cm above a layer of bedding. Once the timer started, mice hung onto the wire with all four limbs (and tail) until exhaustion.

3.2.4 Treadmill Assays of Maximal Running Capacity

To assess maximal running capacity, mice performed a progressive treadmill running assay to exhaustion at baseline and one day (~18-20 hours) prior to sacrifice. The treadmill assay used in this study was a modified version of an assay previously described (156). Two important modifications were: 1) this study did not have permission from the Virginia Tech Institutional Animal Care and Use Committee (IACUC) to use low-grade electric shocks to motivate running, so an alternative prodding approach was used (described below), and 2) we decided to use a higher maximum running speed (0.6m/s vs. 0.46m/s) after troubleshooting the method with a subset of spare mice (described below).

Immediately before each treadmill assay (baseline and final), mice were given ~2 minutes to acclimate to the enclosed treadmill (64cm long belt; 5.5cm wide; TSE Systems) with the belt slowly running (0.1m/s). After acclimation, the belt speed increased 0.1m/s every two minutes up to 0.6m/s (maximum speed reached at 20 minutes); mice were challenged to run at 0.6m/s to exhaustion.

When the mouse slowed down, it approached the back of the treadmill, where the operator attempted to motivate the mouse by prodding it with a cotton-tipped applicator (similar to a Q-tip). To avoid injuring the mouse, if it fell to the back of the treadmill and did not re-engage the treadmill after prodding, the treadmill was briefly stopped (2-5 seconds) to allow the mouse to return to the front of the treadmill before continuing. The test was considered complete when the mouse failed to respond to motivational prodding 3 times (i.e., on the third stop). Typical times to exhaustion did not exceed 30 minutes.

The maximum speed of 0.6m/s was chosen by testing a sample of age-matched, male C57BL10/SnJ mice (n= 4) at baseline. Briefly, after ~2 minutes acclimation at a slow speed (0.1m/s), belt speed was progressively increased by 0.1m/s every minute until the mice could no longer keep pace with the treadmill. All of the mice kept pace at 0.6m/s, but none kept pace when the belt reached 0.7m/s; hence maximum belt speed was limited to 0.6m/s during treadmill fitness assays.

3.2.5 Sacrifice Procedure

One day prior to sacrifice (~18-20 hours), the mice performed the treadmill fitness assay. In the evening (7:00PM; 14-15 hours prior to sacrifice), food was removed and wheels were locked to minimize acute differences in feeding behavior and physical activity prior to sacrifice. Four to six mice were euthanized on each sacrifice date by 1-2 minutes of CO₂ inhalation (100%

CO₂) followed by cervical dislocation. Mice were then exsanguinated by cardiac puncture (27.5 gauge Tuberculin syringe; Beckton-Dickson) and blood was carefully transferred to heparinized tubes (BD Microtainer™ Lithium Heparin tubes; Fisher Scientific) for plasma CK analysis. Next, QUAD muscles were removed, split into red and white components, and homogenized for metabolic assays. Tissues were rapidly excised (heart, GAS, SOL, EDL, TA, hamstrings, and triceps (Tri) muscles), and either minced/freeze-clamped or weighed and mounted on cork for cryosectioning. Next, the carcass was stripped of remaining SkM to isolate satellite cells. Finally, hypothalami and amygdalae were removed and stored in RNA-later solution (LifeTechnologies) until further analysis.

Section 3.3: Assays After Sacrifice

3.3.1 Glucose and Palmitate Oxidation

To determine if RWR promoted metabolic training effects, red and white portions of QUAD muscle were assayed for glycolytic and oxidative capacities. Briefly, muscle portions were homogenized and then placed into Krebs-Henseleit buffer at 30°C (perfused with 95% O₂ and 5% CO₂; pH= 7.4), with 4% fat-free bovine serum albumin (BSA) (Sigma-Aldrich), 5mM glucose, and 1mM palmitate. Once prepared, muscle homogenates were ready for fatty acid oxidation (FAO) and glucose oxidation (GO) assays, which were performed as previously described (189).

To determine FAO rates, 0.75μCi [1-¹⁴C] palmitate was added to the muscle homogenate, and then the mixture was incubated for 1 hour. FAO rates were quantified by measuring the production of ¹⁴CO₂ and 1-¹⁴C-labeled acid-soluble metabolites (ASM) after incubation. To measure ¹⁴CO₂ produced, 1mL of the incubated mixture was removed and placed into a glass

scintillation vial, which contained both 1mL of 1M H₂SO₄ and a micro-centrifuge tube containing 400μL of benzethonium hydroxide. The expelled ¹⁴CO₂ was trapped in the benzethonium hydroxide and counted using the LS6500 Multi-Purpose Scintillation Counter (Beckman-Coulter). Higher ¹⁴CO₂ production and ¹⁴CO₂/ASM ratio indicate higher FAO capacity and efficiency, respectively.

The GO protocol followed the same steps as the FAO protocol above, except [U-¹⁴C] glucose was substituted for [1-¹⁴C] palmitate. Higher ¹⁴CO₂ production in the GO assay indicates higher glycolytic capacity.

3.3.2 Oxidative Enzyme Activity Assays

Maximal activities of citrate synthase (CS) and beta-hydroxyacyl-CoA dehydrogenase (BHAD) were assessed using the same QUAD muscle homogenates as described above (section 3.C.1). Increased activities of CS and BHAD are metabolic markers of endurance training effects in SkM (40).

3.3.2.1 Citrate Synthase Assay

CS catalyzes the formation of citrate and co-enzyme A (CoA-SH) from oxaloacetate and acetyl-CoA (190). Briefly, 10μL of a 1:5 diluted muscle homogenate was added, in duplicate, to 170μL of a solution containing 0.1M Tris buffer (pH= 8.3), 1mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and 0.01M oxaloacetate. Following a 2-minute background reading, the spectrophotometer (BioTek Synergy 2 Microplate Reader (VT Metabolic Phenotyping Core)) was calibrated, and 30μL of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405nm every 12 seconds for 7 minutes at 37°C to determine DTNB reduction by CoA-SH. Maximum CS activity was calculated (reported as μmol/mg protein/min).

3.3.2.2 Beta-Hydroxy-Acyl-CoA Dehydrogenase Assay

BHAD aids in β -oxidation by oxidizing fatty acyl-CoAs to form NADH for aerobic metabolism (191). To determine maximal BHAD activity, 35 μ L of QUAD muscle homogenate (in triplicate) was added to 190 μ L of a buffer containing 0.1M triethanolamine, 5mM ethylenediamine tetra-acetic acid (EDTA) tetra-sodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (BioTek Synergy 2 Microplate Reader (VT Metabolic Phenotyping Core)) was calibrated and 15 μ L of 2mM acetoacetyl-CoA was added to initiate the reaction. Absorbance was measured at 340nm every 12 seconds for 6 minutes at 37°C. Maximum BHAD activity was calculated (reported as μ mol/mg protein/min).

3.3.3 Satellite Cell Assays

3.3.3.1 Satellite Cell Isolation

After removing most of the leg muscles and triceps, Lidan Zhao and Zhenhe Zhang (Rhoads Laboratory, Virginia Tech) stripped each mouse carcass of remaining skeletal muscles, which were pooled together for satellite cell isolation and evaluation. After manually removing excess connective tissue, pooled muscles were minced in phosphate buffered saline (PBS) on ice and centrifuged at 1,500xg for 5 minutes. Next, the supernate was decanted and protease type XIV (1.25 mg/mL; Sigma-Aldrich) was added to digest the samples for 1 hour at 37°C (samples were mixed every 15 minutes). After digestion, the mixture was centrifuged at 1,500xg for 5 minutes (Sorvall™ Legend™ XT; Thermo Scientific), the pellet was suspended in PBS, and then centrifuged at 500xg for 10 minutes; this process was repeated two more times. Supernates were collected and centrifuged at 1,500xg for 5 minutes to pellet cells. Next, the cells were re-suspended into pre-plating medium (Dulbecco modified Eagle's Minimal Essential Medium

(DMEM; Sigma-Aldrich) containing 10% horse serum, 1% Antibiotic-Antimycotic solution (ABAM; Gibco), and 0.5% gentamicin (Gibco)), and then incubated for 2 hours. Isolated satellite cells were collected after centrifugation (1,500xg for 5 minutes), and then plated into 60mm dishes coated with *Erythrina cristagalli* lectin (ECL; Millipore).

3.3.3.2 Evaluating Satellite Cell Differentiation and Proliferation

3.3.3.2.1 Differentiation Assay

Plated cells were incubated in growth medium (F-10 (Gibco) containing 20% fetal bovine serum (FBS; Atlanta Biological), 1% ABAM, and 0.5% gentamicin) for 7 to 10 days. After the initial growth period, cells were either assayed for differentiative or proliferative capacity. To assess differentiative capacity, cells were seeded into 24-well plates at density of $2.5 \times 10^4/\text{cm}^2$. At 80% confluence, cells were incubated in differentiation medium (DMEM containing 2% horse serum, 1% ABAM, and 0.5% gentamicin) for 3 days.

At the end of differentiation, cells were subjected to immunocytochemical staining for myosin heavy chain (mf20 antibody was generously provided by Dr. Sally Johnson, Virginia Tech). Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes and washed twice with ice-cold PBS. Then samples were incubated for 10 minutes in PBS containing 0.25% Triton X-100, and washed in PBS (3 X 5 minutes). Cells were blocked with 1% BSA in 0.05% PBS-Tween solution, and incubated with mf20 antibody overnight at 4°C. The next day, samples were washed in PBS (3 X 5 minutes), and incubated with Dylight 488 secondary antibody (Fisher Scientific) for 1 hour. After 1 hour, samples were washed again in PBS (3 X 5 minutes). Nuclei were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Sigma-Aldrich) in PBS. Images were captured at 20X magnification using a Nikon Eclipse Ti microscope (Nikon).

The DAPI-positive nuclei were counted (>1000 nuclei per sample) and fusion index data (frequency of myotube nuclei relative to total sample nuclei) were presented as a percentage.

3.3.3.2.2 Proliferation Assay

For the proliferation assay, satellite cells were seeded (in triplicate) onto 96-well plates (2,500 cells per well). Proliferative capacity was measured at 0, 24, 48, and 72 hours using a CyQuant NF cell proliferation kit (Life Technologies), following the manufacturer's instructions. Briefly, the CyQuant NF kit quantifies cell proliferation by first incubating cells in a DNA-binding fluorescent dye for 30-60 minutes at 37°C, and then measuring fluorescence intensity using a microplate reader at 485nm (excitation) and 530nm (emission detection). Higher fluorescence indicates higher dye-DNA binding (DNA content), and by extension higher satellite cell proliferation *in-vitro* (192).

3.3.4 Plasma Creatine Kinase

During sacrifice, whole blood was collected in heparinized tubes (BD Microtainer™ Lithium Heparin tubes; Fisher Scientific) for analysis of CK activity. Thirty minutes after sacrifice, samples were centrifuged at 10,000rpm to isolate plasma. Plasma samples were transferred to new microfuge tubes and stored first at 4°C (overnight), followed by -80°C until analysis (at least 1 week). CK activity was determined using the MaxDiscovery Creatine Kinase Enzymatic Assay Kit (BIOO Scientific), according to the manufacturer's instructions. Briefly, serially diluted standards were prepared to ensure kit linearity (340nm absorbance vs. concentration, $R^2 = 0.993$). Duplicate standards and samples (5μL) were pipetted into a 96-well plate, and reagent was added. A baseline reading (340nm) was obtained, followed by a 5 minute reading (340nm) using the BioTek Synergy 2 Microplate Reader (BioTek Instruments, Inc.).

Greater absorbance (5 minute minus baseline reading) indicates higher plasma CK activity, which corresponds to higher levels of cardiac and SkM damage (193, 194).

3.3.5 Sectioning and Histology

3.3.5.1 Cryosectioning

To assess SkM CSA, central nucleation, and MHC distribution, the GAS, SOL, TA, and Tri muscles were cryosectioned. Briefly, muscles were mounted perpendicularly on cork using a mixture of gum tragacanth (Sigma-Aldrich) and Tissue-Tek™ optimal cutting temperature (OCT) gel (Fisher Scientific). The muscle preparations were rapidly frozen in isopentane (Fisher Scientific; cooled by liquid N₂) and stored at -80 °C for later analysis. Ten µm and 7µm thick cross-sections were cut at muscle mid-belly using a Microm 550 cryostat (at -20°C) (Thermo-Fisher Scientific). Sections were mounted on Superfrost Plus® slides (Fisher Scientific) for wheat-germ agglutinin (WGA) and DAPI staining, or for myosin heavy chain staining.

3.3.5.2 Wheat-Germ Agglutinin and DAPI Staining

Muscle cross-sections were stained using wheat germ agglutinin (WGA) conjugate (Alexa Fluor 488®; LifeTechnologies) and DAPI (LifeTechnologies) to determine muscle CSA and central nucleation. Briefly, the sections were fixed in paraformaldehyde for 9 minutes (4% in PBS) and washed in Hank's Basic Salt Solution (HBSS; 3 seconds). Next slides were incubated in WGA (8µg/mL) for 11 minutes to label the sarcolemma for fluorescence microscopy. Then slides were washed again in HBSS (1 second) and immersed in 0.1% Triton X100 (in PBS) for 4 minutes. Slides were washed in PBS (3 seconds) before they were counterstained with DAPI (1µg/mL in dH₂O) for 4 minutes to label nuclei, rinsed again in PBS (3 seconds), and

coverslipped. Fluorescent images (10X magnification) of WGA- and DAPI-labeling were obtained in duplicate (229 ± 9 fibers per image) using standard excitation/emission settings (495nm/519nm and 358nm/461nm, respectively) on a Nikon Eclipse 80i epifluorescence microscope. Using proprietary software, Zizhao Zhang (Yang Laboratory, University of Florida) kindly quantified muscle fiber CSA and the percentage of centrally nucleated fibers were quantified in two non-overlapping fields for each muscle.

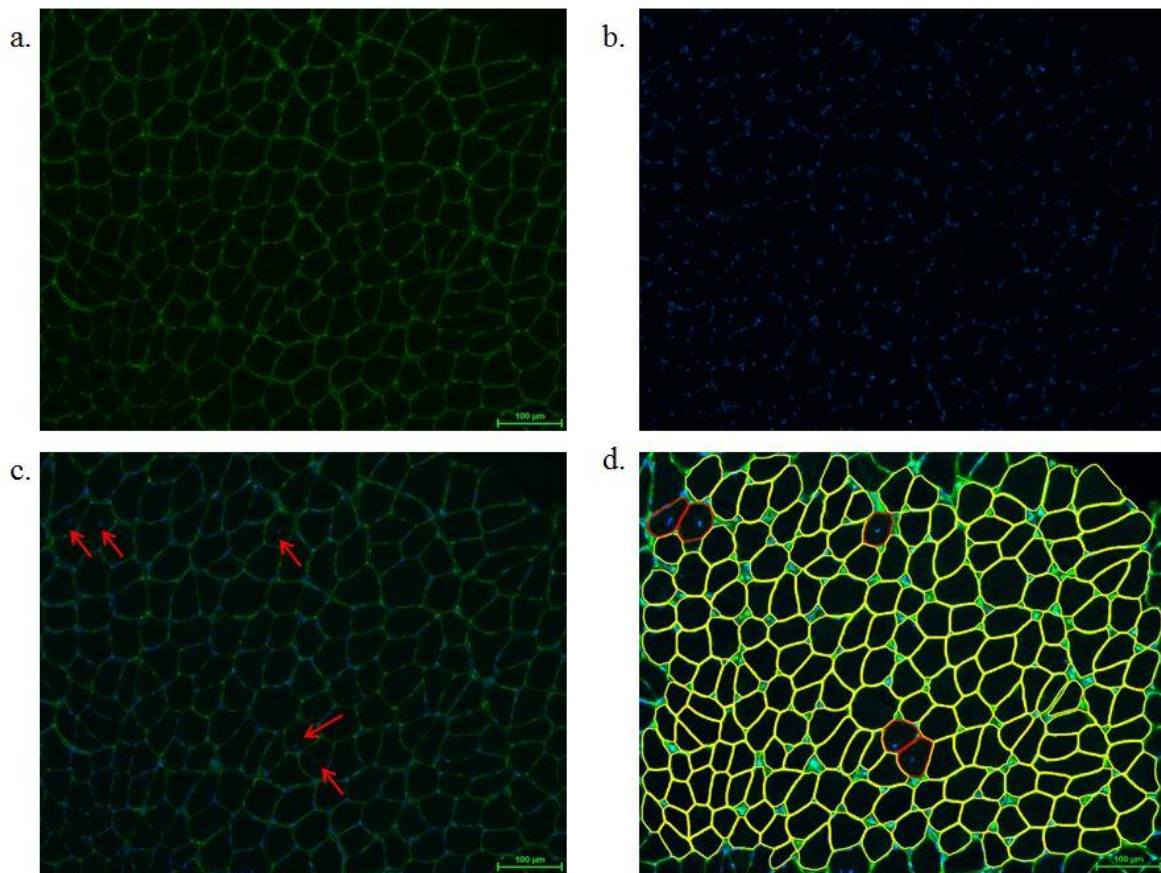


Figure 15. Sample Images of Combined WGA and DAPI Staining.

Muscle cross-sectional area and central nucleation were quantified from 10X images of muscle sections that were stained with WGA and DAPI. **a)** WGA stain; **b)** DAPI stain; **c)** Merged WGA and DAPI; red arrows indicates fibers with centralized nuclei; **d)** Same image after analysis with proprietary software (Dr. Lin Yan, University of Florida). The software outlines fibers with non-centralized nuclei in yellow and outlines fibers with centralized nuclei in red.

3.3.5.3 Myosin Heavy Chain Staining

Muscle cross-sections (TA, Tri, SOL, and GAS) from 4 mice in each group (n= 20 total) were cut 7 μ m thick and were shipped overnight (cooled by dry ice) to Dr. Pam Van Ry and colleagues (University of Nevada, Reno). Using immunohistochemistry, Dr. Van Ry and colleagues (who were blinded to sample conditions) kindly quantified the percentage of type I- and type IIA-positive fibers of muscle cross-sections.

Briefly, sections were fixed using methanol, acetone, 4% paraformaldehyde (PFA) and/or 4% formaldehyde. The Mouse on Mouse (M.O.M.) kit was used with all mouse antibodies for blocking, according to the package instructions (FMK-2201, Vector Laboratories). Mouse primary antibodies, MHC type I (BA-F8, 1:25) and MHC type IIA (SC-71, 1:25; both from the Iowa Hybridoma Bank), were applied overnight and followed by 1 hour incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse-IgG secondary antibody (1:5000; Li-Cor Biosciences). Secondary-only antibody controls were used to test auto-fluorescence. Membranes were labeled with Texas Red®-X conjugated WGA (1:100; LifeTechnologies). Following staining, slides were mounted using Vectashield Hard Set with DAPI (Vector Laboratories Inc.). One to three images (10X) from non-overlapping fields were captured using either a Zeiss Axioskop 2 Plus fluorescent microscope, Zeiss AxioCam HRC digital camera and Axiovision 4.8 software, or with an Olympus FluoviewFV1000 Laser scanning biological confocal microscope and Olympus micro FV10-ASW 3.1 software. ImageJ software was used to quantify total fibers, type-I positive and type IIA-positive fibers.

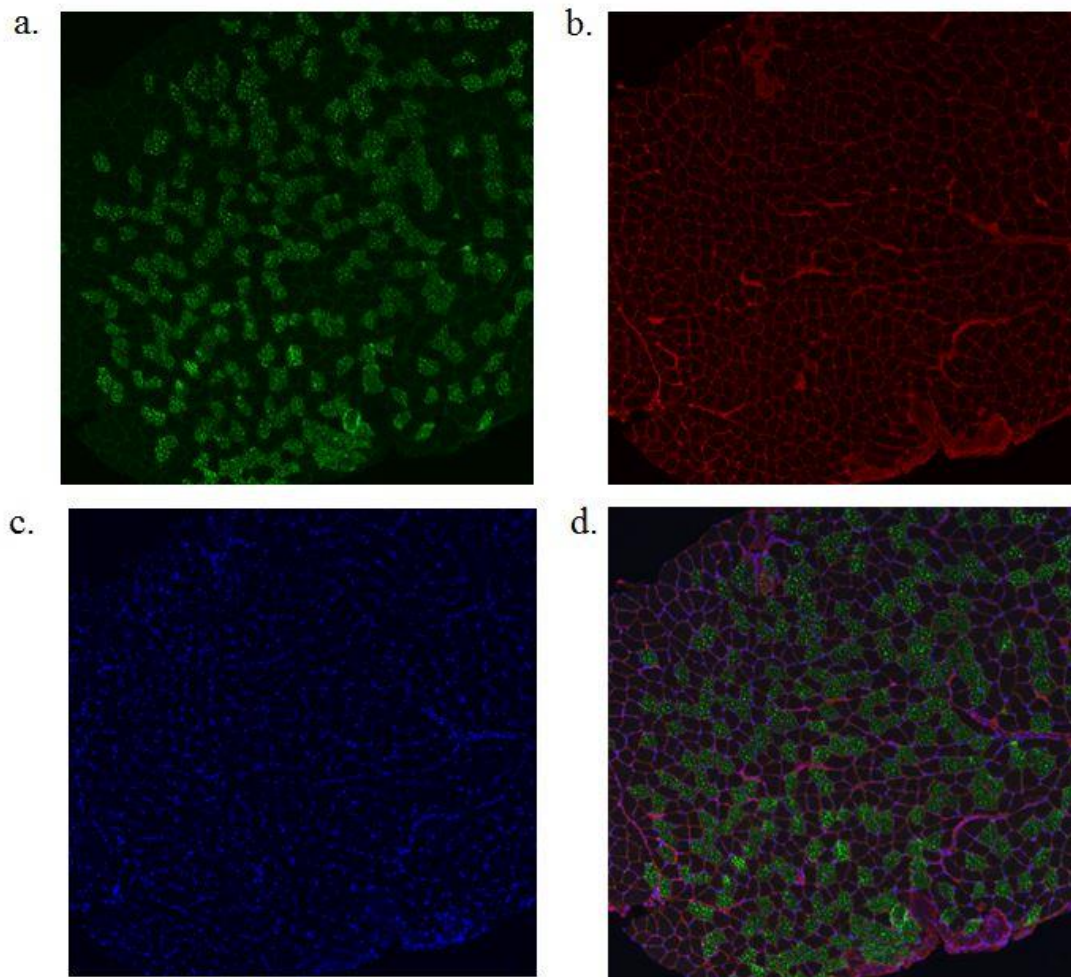


Figure 16. Representative Images of Myosin Heavy Chain Staining.

Myosin heavy chain (MHC) type I-positive and MHC type IIA-positive fibers were quantified from 10X images of muscle cross-sections. **a)** Soleus muscle section stained with type I MHC antibody (type I-positive fibers appear bright green), **b)** Texas Red-conjugated WGA (membranes appear red), and **c)** DAPI (nuclei appear blue); **d)** images a-c merged.

Section 3.4: Statistics

Data were reported as mean $\pm$ SEM. GraphPad Prism 6 software (GraphPad Software, Inc.) was used to perform statistical analyses (significance set to $p < 0.05$). When results were compared between 3 or more groups, either one-way analysis of variance (ANOVA; one main factor) or two-way ANOVA (two main factors) were performed in GraphPad. A mixed model two-way ANOVA was used when comparing multiple measurements between groups over time.

Post-hoc comparisons were only performed when $p < 0.05$ for factor effect(s) (and interaction). Tukey's honestly significant difference (HSD) comparisons were performed following one-way ANOVAs and two-way ANOVAs. Sidak's *post-hoc* comparisons were performed following mixed model two-way ANOVAs. Welch's corrections were used for group comparisons when variances were unequal.

Chapter 4: Results

Section 4.1: Screening

4.1.1 Screening and Mouse Selection

Mice were screened for running status (3 days; no-added resistance) during baseline week; the 30 top runners ($896 \pm 186\text{m/day}$) were used for the remainder of the study. There were no differences in wheel distance between the first and second cohort during screening ($961 \pm 270\text{m/day}$ vs. $803 \pm 261\text{m/day}$, respectively; $p = 0.638$).

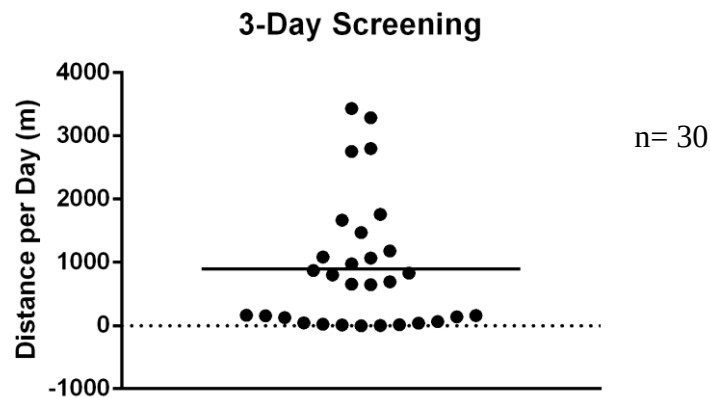


Figure 17. Screening for Running Status at Baseline.

There was a wide distribution in mean daily distance during 3-day screening. Among mice chosen for the study, 29 of 30 exceeded 0m/day during screening. There were no differences in mean daily distance between cohorts ($p > 0.05$). Central solid line indicates mean of all 30 mice (896m/day). Y-axis: Distance per Day (m); X-axis: arbitrary.

Section 4.2: Pre-Training Phase

4.2.1 Wheel Running and Resistance Data during Pre-Training

In the last two weeks of pre-training, three general strata emerged that were used for comparisons. Some mice consistently ran against low ($\sim 0\text{-}5\%$ BM; $n = 11$), medium ($\sim 12\text{-}18\%$

BM; n= 12), or high resistance (~25-28% BM; n= 7). Wheel resistance settings (absolute and relative to BM) were incrementally adjusted over time and significant differences emerged between running strata after 4 weeks ($p < 0.05$) (Figure 18a,b). There was a main effect of time on wheel distance ($p < 0.001$), but no effect of running stratum ($p = 0.06$) (Figure 18c). After 3 weeks, differences in calculated wheel work emerged between groups ($p < 0.05$) (Figure 18d); however, there were no differences between the low resistance and medium resistance strata ($p > 0.05$). Note that issues with the stepper motors (and wheel resistances) were not discovered until the ~16.5 weeks into the study, so we were unaware of potential deviations in wheel resistance during pre-training, despite frequent recalibrations (~2X per week).

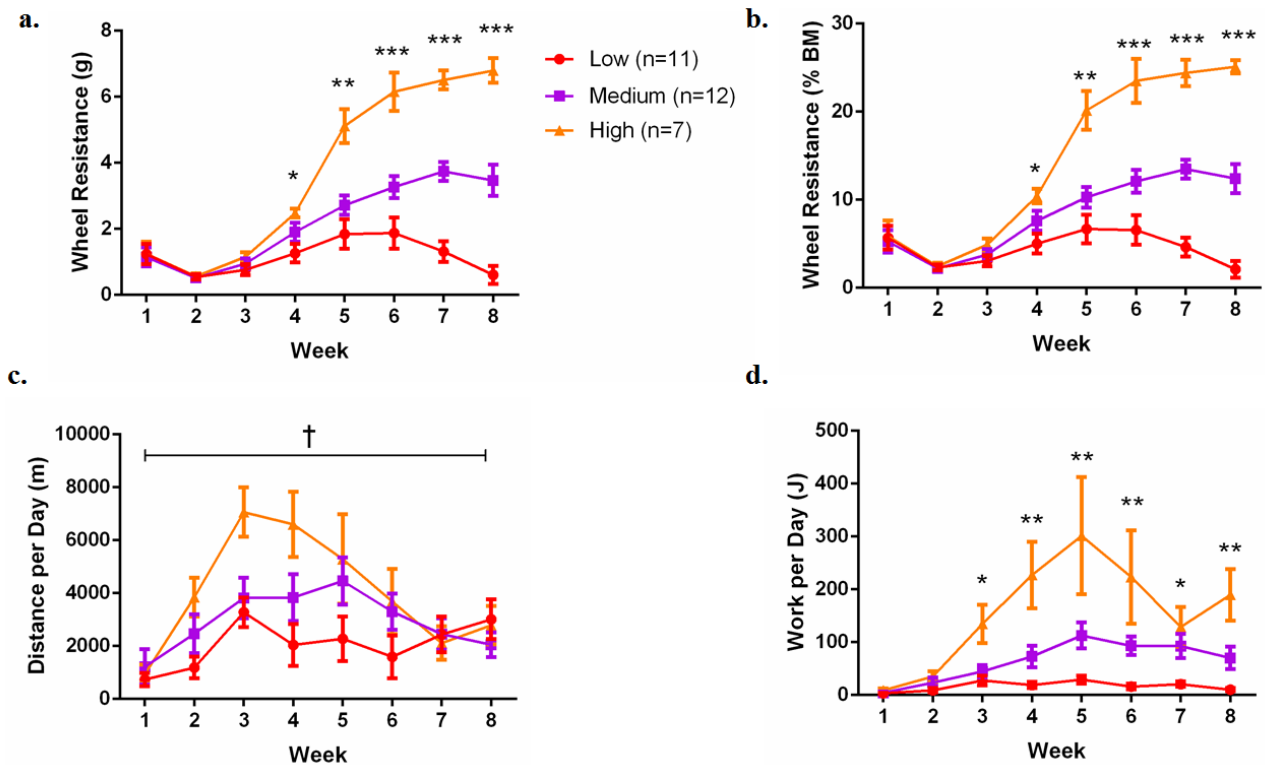


Figure 18. Wheel Resistance and Running Performance during Pre-Training.

Based on running performance, wheel resistances were adjusted over time; wheel distance and work were recorded. **a)** There were differences in absolute wheel resistance (in grams) between groups, which reached significance after 4 weeks ($p < 0.05$); **b)** the same pattern was observed for relative wheel resistance (% BM). **c)** There was a main effect of time on wheel distance ($p < 0.001$), but no effect of running stratum ($p = 0.06$). **d)** Significant differences in calculated wheel work were observed after 3 weeks. * Indicates difference from Low stratum ($p < 0.001$); ** Indicates High stratum > Low and Medium strata ($p < 0.05$); *** Indicates differences between all three strata ($p < 0.05$); † Indicates main effect of time ($p < 0.001$).

4.2.2 Assessment of Plantar Flexor Function at Baseline and during Pre-Training

4.2.2.1 Twitches and Tetany

After 6 weeks of pre-training, PFT properties were assayed *in-vivo* to test for differences in plantar flexor function between running strata. Tables 8 and 9 summarize baseline and 6-week torque properties and temporal properties of plantar flexor twitches and tetany.

Table 8. Plantar Flexor Torque Properties at Baseline and after 6 Weeks of Pre-Training				
Description		Baseline Measures		
		Low (n= 11)	Medium (n= 12)	High (n= 7)
Twitch	Absolute Torque (N*cm)	0.13 ± 0.0	0.13 ± 0.0	0.15 ± 0.0
	Relative Torque (N*mm/kg)	76 ± 6.1	80 ± 3.6	87 ± 3.6
Tetanus	Absolute Torque (N*cm)	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
	Relative Torque (N*mm/kg)	471 ± 16	481 ± 15	489 ± 15
Ratio	Twitch:Tetanus (Unit-less)	0.16 ± 0.0	0.17 ± 0.0	0.18 ± 0.0
Description		6 Weeks (Pre-Training)		
		Low (n= 11)	Medium (n= 12)	High (n= 7)
Twitch	Absolute Torque (N*cm)	0.36 ± 0.0*	0.35 ± 0.0*	0.36 ± 0.0*
	Relative Torque (N*mm/kg)	125 ± 8.0*	129 ± 6.4*	134 ± 7.3*
Tetanus	Absolute Torque (N*cm)	2.0 ± 0.1*	1.9 ± 0.1*	2.0 ± 0.1*
	Relative Torque (N*mm/kg)	703 ± 27*	680 ± 22*	760 ± 27*
Ratio	Twitch:Tetanus (Unit-less)	0.18 ± 0.0	0.19 ± 0.0	0.18 ± 0.0

Relative Torque= Absolute torque divided by body mass (expressed in N*mm/kg); * Indicates main effect of time (p < 0.05).

When the low-, medium-, and high-resistance strata were compared at baseline, there were no absolute or relative differences in PFT of twitches or tetany (all p > 0.05), which indicates that baseline plantar flexor strength was not different between strata (Table 8). Note that there were also no differences in body mass between running strata at baseline and 6-weeks (section 4.B.3). When baseline PFT properties were compared to 6 weeks using mixed model two-way ANOVA, there were time effects on twitch and tetanic torque production (absolute and relative (all p < 0.05)), but there was no time effect on twitch: tetanus ratio (p > 0.05).

Table 9. Plantar Flexor Temporal Properties at Baseline and after 6 Weeks of Pre-Training				
Parameter		Baseline		
		Low (n= 11)	Medium (n= 12)	High (n= 7)
Twitch	TPT (ms)	22.4 ± 0.6	22.0 ± 0.4	22.0 ± 0.5
	Max dT/dt (N*cm/s)	8.8 ± 1.0	9.1 ± 0.7	10.0 ± 1.0
	HRT (ms)	12.8 ± 0.3	12.7 ± 0.3	12.5 ± 0.3
	TTI (N*cm*ms)	3.1 ± 0.3	3.2 ± 0.2	3.4 ± 0.4
Tetanus	TPT (ms)	365 ± 47	312 ± 21	397 ± 52
	Max dT/dt (N*cm/s)	17.6 ± 1.8	18.8 ± 1.4	18.3 ± 1.2
	HRT (ms)	579 ± 47	632 ± 20	548 ± 52
	TTI (N*cm*ms)	709 ± 56	734 ± 55	733 ± 67
Parameter		6 Weeks (Pre-Training)		
		Low (n= 11)	Medium (n= 12)	High (n= 7)
Twitch	TPT (ms)	22.1 ± 0.4	22.6 ± 0.5	22.6 ± 0.5
	Max dT/dt (N*cm/s)	23.0 ± 1.6*	22.7 ± 1.2*	22.8 ± 1.6*
	HRT (ms)	13.2 ± 0.3*	14.0 ± 0.4	13.5 ± 0.5
	TTI (N*cm*ms)	9.2 ± 0.8*	9.2 ± 0.7*	9.4 ± 0.8*
Tetanus	TPT (ms)	415 ± 67	386 ± 54	323 ± 60
	Max dT/dt (N*cm/s)	46.1 ± 2.8*	41.4 ± 1.4*	44.5 ± 2.7*
	HRT (ms)	536 ± 67	567 ± 54	629 ± 60
	TTI (N*cm*ms)	1819 ± 103*	1686 ± 72*	1824 ± 67*

TPT= Time to Peak Torque; Max dT/dt= Maximum rate of Torque Production; HRT= Half Relaxation Time; TTI= Torque-Time Integral; * Indicates main effect of time ($p < 0.05$).

Similarly, there were no differences in temporal properties between strata at baseline (all $p < 0.05$) (Table 9). When baseline PFT properties were compared to 6 weeks using mixed model two-way ANOVA (with repeated measures), there were time effects on maximum rate of torque production (Max dT/dt) and torque-time integral (TTI) for twitches and tetany (all $p < 0.05$), but there were no effects of running strata on these variables ($p > 0.05$). Half relaxation time (HRT) of twitches (but not tetany), were also different at 6 weeks compared to baseline ($p = 0.01$), but again there was no effect of running strata ($p > 0.05$). Time to peak torque (TPT) was unchanged for twitches and tetany after 6 weeks of pre-training ($p > 0.05$).

4.2.2.2 Torque-Frequency Profiles

Following twitches (1Hz, minimum response) and tetany (150Hz, maximum response), plantar flexor torque was assessed through a range of stimulation frequencies. Plantar flexor torque-frequency profiles (absolute and relative) were not different between running strata at baseline and after 6 weeks of pre-training (two way ANOVAS showed all $p > 0.05$) (Figure 19).

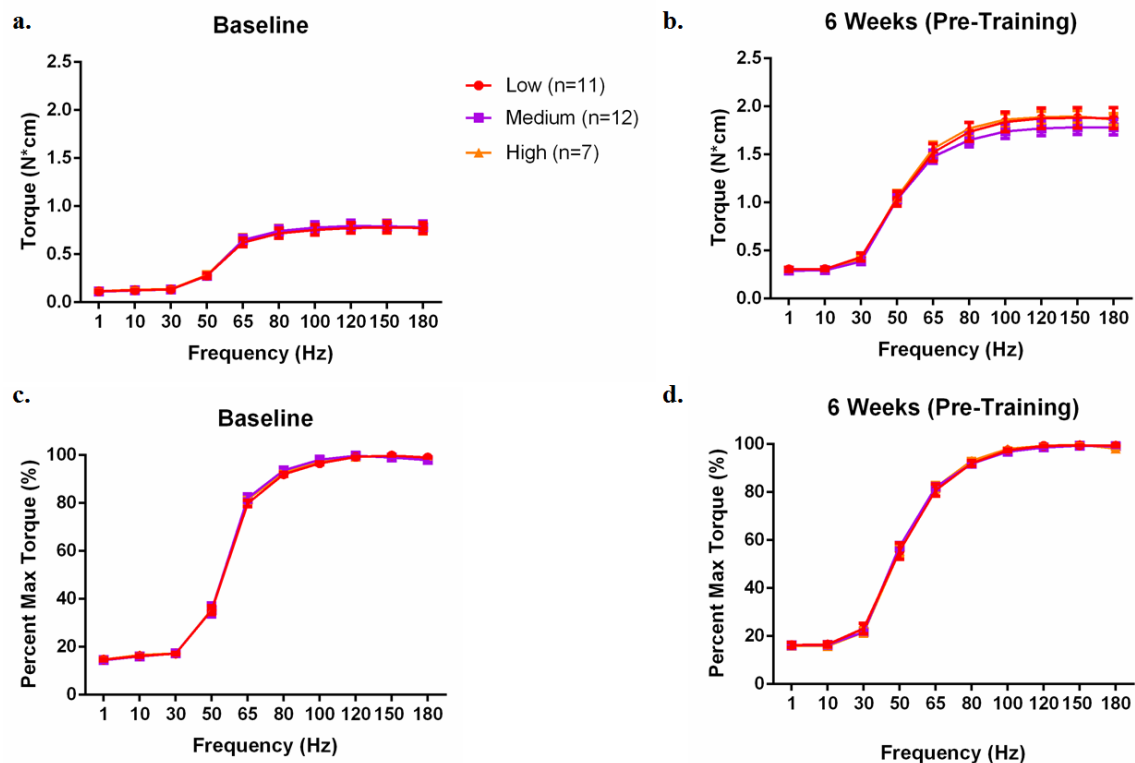


Figure 19. Plantar Flexor Torque-Frequency Profiles at Baseline and after 6-Weeks of Pre-Training.

Plantar flexor torque (PFT) was measured at increasing stimulation frequencies (1-180Hz). There were no differences in PFT-frequency profiles between running strata (low, medium, and high) after 6-weeks of pre-training (all $p > 0.05$). **a)** Baseline torque-frequency profiles with torque expressed in absolute units (N*cm); **b)** 6-week torque-frequency profiles expressed in absolute units (N*cm); **c)** baseline profiles with torque expressed relative to maximum torque (%); **d)** 6-week profiles expressed relative to maximum torque (%).

4.2.2.3 Fatigue Profiles

After the torque-frequency measures were performed, plantar flexor muscles were stimulated to fatigue (100Hz stimulation every 5 seconds for 5 minutes) to determine if there were differences in plantar flexor fatigue profiles. There were no differences in absolute or relative fatigue profiles between running strata at baseline or after 6 weeks of pre-training (all $p > 0.05$) (Figure 20).

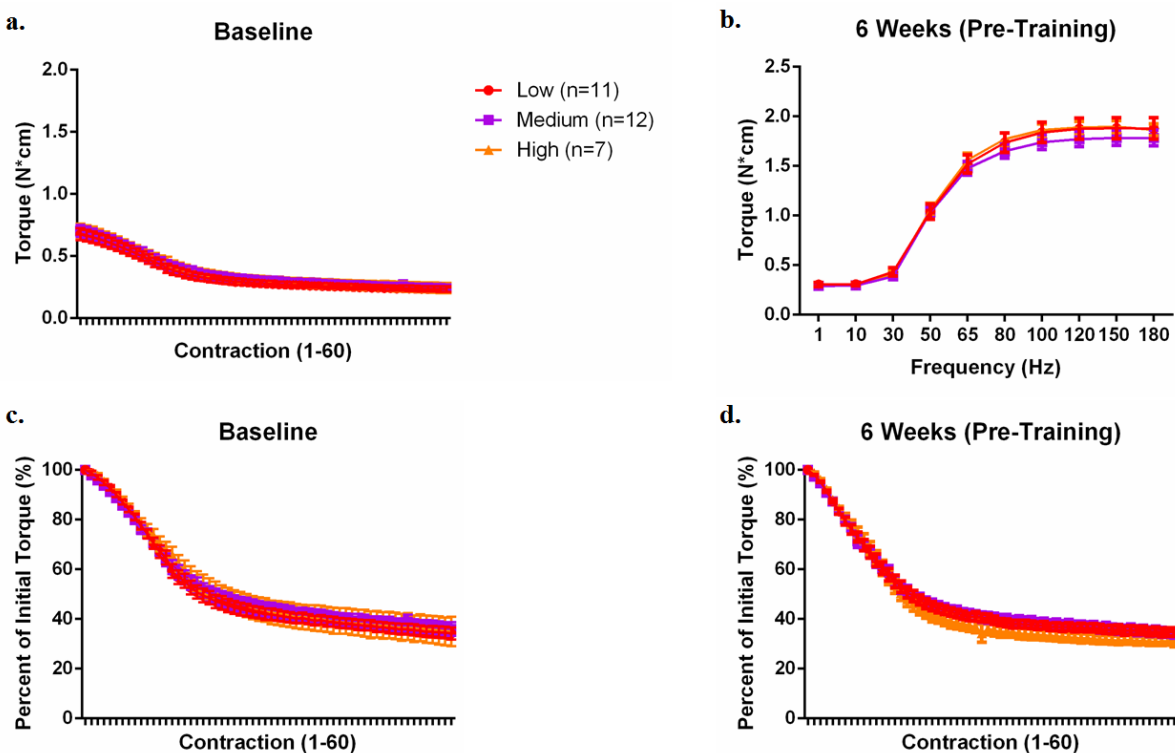


Figure 20. Plantar Flexor Fatigue Profiles at Baseline and after 6-Weeks of Pre-Training.

When plantar flexors were stimulated to fatigue (100Hz stimulation every 5 seconds, for 5 minutes), there were no differences in fatigue profiles between running strata at baseline or after 6-weeks of pre-training (all $p > 0.05$). **a)** Baseline fatigue profiles with torque expressed in absolute units (N*cm); **b)** 6-week measures expressed in absolute units (N*cm); **c)** baseline fatigue profiles with torque responses expressed relative to torque produced during initial stimulation (%); **d)** 6 week measures expressed relative to initial torque (%).

4.2.3 Body Mass, Body Composition, and Food Consumption

There were no differences in body mass between strata during pre-training (all $p > 0.05$), but there was a main effect of time on body mass ($p < 0.001$) (Figure 21a). There were main effects of time ($p < 0.001$) and running stratum ($p = 0.04$) on food consumption, but no interaction ($p = 0.696$) (Figure 21b).

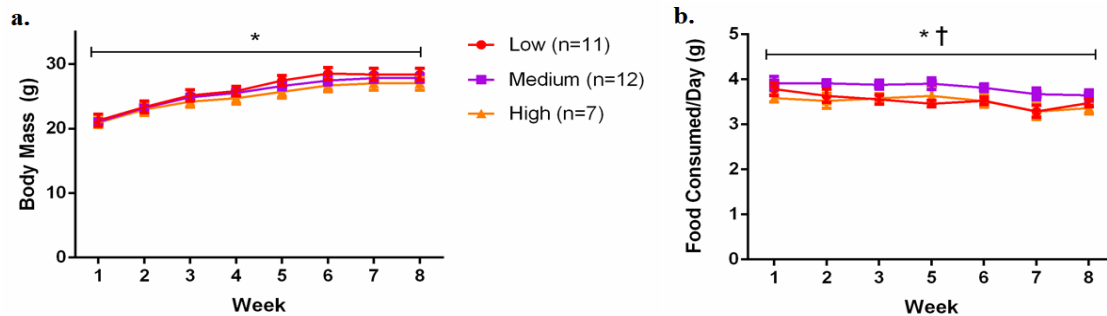


Figure 21. Body Mass and Food Consumption during Pre-Training.

According to two-way ANOVAs with repeated measures, **a)** there was a main effect of time on body mass during pre-training ($p < 0.05$); and **b)** there were main effects of time and running stratum on food consumption during pre-training (both $p < 0.05$), but no interaction ($p > 0.05$). Note that food consumption was not recorded during week 4. * Indicates main effect of time ($p < 0.05$); † Indicates main effect of stratum ($p < 0.05$).

Body composition (lean, fat, and fluid mass) was assessed by NMR on the same day as plantar flexor torque assays. At baseline, there were no differences in lean, fat, or fluid mass (absolute or relative to BM) between running strata (one-way ANOVAs showed all $p > 0.05$). Despite differences in wheel activity during pre-training, there were no differences in body composition between resistance running strata after 6 weeks (one-way ANOVAs showed all $p > 0.05$), but there were main effects of time on body composition (two-way ANOVAs showed all $p < 0.05$ for time effects) (Figure 22).

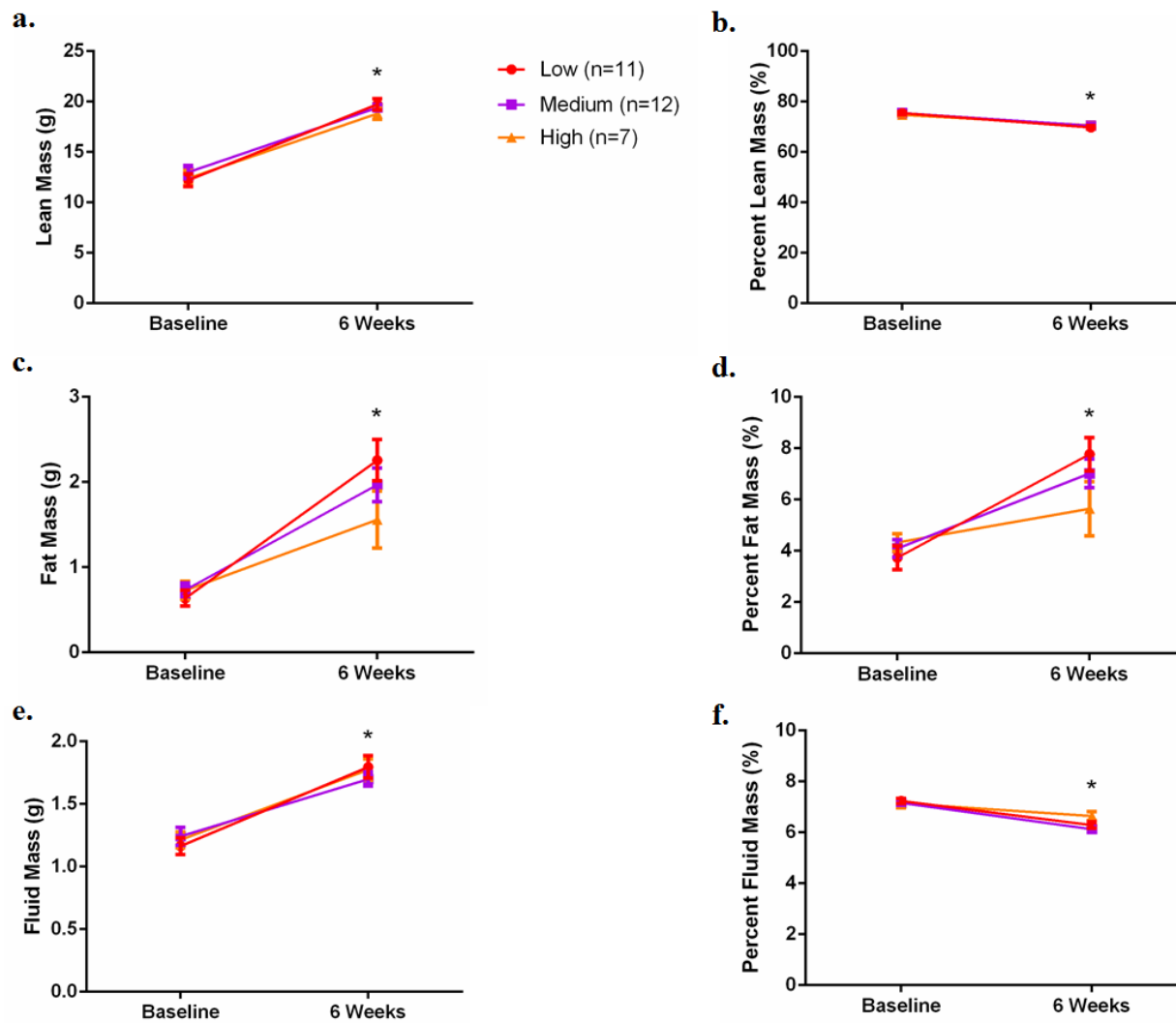


Figure 22. Body Composition at Baseline and after 6 Weeks of Pre-Training.

There were no differences in body composition parameters (absolute or relative) between resistance running strata at baseline or after 6-weeks of pre-training (all $p > 0.05$). However, there were main effects of time on **a)** lean body mass, **b)** percent lean mass (relative to body mass (BM)), **c)** fat mass, **d)** percent fat mass, **e)** fluid mass, and **f)** percent fluid mass (two-way ANOVAs showed all $p < 0.05$). * Indicates main effect of time ($p < 0.05$).

Section 4.3: Static Resistance Training Phase

4.3.1 Wheel Running and Resistance

After pre-training, mice were tasked to run against static resistance (load set to constant % BM), based on their performance during pre-training (Low- 0% BM (n= 9), Med- 15% BM (n= 9), or High- 25% BM (n= 7)), and wheels were locked for cage-activity control mice (CA (n= 5)). Although mice pre-trained against similar resistances, wheel distance appeared to decline during the latter half of static training for the majority of mice running against resistance (13 of 16) (first half= 1984 ± 349 m/day vs. second half= 1420 ± 324 m/day; $p = 0.12$) (Figure 23b). When analyzed, there was an effect of time on wheel distance and work (both $p < 0.05$). There was also an effect of group on calculated wheel work ($p < 0.05$), but no interaction ($p > 0.05$) (Figure 23c).

However, several mice running against added-resistance (3 of 16; all 15% BM) performed well throughout static training (first half= 1992 ± 544 m/day vs. second half= 2040 ± 1036 m/day; $p = 0.97$). Note that these observations were used to determine training conditions for the dynamic training phase.

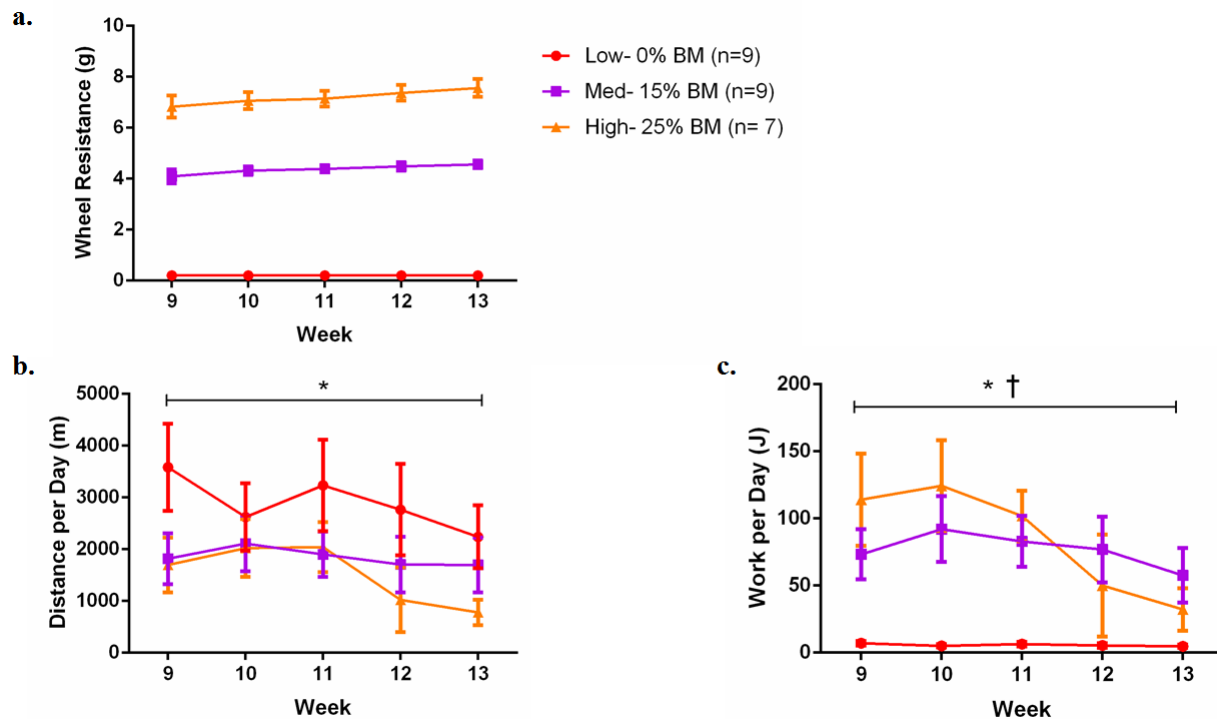


Figure 23. Wheel Resistance and Running Performance during Static Training.

Wheel resistances were set to constant load for running strata during static training: low (0% BM), medium (15% BM), or high (25% BM) (cage-activity controls had locked wheels; not shown). **a)** This panel shows wheel resistance settings for running strata (in grams) during the 5 week static training period. **b)** Distance per day generally declined during static training, despite similar absolute and relative resistance settings over time (time effect: $p < 0.05$). **c)** As a result, calculated wheel work also declined during static training; there main effects of time and group on wheel work (both $p < 0.05$), but no interaction ($p = 0.13$). BM= body mass; * Indicates main effect of time ($p < 0.05$); † Indicates main effect of stratum ($p < 0.05$).

4.3.2 Assessment of Plantar Flexor Function during Static Training

4.3.2.1 Twitches and Tetany

In addition to (apparent) declines in wheel performance, when we performed PFT measures at 12 weeks (3 weeks into static training), there were no differences in twitch or tetanic torque properties (absolute or relative) between running strata and CA-controls (one-way ANOVAs showed all $p > 0.05$) (Table 10).

Table 10. Plantar Flexor Torque Properties during Static Training					
Parameter		12 Weeks (Static Training)			
		CA (n= 5)	0% BM (n= 9)	15% BM (n= 9)	25% BM (n= 7)
Twitch	Absolute Torque (N*cm)	0.34 ± 0.0	0.32 ± 0.0	0.28 ± 0.0	0.29 ± 0.0
	Relative Torque (N*mm/kg)	103 ± 17	102 ± 6	89 ± 4	96 ± 8
Tetanus	Absolute Torque (N*cm)	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
	Relative Torque (N*mm/kg)	638 ± 17	669 ± 19	631 ± 19	671 ± 28
Ratio	Twitch : Tetanus (unitless)	0.16 ± 0.0	0.15 ± 0.0	0.14 ± 0.0	0.14 ± 0.0

Relative Torque= Absolute torque divided by body mass (expressed in N*mm/kg); BM= body mass; CA= cage-activity controls

Additionally, there were no differences in temporal properties between running strata and CA-controls (one-way ANOVA showed all $p > 0.05$) (Table 11).

Table 11. Plantar Flexor Temporal Properties during Static Training					
Parameter		12 Weeks (Static Training)			
		CA (n= 5)	0% BM (n= 9)	15% BM (n= 9)	25% BM (n= 7)
Twitch	TPT (ms)	21 ± 0.8	21 ± 0.4	21 ± 0.2	21 ± 0.4
	Max dT/dt (N*cm/s)	23 ± 2.5	22 ± 1.2	19 ± 1.0	20 ± 1.4
	HRT (ms)	13 ± 0.4	12 ± 0.2	12 ± 0.1	12 ± 0.2
	TTI (N*cm*ms)	8.2 ± 1.5	7.2 ± 0.5	6.2 ± 0.4	6.8 ± 0.6
Tetanus	TPT (ms)	270 ± 52	291 ± 44	262 ± 36	313 ± 54
	Max dT/dt (N*cm/s)	49 ± 1.4	49 ± 1.9	46 ± 1.1	47 ± 2.0
	HRT (ms)	676 ± 52	654 ± 45	682 ± 37	633 ± 54
	TTI (N*cm*ms)	1842 ± 91	1857 ± 77	1750 ± 42	1824 ± 56

TPT= Time to Peak Torque; Max dT/dt= Maximum rate of Torque Production; HRT= Half Relaxation Time; TTI= Torque-Time Integral; BM= body mass; CA= cage-activity controls

4.3.2.2 Torque-Frequency Profiles

There were also no differences in absolute or relative torque-frequency profiles at 12 weeks between running strata and CA-controls (two-way ANOVAs showed all $p > 0.05$) (Figure 24).

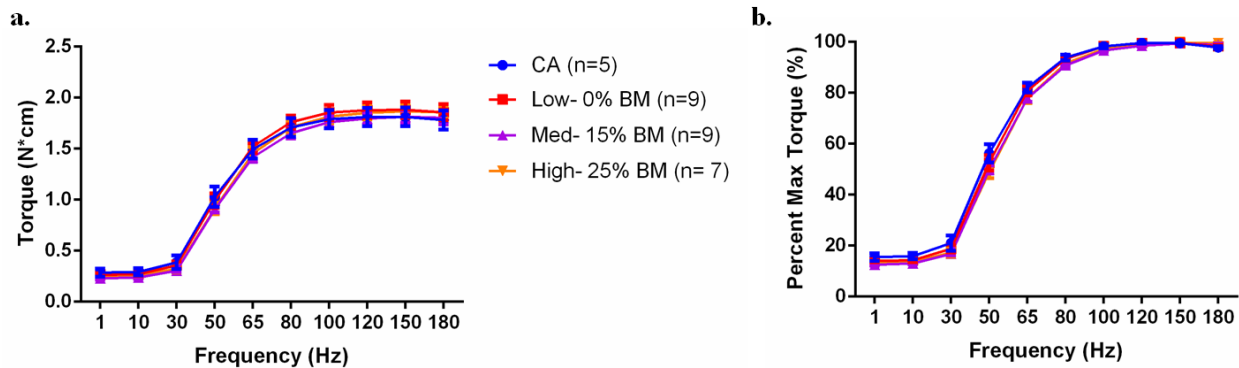


Figure 24. Plantar Flexor Torque-Frequency Profiles during Static Training.

There were no differences in plantar flexor torque-frequency profiles between running strata (low 0% BM, medium 15% BM, high 25% BM) and CA-controls at 12 weeks (3 weeks into static training) (all $p > 0.05$). **a)** Torque-frequency profiles with torque expressed in absolute units (N*cm); **b)** Torque-frequency profiles with torque expressed relative to maximum torque (%). BM= body mass; CA= cage-activity controls.

4.3.2.3 Fatigue Profiles

Similarly, there were no differences in absolute or relative plantar flexor fatigue profiles between running strata and CA-controls (two-way ANOVAs showed all $p > 0.05$) (Figure 25).

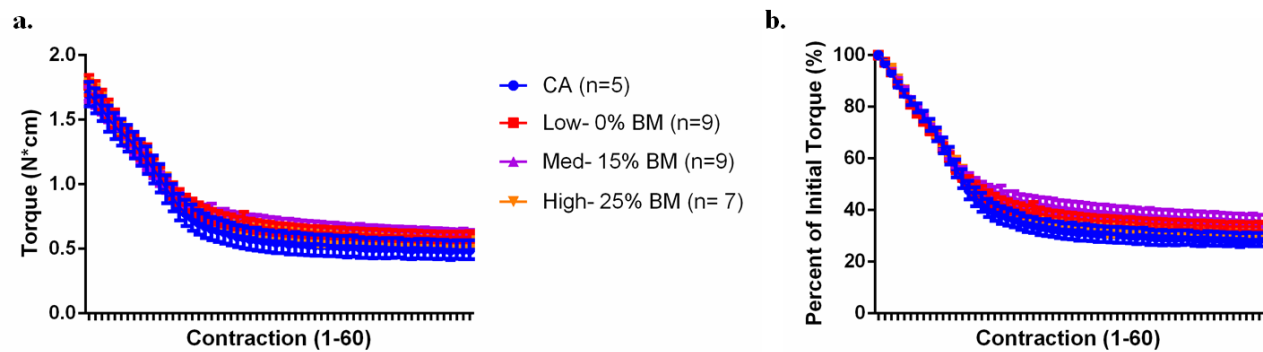


Figure 25. Plantar Flexor Fatigue Profiles during Static Training.

There were no differences in fatigue profiles between running strata and CA-controls at 12 weeks (3 weeks into static training) (all $p > 0.05$). **a)** Fatigue profiles with torque expressed in absolute units (N*cm); **b)** fatigue profiles with torque expressed relative to torque produced during initial stimulation (%). BM= body mass; CA= cage-activity controls.

4.3.3 Body Mass, Food Consumption, and Body Composition

Throughout static straining were no differences in body mass or food consumption between running strata and CA-controls (all $p > 0.05$), but there were significant time effects on body mass and food consumption (both $p < 0.05$) (Figure 26). Note that food consumption was not recorded during week 10.

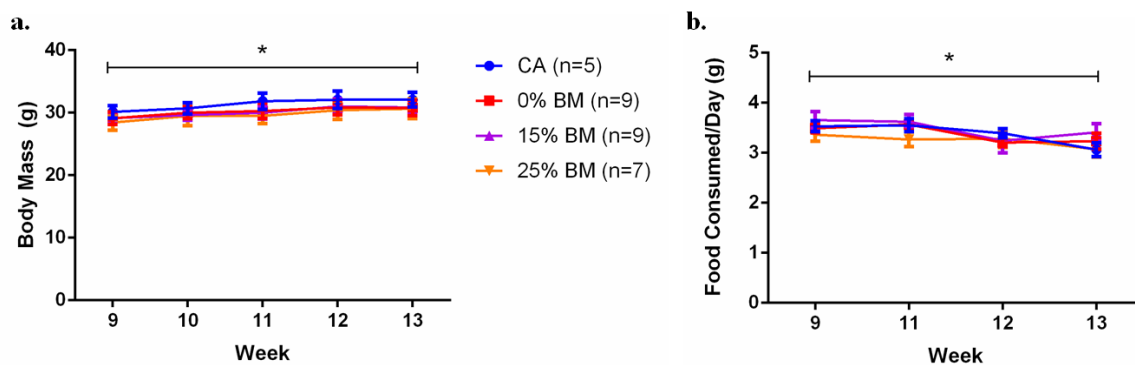


Figure 26. Body Mass and Food Consumption during Static Training.

a) Body masses were not different between running strata and CA-controls throughout static training ($p > 0.05$). **b)** Additionally, food consumption was not different between groups ($p > 0.05$). However, there was a main effect of time on each of these parameters, according to two-way ANOVAs (both $p < 0.05$). Note that food consumption was not recorded during week 10. BM= body mass; CA= cage-activity controls; * Indicates main effect of time ($p < 0.05$).

Further, after 12 weeks, there were no differences in body composition between running strata and CA-controls (one-way ANOVAs showed all $p > 0.05$) (Figure 27).

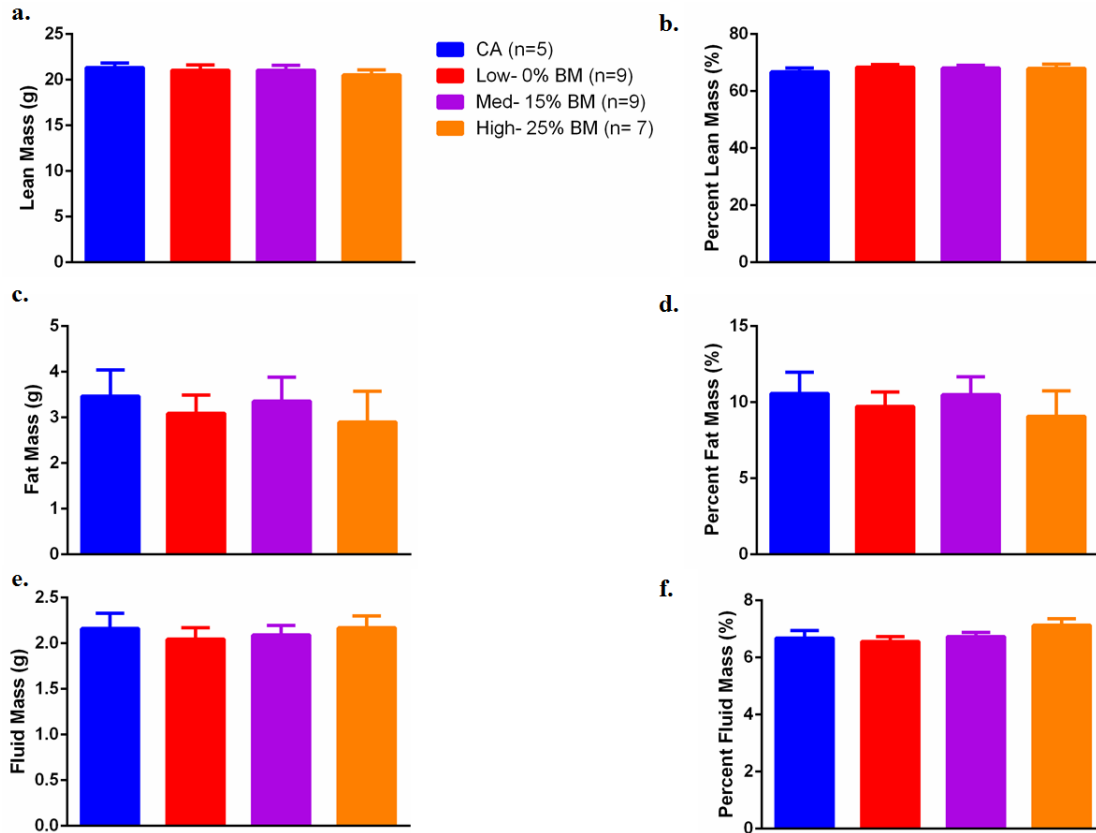


Figure 27. Body Composition during Static Training.

There were no differences in body composition parameters (absolute or relative) between running strata and CA-controls 3 weeks into static training (two-way ANOVAs showed all $p < 0.05$). Body composition parameters include **a)** lean body mass, **b)** percent lean mass (relative to BM), **c)** fat mass, **d)** percent fat mass, **e)** fluid mass, and **f)** percent fluid mass. BM= body mass; CA= cage-activity controls.

Section 4.4: Dynamic Resistance Phase

Since wheel performances declined for most Med- 15% BM and High- 25% BM resistance runners (13 of 16) during static training, and there were no differences in PFT properties or body composition between running strata and CA-controls, we hypothesized that

runners had not performed enough work to drive functional adaptations, so we implemented a new kind of training program, dynamic resistance training, which modulated wheel resistance in real-time to attempt to maximize wheel work. Thirteen of 16 mice from the 15% and 25% BM running strata underwent dynamic training, while the other 3 stayed on their static programs (all 15% BM). Additionally, several mice (4 of 9) that were running against 0% BM were shifted to dynamic training (the other 5 remained on their free-wheel running program (i.e., no-added resistance) (FWR 0%BM).

The dynamic training phase lasted 7 weeks for the first cohort and 10 weeks for the second cohort. Two weeks prior to sacrifice, final PFT and body composition measures were obtained for each cohort (Cohort 1= 18 weeks; Cohort 2= 21 weeks). Resistance runners within each cohort were split into sub-groups (tertiles) based on work accomplished after the stepper motor correction. Because of these differences between cohorts, data presented in sections 4D-4F are split by cohort, which decreased statistical power for comparisons. Most tables and figures only report sub-group results for each cohort, but additional comparisons within/between cohorts are described in-text.

It is also important to note that, throughout the study, we observed considerable variability in running within and between cohorts. For example, cumulative distance of mice in the second cohort was ~100% greater than mice in cohort 1, even when compared on the same timescale (i.e., only compared up to 20 weeks) (Table 12). Table 12 summarizes wheel distances for each cohort (as a whole) across training phases.

Table 12. Comparisons of Running Between Cohorts Across Training Phases			
Description	Cohort 1	Cohort 2	T-Test Comparison
Pre-Training (m/day)	2093 ± 224 (n= 16)	3711 ± 541 (n= 14)	P= 0.013*
Static Training (m/day)	1498 ± 244 (n= 13)	2549 ± 573 (n= 12)	P= 0.024*
Dynamic Training (m/day)	1183 ± 228 (n= 13)	2201 ± 458 (n= 12)	P= 0.053
Cumulative Distance Up to 20 Weeks (km)	221 ± 25 (n= 13)	435 ± 54 (n= 12)	P= 0.001*
Cumulative Distance Overall (km)	221 ± 25 (n= 13)	486 ± 62 (n= 12)	P < 0.001*

Data expressed as mean ± SEM (n= X; number of runners in cohort); * Indicates cohort 2 > cohort 1.

4.4.1 Post-Hoc Grouping

Resistance runners within each cohort were grouped *post-hoc* according to work accomplished after the stepper motor correction. Figure 28 illustrates that average wheel distance and calculated wheel work were similar for resistance running sub-groups (for both cohorts), before and after the stepper motor correction (two-way ANOVAs showed all $p > 0.05$). Figure 28 also illustrates the differences in wheel distance and work between resistance running sub-groups (i.e., Low-Work vs. Med-Work vs. High-Work); but differences between sub-groups were not surprising because the sub-groupings were determined *post-hoc*, based on wheel work data.

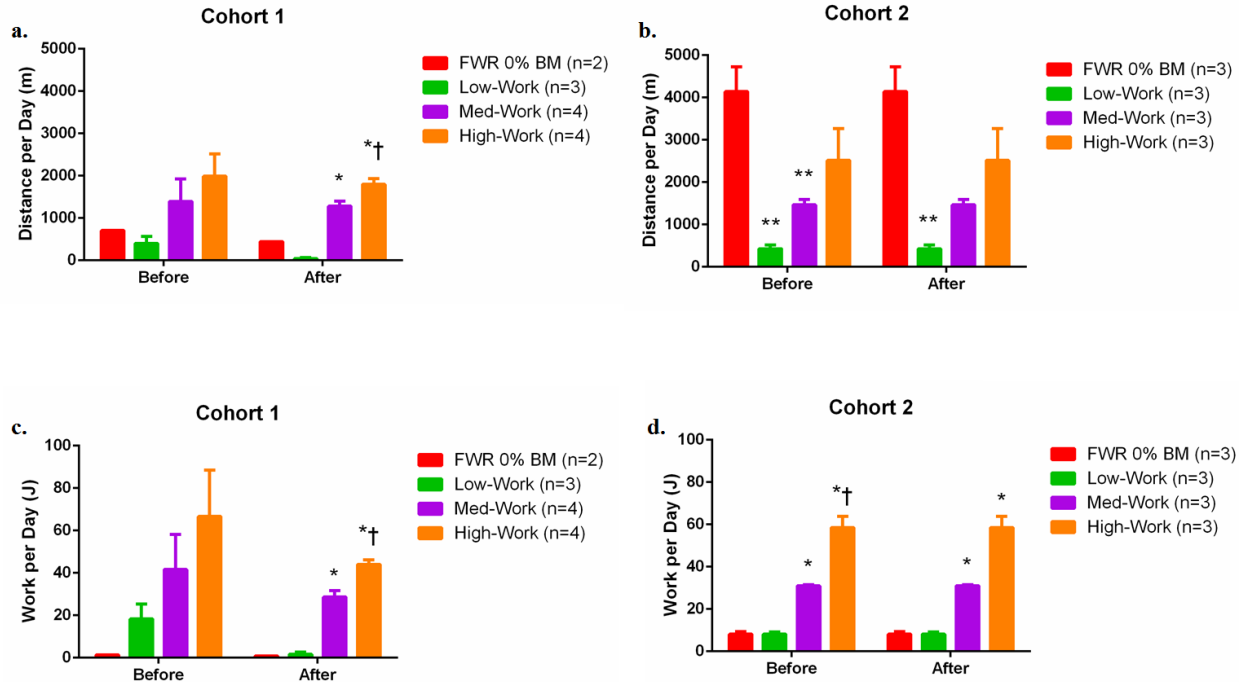


Figure 28. Wheel Running Data Used for *Post-Hoc* Grouping.

Wheel distance and work data are shown for each sub-group (in each cohort) before and after the stepper motor correction. For *post-hoc* grouping, 12-17 days of running data were available for mice cohort 1 (before sacrifice), while ~6.5 weeks of running data were available for mice in cohort 2. Note that for all sub-groups (in both cohorts) there were no differences in wheel distance or calculated wheel work before and after the stepper motor correction. **a)** In cohort 1, after the stepper motor correction, the High-Work sub-group ran further than all other sub-groups ($p < 0.05$) and the Med-Work sub-group ran further than FWR 0% BM and Low-Work ($p < 0.05$). **b)** In cohort 2, before the stepper motor correction, FWR 0% BM ran further than Low-Work and Med-Work ($p < 0.05$), but after the correction, FWR 0% BM only ran further than Low-Work ($p < 0.05$). **c)** In cohort 1, there were no differences in calculated wheel work before the stepper motor correction (all $p > 0.05$), but after the correction, the High-Work group performed more work than all other sub-groups (all $p < 0.05$), while the Med-Work group was only greater than the FWR 0% BM and Low-Work groups ($p < 0.05$). **d)** In cohort 2, before the stepper motor correction, the High-Work sub-group performed more work than all other sub-groups ($p < 0.05$) and the Med-Work sub-group performed more work than FWR 0% BM and Low-Work ($p < 0.05$). However, after the stepper motor correction, the High-Work sub-group and Med-Work sub-group similarly performed more work than Low-Work and FWR 0% BM ($p < 0.05$). Data expressed as mean $\pm$ SEM; mean was reported when $n < 3$, but not SEM. FWR= free-wheel runners; * Indicates $>$ Low-Work and FWR 0% BM sub-groups ($p < 0.05$); † Indicates $>$ Med-Work sub-group ($p < 0.05$); ** Indicates $<$ FWR 0% BM ($p < 0.05$).

4.4.2 Wheel Resistance and Running Data

The purpose of implementing dynamic training was to determine if we could increase the load against which the mice would voluntarily run, in an effort to maximize wheel work. We hypothesized that mice would run more (and at higher loads) if wheel resistances were increased during individual exercise bouts (i.e., +2% BM per 1J work goal), compared to static load conditions. Table 13 summarizes wheel resistances (after the stepper motor correction) achieved by mice during dynamic training. There were no differences in dynamic minimum, maximum, or average wheel resistances (absolute and relative) between cohorts (all $p > 0.05$). Note that sub-group sample sizes were smaller for cohort 1 Low-Work ($n = 1$ of 3) and cohort 2 High-Work ($n = 2$ of 3); mice excluded from table 13 were those that ran continued to run against 15% BM (static resistance) throughout the dynamic training phase ($n = 3$; $5.1 \pm 0.4g$ on average).

Table 13. Wheel Resistances for Dynamic Runners After the Stepper Motor Correction							
Sub-Group (n)	Body Mass (g)	Absolute Resistance (g)			Relative Resistance (% BM)		
		Minimum Resistance	Maximum Resistance	Average Resistance	Minimum Resistance	Maximum Resistance	Average Resistance
Cohort 1							
Low-Work (1)	37.9	2.0	2.0	2.0	5.0	5.0	5.0
Med-Work (4)	32.8 ± 3.2	1.6 ± 0.2	3.9 ± 0.5	2.4 ± 0.2	5.0	11.9 ± 1.2	7.4 ± 0.5
High-Work (4)	33.2 ± 2.4	1.7 ± 0.1	4.8 ± 0.3	2.7 ± 0.2	5.0	14.7 ± 1.4	8.3 ± 0.4
Cohort 2							
Low-Work (3)	34.5 ± 3.0	1.7 ± 0.1	3.1 ± 0.4	2.2 ± 0.3	5.0	8.8 ± 0.8	6.5 ± 0.3
Med-Work (3)	31.4 ± 0.9	1.6 ± 0.1	4.7 ± 0.6	2.6 ± 0.2	5.0	14.9 ± 1.5	8.3 ± 0.6
High-Work (2)	27.1	1.4	4.6	2.3	5.0	16.8	8.6

Data expressed as mean $\pm$ SEM; mean was reported when $n < 3$, but not SEM. BM= body mass

Figure 29 shows wheel distance and work data throughout dynamic training for both cohorts. There was considerable variability in voluntary wheel running within and between sub-groups (and cohorts). However, high SEM in the results can partially be explained by small sub-group sizes within cohorts ($n = 2-4$ mice, each).

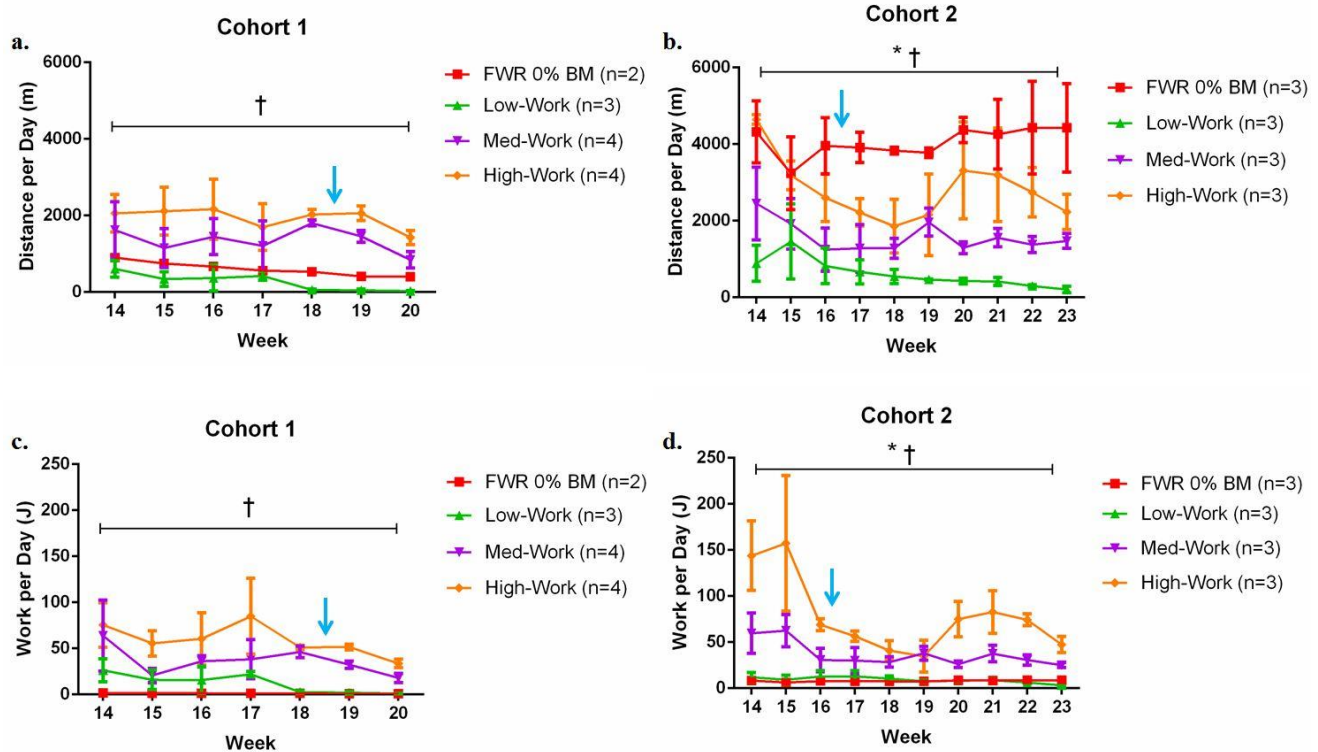


Figure 29. Weekly Running Performance during Dynamic Training.

The dynamic training phase lasted 7 weeks for cohort 1 (weeks 14-20) and 10 weeks for cohort 2 (weeks 14-23). Several weeks into dynamic training (~5.5 weeks for cohort 1; ~3.5 weeks for cohort 2), issues with the stepper motors were resolved (light blue arrows); resistance runners were sub-grouped *post-hoc* based on work data after the stepper motor correction. **a)** Wheel distance for mice in cohort 1 did not significantly change over time during dynamic training ($p > 0.05$), but there was a significant effect of group ($p < 0.05$). **b)** Wheel distance for mice in cohort 2 significantly changed over time and there was a main effect of sub-group (both $p < 0.05$), but there was no interaction ($p > 0.05$). **c)** Similar to the first cohort's wheel distance data, there was a main effect of sub-group on wheel work ($p < 0.05$), but no effect of time ($p > 0.05$). **d)** Similar to the second cohort's wheel distance data, there were significant effects of time and group on wheel work (both $p < 0.05$), but no interaction ($p > 0.05$). Data expressed as mean $\pm$ SEM; mean was reported when $n < 3$, but not SEM. FWR= free-wheel runners; † Indicates main effect of sub-group ($p < 0.05$); * Indicates main effect of time ($p < 0.05$).

4.4.3 Assessment of Plantar Flexor Function During Dynamic Training

Two weeks prior to sacrifice, we assessed plantar flexor function (18 weeks for cohort 1; 21 weeks for cohort 2). Comparisons were made within cohorts (i.e., between sub-groups) and between cohorts (i.e., pooled data from all mice in each cohort).

4.4.3.1 Twitches and Tetany

There were no differences in twitch or tetanic torque properties between sub-groups in cohort 1 (one-way ANOVAs showed all $p > 0.05$), and there were no differences between all runners ($n= 13$) vs. CA ($n= 3$) (all $p > 0.05$). One-way ANOVA showed that twitch to tetanus ratio was different between sub-groups in cohort 2 ($p= 0.01$); multiple comparisons showed that the twitch to tetanus ratio was only significantly different between cohort 2's High-Work sub-group and Low-Work sub-group ($p= 0.02$) (Table 14). When all runners in cohort 2 ($n= 12$) were compared to the CA sub-group ($n= 2$), running ($\pm$ added-resistance) increased tetanic torque relative to BM ($662 \pm 20\text{N*mm/kg}$ vs. 596N*mm/kg ; $p= 0.007$) (Table 14).

Further, when cohort 1 was compared to cohort 2, there were significant differences in torque between cohorts: tetanus relative to BM ($585 \pm 18\text{N*mm/kg}$ vs. $652 \pm 18\text{N*mm/kg}$; $p= 0.02$), absolute twitch torque ($0.32 \pm 0.0\text{N*cm}$ vs. $0.37 \pm 0.0\text{N*cm}$; $p= 0.009$), relative twitch torque ($93 \pm 4\text{N*mm/kg}$ vs. $113 \pm 4\text{N*mm/kg}$; $p= 0.002$), and twitch to tetanus ratio (0.16 ± 0.0 vs. 0.17 ± 0.0 ; $p= 0.04$). Final PFT measures for cohort 2 were performed 3 weeks later than cohort 1 (21 weeks vs. 18 weeks, respectively), which could indicate that additional training had beneficial effects on plantar flexor function.

Table 14. Plantar Flexor Torque Properties during Dynamic Training						
Description		Cohort 1 Final (18 Weeks)				
		CA (n= 3)	FWR 0% BM (n= 2)	Low-Work (n= 3)	Med-Work (n= 4)	High-Work (n= 4)
Twitch	Absolute Torque (N*cm)	0.30 ± 0.0	0.29	0.34 ± 0.1	0.33 ± 0.0	0.32 ± 0.0
	Relative Torque (N*mm/kg)	82 ± 12	84	93 ± 8	101 ± 6	98 ± 5
Tetanus	Absolute Torque (N*cm)	1.9 ± 0.1	2.2	2.1 ± 0.3	1.9 ± 0.1	2.0 ± 0.1

	Relative Torque (N*mm/kg)	527 ± 31	637	577 ± 30	600 ± 40	598 ± 41
Ratio	Twitch : Tetanus (unitless)	0.15 ± 0.0	0.13	0.16 ± 0.0	0.17 ± 0.0	0.16 ± 0.0
Description		Cohort 2 Final (21 Weeks)				
		CA (n= 2)	FWR 0% BM (n= 3)	Low-Work (n= 3)	Med-Work (n= 3)	High-Work (n= 3)
Twitch	Absolute Torque (N*cm) ‡	0.38	0.38 ± 0.0	0.33 ± 0.0	0.37 ± 0.0	0.38 ± 0.0
	Relative Torque (N*mm/kg)	117	113 ± 7	94 ± 11	115 ± 3	128 ± 9
Tetanus	Absolute Torque (N*cm)	2.0	2.3 ± 0.1	2.2 ± 0.2	2.2 ± 0.0	2.0 ± 0.2
	Relative Torque (N*mm/kg) ‡	596†	682 ± 21	629 ± 85	674 ± 12	664 ± 14
Ratio	Twitch : Tetanus (unitless) ‡	0.20	0.17 ± 0.0	0.15 ± 0.0	0.17 ± 0.0	0.19 ± 0.0*

Data expressed as mean ± SEM; mean was reported when n < 3, but not SEM. CA= cage-activity controls; FWR= free-wheel runners; Relative Torque= Absolute torque divided by body mass (expressed in N*mm/kg); * Indicates > Low-Work group in cohort 2 (p < 0.05). † Indicates all runners in cohort 2 > CA control (p < 0.05). ‡ Indicates cohort 2 > cohort 1 (p < 0.05).

In cohort 1, there were no differences in twitch or tetanic temporal properties between running sub-groups, or between all runners (n= 13) vs. CA (n= 3) (all p > 0.05). However, one-way ANOVA showed that twitch half-relaxation times of sub-groups in cohort 2 were different during their final PFT measures (p= 0.02). Multiple comparisons revealed that twitch HRT of mice in cohort 2's High-Work sub-group were slightly longer than those of mice in the Low-Work and Med-Work sub-groups (~1.5ms difference; both p < 0.05) (Table 15). There were no differences in temporal properties between all runners (n= 12) and CA controls (n= 2) (all p > 0.05).

When data were compared between cohorts, there were no differences in tetanic temporal properties, but there were small differences in twitch temporal properties between cohort 1 and cohort 2: time to peak torque ($21 \pm 0.2\text{ms}$ vs. $22 \pm 0.2\text{ms}$; $p < 0.001$), half-relaxation time ($12 \pm 0.1\text{ms}$ vs. $13 \pm 0.2\text{ms}$; $p = 0.002$), and torque-time integral ($7.3 \pm 0.4\text{N*cm*ms}$ vs. $9.0 \pm 0.3\text{N*cm*ms}$; $p = 0.002$).

Table 15. Plantar Flexor Temporal Properties during Dynamic Training						
Description		Cohort 1 Final (18 Weeks)				
		CA (n= 3)	FWR 0% BM (n= 2)	Low-Work (n= 3)	Med-Work (n= 4)	High-Work (n= 4)
Twitch	TPT (ms)	20 ± 0.4	21	20 ± 0.2	21 ± 0.1	21 ± 0.3
	Max dT/dt (N*cm/s)	21 ± 1.7	20	24 ± 3.3	22 ± 1.0	22 ± 0.8
	HRT (ms)	12 ± 0.3	12	12 ± 0.2	13 ± 0.2	12 ± 0.1
	TTI (N*cm*ms)	6.7 ± 0.8	6.7	8.0 ± 1.3	7.7 ± 0.3	7.3 ± 0.3
Tetanus	TPT (ms)	196 ± 10	333	287 ± 75	259 ± 20	299 ± 42
	Max dT/dt (N*cm/s)	51 ± 2	51	53 ± 7	45 ± 3	49 ± 1
	HRT (ms)	751 ± 10	614	658 ± 76	688 ± 19	650 ± 42
	TTI (N*cm*ms)	1744 ± 46	2013	1925 ± 248	1746 ± 46	1787 ± 81
Description		Cohort 2 Final (21 Weeks)				
		CA (n= 2)	FWR 0% BM (n= 3)	Low-Work (n= 3)	Med-Work (n= 3)	High-Work (n= 3)
Twitch	TPT $\pm$ (ms)	22	22 ± 0.6	22 ± 0.6	22 ± 0.2	23 ± 0.2
	Max dT/dt (N*cm/s)	26	25 ± 0.7	22 ± 1.2	24 ± 0.2	24 ± 1.8
	HRT $\pm$ (ms)	13	13 ± 0.3	$12 \pm 0.3^*$	$13 \pm 0.3^*$	14 ± 0.3
	TTI $\pm$ (N*cm*ms)	9.3	9.3 ± 0.5	7.7 ± 0.9	8.9 ± 0.2	9.9 ± 0.9
Tetanus	TPT (ms)	268	367 ± 83	287 ± 34	280 ± 33	331 ± 43
	Max dT/dt (N*cm/s)	47	50 ± 3	48 ± 2	48 ± 2	40 ± 5
	HRT (ms)	681	585 ± 84	661 ± 34	668 ± 33	623 ± 44

	TTI (N*cm*ms)	1749	2077 ± 131	1964 ± 211	1947 ± 34	1761 ± 155
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Data expressed as mean ± SEM; mean was reported when n < 3, but not SEM. TPT= Time to Peak Torque; Max dT/dt= Maximum rate of Torque Production; HRT= Half Relaxation Time; TTI= Torque-Time Integral. CA= cage-activity controls; FWR= free-wheel runners; * Indicates < High-Work subgroup in cohort 2 (p < 0.05). ‡ Indicates cohort 2 ≠ cohort 1 (p < 0.05).

4.4.3.2 Torque-Frequency

According to two-way ANOVAs, there were no differences in torque frequency profiles between sub-groups within cohorts (absolute, relative to BM, and percent of maximum; all p > 0.05) (Figure 30). However, there were differences in torque-frequency profiles between cohorts: mice in cohort 2 produced more relative torque at high frequencies ($\geq 50\text{Hz}$) than mice in cohort 1 (data not shown). Note there were no cohort differences in body mass or lean mass during final PFT measures (section 4.D.4).

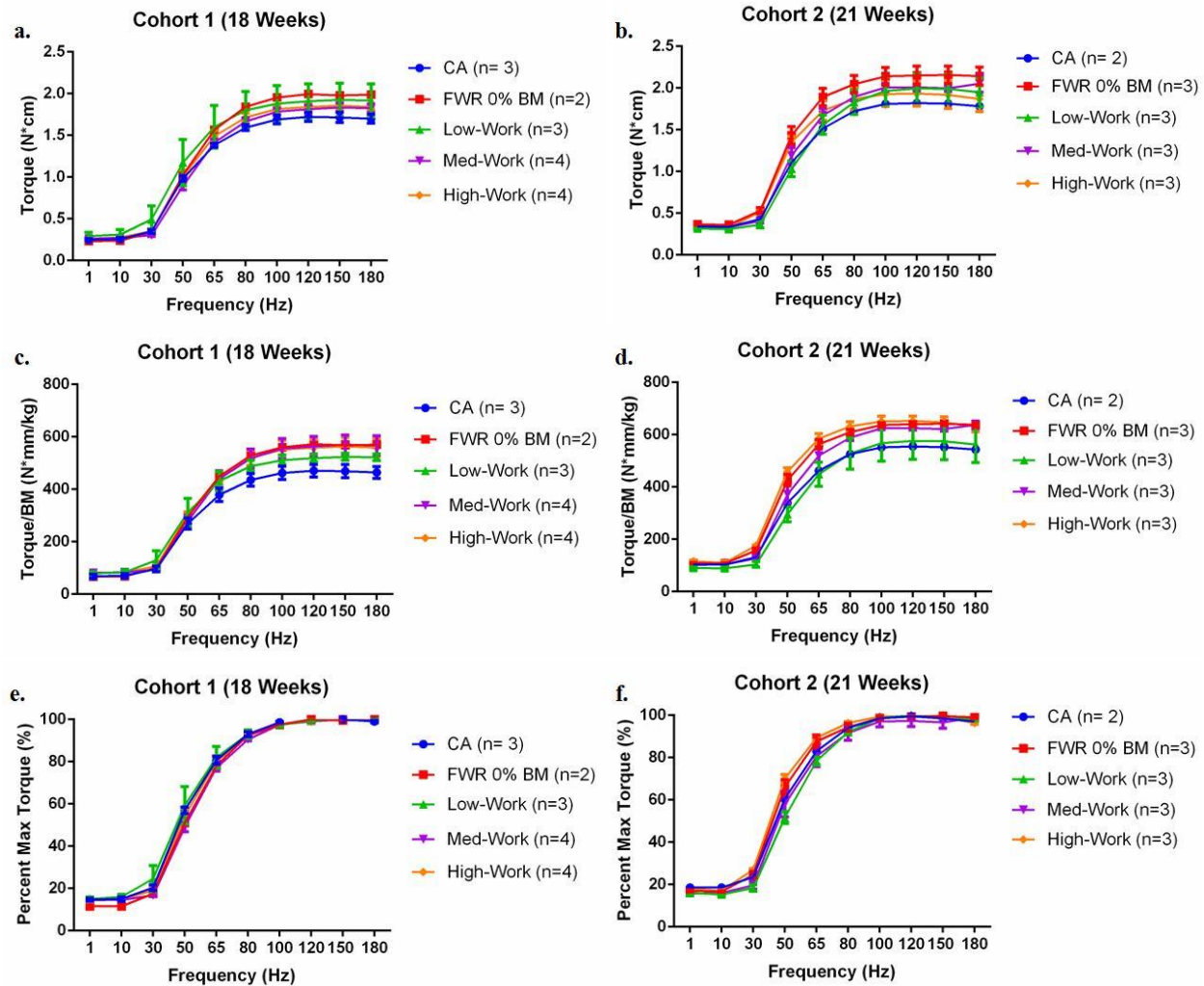


Figure 30. Plantar Flexor Torque-Frequency Profiles of Each Cohort during Dynamic Training.

After 18 weeks there were no differences in torque-frequency profiles (absolute, relative to BM, or percent maximum torque) between sub-groups in cohort 1 (**panels a., c., e.,** respectively) (two-way ANOVAs showed all $p > 0.05$). Similarly, after 21 weeks there were no differences in torque-frequency profiles (absolute, relative to BM, or percent maximum torque) between sub-groups in cohort 2 (**panels b., d., f.,** respectively) (two-way ANOVAs showed all $p > 0.05$). CA= cage-activity controls; FWR= free-wheel runners.

4.4.3.3 Fatigue Profiles

When cohort 1 plantar flexors were stimulated to fatigue during final measures (18 weeks), absolute and relative fatigue profiles were no different between sub-groups. When cohort 2 plantar flexors were stimulated to fatigue during final measures (21 weeks), there were

sub-group differences in absolute torque, but not relative torque (two-way ANOVA showed significant group effect, $p < 0.05$) (Figure 31). However, when cohorts were compared to each other (by area under the curve), mice in cohort 1 produced more relative torque (sum of percent initial torques) during the fatigue protocol than mice in cohort 2 ($2884 \pm 72\%$ vs. $2646 \pm 78\%$, respectively; $p = 0.03$). These results were surprising considering that mice in cohort 1 had not trained as long as mice in cohort 2 (3 weeks shorter). Yet, when sum percent of initial torques were compared between all runners ($n = 13$) and CA ($n = 3$) in cohort 1, there were no differences ($2929 \pm 64\%$ vs. $2688 \pm 280\%$; $p = 0.48$); but in cohort 2, runners ($n = 12$) produced more relative torque than CA ($n = 2$) ($2698 \pm 81\%$ vs. 2327% ; $p = 0.036$).

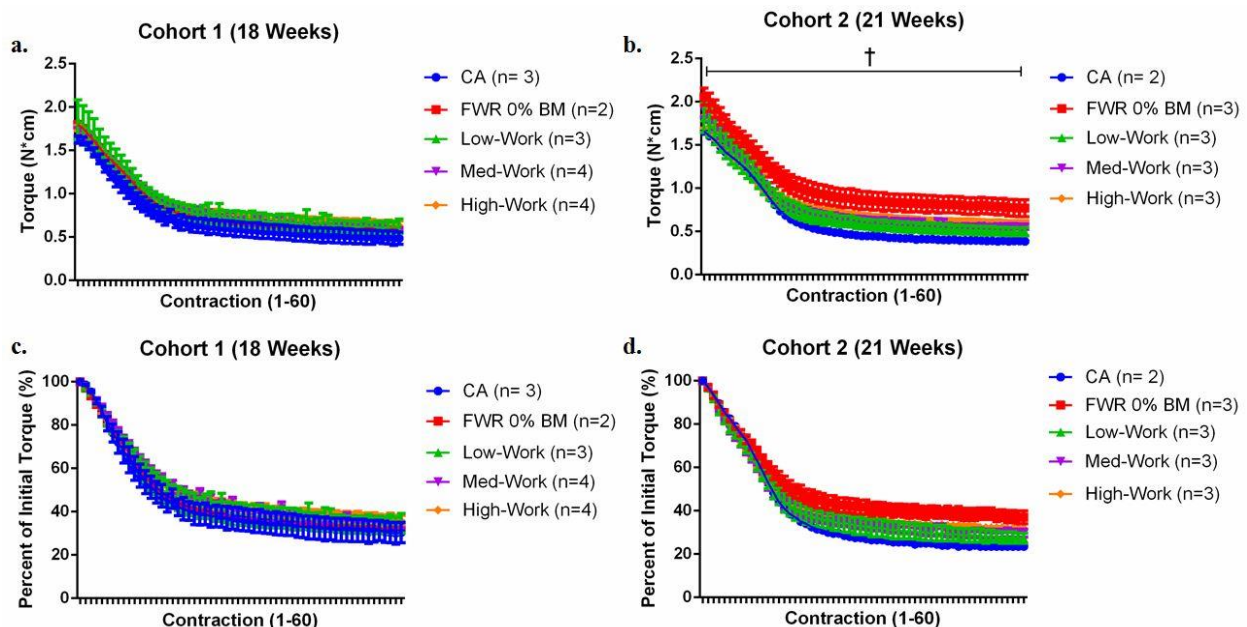


Figure 31. Plantar Flexor Fatigue Profiles during Dynamic Training.

Final PFT measures were obtained for cohort 1 at 18 weeks and for cohort 2 at 21 weeks. For cohort 1 sub-groups, there were no differences in absolute ($N \cdot cm$) or relative fatigue profiles (% of initial torque) (**panels a. and c.**). For cohort 2, there was an effect of sub-group on absolute fatigue profiles (**panel b.**) (sub-group effect: $p < 0.05$), but no interaction ($p > 0.05$). However, there were no differences in relative fatigue profiles between sub-groups in cohort 2 (**panel d.**) ($p > 0.05$). CA= cage-activity controls; FWR= free-wheel runners; † Indicates main effect of sub-group within cohort 2 ($p < 0.05$).

4.4.4 Body Mass, Food Consumption, and Body Composition

There were no body mass or food consumption differences between sub-groups within cohorts during dynamic training, nor were there differences between cohorts (all $p > 0.05$). Note that food consumption was not recorded during week 22 for cohort 2 (Figure 32)

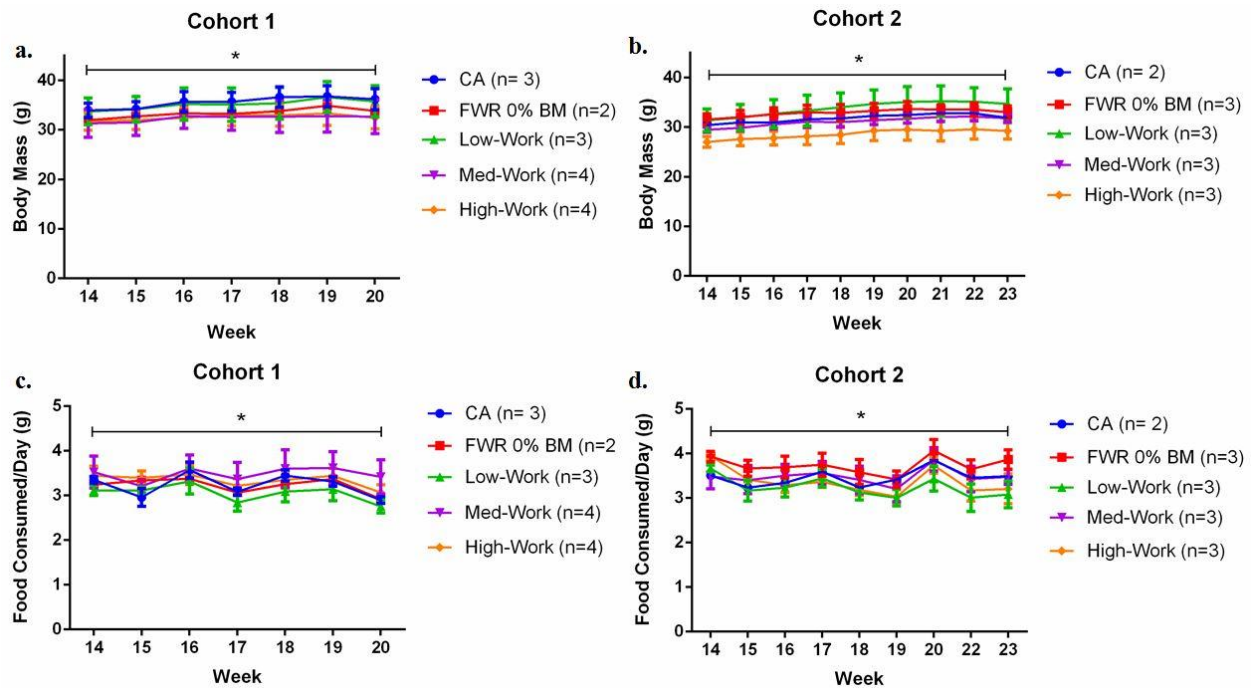


Figure 32. Body Mass and Food Consumption during Dynamic Training.

According to two-way ANOVAs with repeated measures, there were no differences in body mass (**panels a. and c.**) or food consumption (**panels b. and d.**) between sub-groups within cohorts during dynamic training (all $p > 0.05$). There were main effects of time on body mass and food consumption for both cohorts (all $p < 0.05$), but there were no differences in body mass or food consumption between cohorts over time ($p > 0.05$). CA= cage-activity controls; FWR= free wheel runners; * Indicates main effect of time ($p < 0.05$).

Importantly, there were no differences in body mass between cohort 1 ($34.6 \pm 1.2\text{g}$) and cohort 2 ($32.7 \pm 0.9\text{g}$) when final PFT measures were performed for each cohort (18 weeks and 21 weeks, respectively; $p = 0.19$). There were also no differences in absolute or relative lean, fat, and fluid mass between cohorts (all $p > 0.05$) (Figure 33).

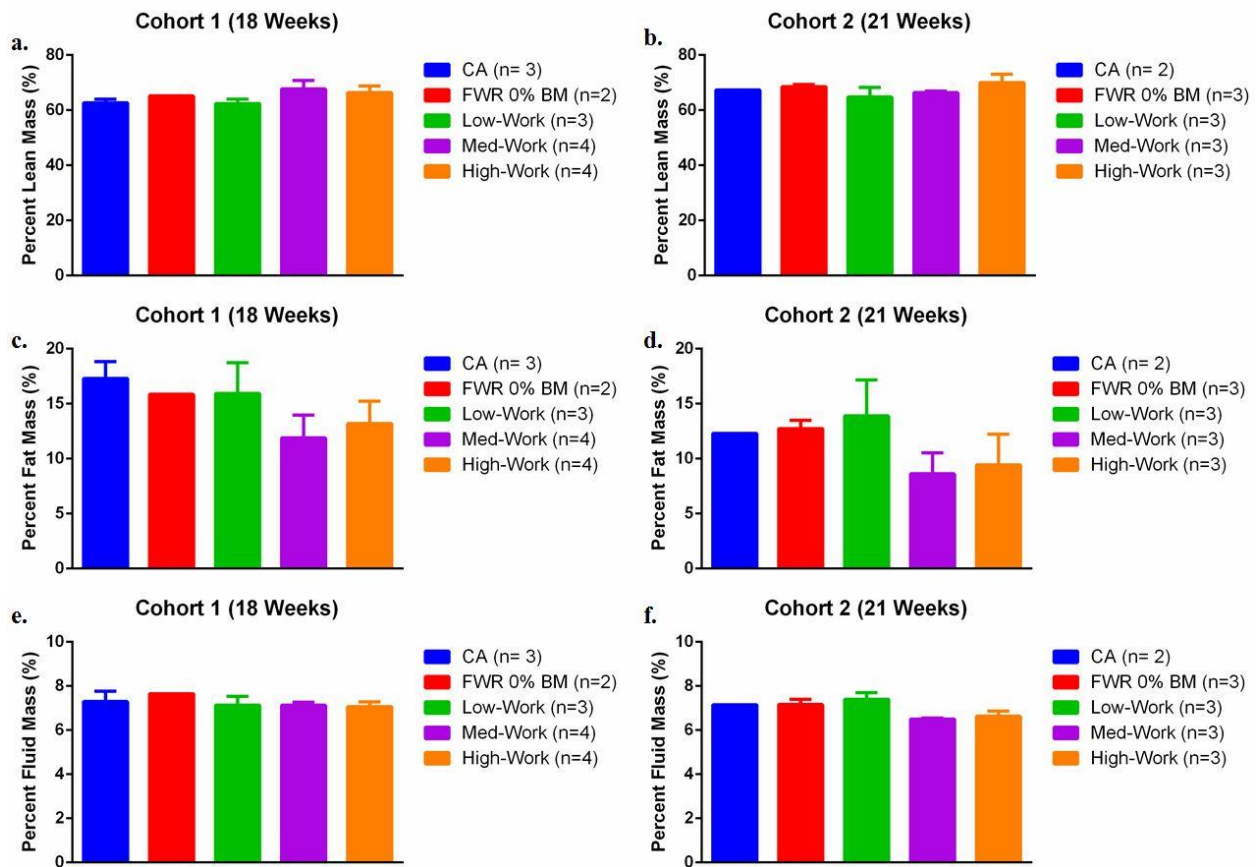


Figure 33. Body Composition for Both Cohorts during Dynamic Training.

There were no differences in absolute or relative lean, fat, or fluid mass between sub-groups within cohorts (only relative data shown) (all $p > 0.05$). Additionally, there were no differences in body mass or composition between cohorts (all $p > 0.05$). **a)** Percent lean mass of cohort 1 sub-groups at 18 weeks; **b)** percent lean mass of cohort 2 sub-groups at 21 weeks; **c)** percent fat mass for cohort 1 sub-groups; **d)** percent fat mass of cohort 2 sub-groups; **e)** percent fluid mass of cohort 1 sub-groups; **f)** percent fluid mass of cohort 2 sub-groups. CA= cage-activity controls; FWR= free-wheel runners.

Section 4.5: Assessments of Animal Fitness

Isometric muscular (grip) endurance and maximal running capacity were estimated using hanging wire tests and treadmill fitness assays, respectively. The hanging wire tests were performed two days prior to sacrifice (weeks 20 or 23), but the treadmill fitness assays were performed at baseline (week 0) and one-day prior to sacrifice (weeks 20 or 23).

There were no differences in body mass or hanging wire test performance (hang time or impulse) between cohorts (all $p > 0.05$). There were no differences in hanging wire performance between sub-groups within cohort 1, or between all runners ($n = 13$) vs. CA ($n = 3$) in cohort 1 (all $p > 0.05$). However, there were differences within cohort 2; the High-Work sub-group performed better than other sub-groups (CA, FWR 0% BM, Low-Work) (Table 16). But all runners ($n = 12$) were not different than CA ($n = 2$) in cohort 2 (all $p > 0.05$).

Because sub-groups were not formed until after dynamic training, baseline treadmill fitness data are reported for each cohort, as a whole (Table 16). At baseline, there were no differences in treadmill running fitness between cohorts ($p > 0.05$). However, during final measures, cohort 2 performed better than cohort 1 ($p < 0.05$).

When all runners were compared to CA controls in each cohort, runners ($n = 13$) from cohort 1 performed significantly better than CA ($n = 3$) ($1233 \pm 92s$ vs. $572 \pm 43s$; $p < 0.001$), and similar results were obtained for runners ($n = 12$) vs. CA ($n = 2$) in cohort 2 ($1568 \pm 69s$ vs. $961s$; $p = 0.008$).

One-way ANOVAs showed significant differences between sub-groups within both cohorts (both $p < 0.05$). In cohort 1, the Med-Work and High-Work sub-groups performed better than CA-controls (both $p < 0.05$). Also, cohort 1, all runners ($n = 13$) performed better than CA ($n = 3$) ($1233 \pm 92s$ vs. $572 \pm 43s$; $p < 0.001$). Surprisingly, in cohort 2, only the FWR 0% BM sub-group performed better than CA-controls ($p = 0.036$), and all runners ($n = 12$) did not perform significantly better than CA ($n = 2$) ($1568 \pm 69s$ vs. $961s$; $p = 0.25$) (Table 16).

Table 16. Hanging Wire Tests Prior to Sacrifice and Maximal Treadmill Running Capacity at Baseline and Prior to Sacrifice

	Cohort 1					
	CA (n= 3)	FWR 0% BM (n= 2)	Low-Work (n= 3)	Med-Work (n= 4)	High-Work (n= 3-4)	All (n= 15- 16)
Hanging Wire Time (s)	53 ± 13	60	39 ± 14	124 ± 60	62 ± 20	72 ± 18
Hanging Wire Impulse (g*s)	1910 ± 491	2023	1345 ± 495	3862 ± 1657	1914 ± 606	2333 ± 517
Baseline Treadmill (s)	-	-	-	-	-	724 ± 37
Final # Treadmill (s)	572 ± 43	1227	935 ± 223	1334 ± 115‡	1359 ± 198‡	1109 ± 100
	Cohort 2					
	CA (n= 2)	FWR 0% BM (n= 3)	Low-Work (n= 3)	Med-Work (n= 3)	High-Work (n= 3)	All (n= 14)
Hanging Wire Time (s)	45*	34 ± 7*	37 ± 9*	64 ± 7	105 ± 16	58 ± 9
Hanging Wire Impulse (g*s)	1466	1168 ± 277*	1348 ± 392*	2026 ± 218	3100 ± 493	1846 ± 242
Baseline Treadmill (s)	-	-	-	-	-	693 ± 56
Final # Treadmill (s)	961	1747 ± 74**	1348 ± 155	1624 ± 160	1553 ± 81	1481 ± 88†

Data expressed as mean ± SEM; mean was reported when n < 3, but not SEM. Note that one mouse from cohort 1 (High-Work) was non-compliant during its hanging wire tests (excluded from analysis). CA= cage-activity controls; FWR= free-wheel runners; ‡ Indicates > CA-controls in cohort 1 (p < 0.05); * Indicates < High-Work sub-group in cohort 2 (p < 0.05); ** Indicates > CA-controls in cohort 2 (p < 0.05); † Indicates cohort 2 > cohort 1 (p < 0.05); # Indicates all runners > CA-controls (p < 0.05).

Section 4.6: Data Collected After Sacrifice

4.6.1 Muscle Mass and Cross-Sectional Area

Muscles masses and body masses were recorded during sacrifice. There were no BM differences between cohorts at sacrifice ($34.0 \pm 1.2\text{g}$; $32.2 \pm 0.9\text{g}$; $p = 0.318$). According to one-way ANOVAs, there were no differences in absolute or BM-normalized muscle masses (GAS, SOL, TA, and Tri) between sub-groups within cohorts (all $p > 0.05$) (Table 17). Additionally, there were no differences in absolute or relative muscle masses between runners vs. CA controls within each cohort (all $p > 0.05$).

However, when cohorts were compared, absolute TA masses of mice in cohort 2 were greater than TA masses of mice in cohort 1 ($52.7 \pm 3.3\text{mg}$ vs. $36.5 \pm 2.6\text{mg}$, respectively; $p < 0.001$). BM-normalized masses of TA and GAS muscles were also greater in the second cohort compared to the first cohort (+53% and +11%, respectively; both $p < 0.01$).

Table 17. Absolute and Relative Masses of Selected Running Muscles								
Sub-Group	Cohort 1 (20 Weeks)							
	Absolute Muscle Mass (mg)				Relative Muscle Mass (mg/g)			
	GAS	SOL	TA	Tri	GAS	SOL	TA	Tri
CA (n= 3)	169 $\pm$ 13	10.7 $\pm$ 0.6	38.2	142 $\pm$ 10	4.7 $\pm$ 0.2	0.30 $\pm$ 0.0	1.01	4.0 $\pm$ 0.5
FWR (n= 2)	174	23	26.6	146	5.2	0.68	0.79	4.3
Low-Work (n= 2-3)	192 $\pm$ 15	10.7 $\pm$ 1.8	40.2 $\pm$ 7.4	111	5.4 $\pm$ 0.1	0.31 $\pm$ 0.0	1.13 $\pm$ 0.2	3.4
Med-Work (n= 4)	172 $\pm$ 10	10.6 $\pm$ 1.3	36.7 $\pm$ 4.7	126 $\pm$ 9	5.3 $\pm$ 0.3	0.32 $\pm$ 0.0	1.13 $\pm$ 0.1	3.9 $\pm$ 0.4
High-Work (n= 4)	178 $\pm$ 11	11.2 $\pm$ 1.3	37.6 $\pm$ 1.0	135 $\pm$ 4	5.5 $\pm$ 0.3	0.35 $\pm$ 0.1	1.17 $\pm$ 0.0	4.2 $\pm$ 0.2
Sub-Group	Cohort 2 (23 Weeks)							
	Absolute Muscle Mass (mg)				Relative Muscle Mass (mg/g)			
	GAS	SOL	TA †	Tri	GAS †	SOL	TA †	Tri
CA (n= 2)	208	15.6	45.1	137	6.6	0.49	1.43	4.3
FWR (n= 3)	200 $\pm$ 20	13.3 $\pm$ 0.8	63.0 $\pm$ 9.2	159 $\pm$ 14	6.1 $\pm$ 0.5	0.40 $\pm$ 0.0	1.90 $\pm$ 0.2	4.8 $\pm$ 0.4
Low-Work (n= 3)	191 $\pm$ 18	13.1 $\pm$ 4.2	58.0 $\pm$ 4.9	137 $\pm$ 4	5.6 $\pm$ 0.9	0.39 $\pm$ 0.1	1.72 $\pm$ 0.3	4.0 $\pm$ 0.2
Med-Work (n= 3)	199 $\pm$ 17	12.6 $\pm$ 3.0	52.0 $\pm$ 5.9	146 $\pm$ 10	6.3 $\pm$ 0.5	0.40 $\pm$ 0.1	1.64 $\pm$ 0.2	4.6 $\pm$ 0.3
High-Work (n= 3)	174 $\pm$ 18	10.8 $\pm$ 0.9	43.0 $\pm$ 6.6	143 $\pm$ 5	5.9 $\pm$ 0.4	0.37 $\pm$ 0.0	1.46 $\pm$ 0.1	4.7 $\pm$ 0.2

Data expressed as mean $\pm$ SEM; mean was reported when $n < 3$, but not SEM. CA= cage-activity; FWR= free-wheel runners; Relative Muscle Mass= muscle mass (mg)/ body mass (g); GAS= gastrocnemius; SOL= soleus; TA= tibialis anterior; Tri= triceps. † Indicates cohort 2 > cohort 1 ($p < 0.05$).

Despite some differences in muscle mass between cohorts, there were no differences in fiber CSA between cohorts (all $p > 0.05$). Additionally, one-way ANOVAs showed there were no differences in fiber CSA between sub-groups within cohorts (all $p > 0.05$) (Table 18). There were no differences between all runners ($n= 13$) and CA ($n= 3$) in cohort 1, but surprisingly in

cohort 2, GAS muscle CSA was larger in CA (n= 2) vs. all runners (n= 12) ($2057\mu\text{m}^2$ vs. $1782 \pm 115 \mu\text{m}^2$; $p = 0.005$).

Table 18. Cross-Sectional Area of Selected Running Muscles					
Muscle	Cohort 1 (20 Weeks)				
	CA (n= 3)	FWR 0% BM (n= 1-2)	Low-Work (n= 3)	Med-Work (n= 3-4)	High-Work (n= 4)
GAS (μm^2)	1910 $\pm$ 211	1593	1510 $\pm$ 67	1609 $\pm$ 179	1655 $\pm$ 182
SOL (μm^2)	1539 $\pm$ 129	1248*	1218 $\pm$ 296	1311 $\pm$ 88	1558 $\pm$ 140
TA (μm^2)	1590 $\pm$ 156	1486	1497 $\pm$ 187	1413 $\pm$ 93	1446 $\pm$ 118
Tri (μm^2)	1852 $\pm$ 241	1994	1644 $\pm$ 165	1928 $\pm$ 271	1595 $\pm$ 128
Muscle	Cohort 2 (23 Weeks)				
	CA (n= 2)	FWR 0% BM (n= 3)	Low-Work (n= 3)	Med-Work (n= 2-3)	High-Work (n= 2-3)
GAS (μm^2)	2057 †	2100 $\pm$ 252	1558 $\pm$ 117	1426	1926 $\pm$ 206
SOL (μm^2)	1519	1340 $\pm$ 124	1224 $\pm$ 113	1614 $\pm$ 278	1800 $\pm$ 221
TA (μm^2)	1633	1617 $\pm$ 222	1488 $\pm$ 295	1406 $\pm$ 160	1710
Tri (μm^2)	1961	1765 $\pm$ 243	1512 $\pm$ 151	1586 $\pm$ 114	1804 $\pm$ 208

Data expressed as mean $\pm$ SEM; mean was reported when $n < 3$, but not SEM. CA= cage-activity controls; FWR= free-wheel runners; GAS= gastrocnemius; SOL= soleus; TA= tibialis anterior; Tri= triceps; * Excluded from one-way ANOVA because $n = 1$. † Indicates $>$ all runners in cohort 2 ($p < 0.05$).

4.6.2 Myosin Heavy Chain Distributions

To determine if RWR promoted fiber type shifts, a subset of mouse muscles were sent to Dr. Pam Van Ry (University of Nevada, Reno) for type-I and type-IIA myosin heavy chain staining ($n = 20$ of 30), so MHC-staining data from each cohort were pooled by sub-groups (e.g., data from CA mice in both cohorts were pooled). When MHC data from the first cohort ($n = 9$) were compared to the second cohort ($n = 11$), there was only one significant difference: the percent of type IIA-positive fibers in triceps muscles ($4.1 \pm 1.2\%$ vs. $9.5 \pm 2.1\%$, respectively; $p = 0.049$). According to one-way ANOVAs, there were no differences in the percent of type I-positive or type IIA-positive fibers between pooled sub-groups from both cohorts (all $p > 0.05$) (Table 19).

Table 19. Myosin Heavy Chain Expression: Data Pooled Between Cohorts by Sub-group					
Muscle	Type I-Positive Fibers (%)				
	CA (n= 4)	FWR 0% BM (n= 3-4)	Low-Work (n= 3-4)	Med-Work (n= 4)	High-Work (n= 4)
GAS	0.67 ± 0.5	0.29 ± 0.3	2.61 ± 1.6	0.21 ± 0.1	0.41 ± 0.3
SOL	40.3 ± 5.7	25.7 ± 3.6	58.7 ± 3.7	52.1 ± 12.0	54.8 ± 9.1
TA	0.00 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.03 ± 0.0	0.01 ± 0.0
Tri	0.18 ± 0.1	0.30 ± 0.3	1.13 ± 0.4	0.48 ± 0.4	0.03 ± 0.0
Muscle	Type IIA-Positive Fibers (%)				
	CA (n= 4)	FWR 0% BM (n= 3-4)	Low-Work (n= 4)	Med-Work (n= 4)	High-Work (n= 4)
GAS	9.63 ± 3.0	4.51 ± 3.6	3.65 ± 1.5	7.41 ± 2.7	2.07 ± 0.8
SOL	12.6 ± 4.8	22.0 ± 14.7	18.4 ± 5.5	4.88 ± 1.4	13.0 ± 2.8
TA	2.99 ± 1.5	2.84 ± 0.6	4.08 ± 1.6	2.10 ± 0.5	1.77 ± 1.4
Tri	5.28 ± 3.4	5.34 ± 4.0	6.54 ± 1.0	11.76 ± 3.9	6.28 ± 2.6

Muscles from a sub-set of mice (n= 20 of 30) were stained for type I and type IIA myosin heavy chain expression. Data from all sub-groups were pooled between cohorts. CA= cage-activity controls; FWR= free-wheel runners; GAS= gastrocnemius; SOL= soleus; TA= tibialis anterior; Tri= triceps.

4.6.3 Quadriceps Muscle Metabolism Data

4.6.3.1 *In-vitro* Fatty Acid and Glucose Oxidation in Red and White Portions of Quadriceps Muscles

During sacrifice, quadriceps muscles were separated into red portions (slower, more oxidative fibers) and white portions (faster, more glycolytic fibers) for *in-vitro* assessment of glucose oxidation and fatty acid oxidation. For both cohorts, red portions of QUAD muscles had higher FAO capacity (i.e., higher CO₂ and acid soluble metabolite production rates) and were more efficient (i.e., higher CO₂:ASM ratio) than white-QUAD (all p < 0.001). Additionally, red QUADs had higher GO capacity than white QUADs (p < 0.001).

There were no differences in FAO or GO capacity between cohorts (all p > 0.05). In cohort 2, but not cohort 1, all runners (n= 12) exhibited greater red-QUAD FAO efficiency than

CA-controls (n= 2) (0.83 ± 0.01 vs. 0.63 ; $p = 0.02$). However, comparisons of sub-groups in cohort 2 showed that white-QUAD muscles in CA-control mice produced more CO_2 than mice in the Low-Work and High-Work sub-groups (both $p < 0.05$) (Table 20).

Table 20. <i>In-vitro</i> Substrate Metabolism in Red and White Portions of Quadriceps Muscles							
Assay	Portion of QUAD	Parameter	Cohort 1 (20 Weeks)				
			CA (n= 3)	FWR (n= 2)	Low-Work (n= 3)	Med-Work (n= 4)	High-Work (n= 4)
Glucose Oxidation	Red	CO_2 Produced	17.7 ± 7.7	14.3	20.5 ± 3.2	19.0 ± 5.0	16.2 ± 2.0
	White	CO_2 Produced	8.7 ± 2.1	8.5	7.5 ± 1.4	6.4 ± 1.1	7.7 ± 1.4
Fatty Acid Oxidation	Red	CO_2 Produced	18.1 ± 2.9	25.1	23.2 ± 3.3	22.7 ± 2.9	24.4 ± 1.7
	Red	ASM Produced	25.2 ± 5.9	22.3	31.3 ± 2.6	27.1 ± 7.0	23.7 ± 2.3
	Red	$\text{CO}_2 + \text{ASM}$	43.3 ± 4.8	47.4	54.5 ± 1.5	49.8 ± 4.6	48.1 ± 2.3
	Red	$\text{CO}_2:\text{ASM}$	0.82 ± 0.2	1.14	0.77 ± 0.2	1.04 ± 0.29	1.06 ± 0.2
	White	CO_2 Produced	2.0 ± 0.5	1.4	1.0 ± 0.1	1.9 ± 0.2	1.8 ± 0.2
	White	ASM Produced	5.6 ± 1.0	4.0	3.2 ± 0.4	6.2 ± 0.7	5.4 ± 0.5
	White	$\text{CO}_2 + \text{ASM}$	7.6 ± 1.5	5.4	4.2 ± 0.4	8.1 ± 0.9	7.2 ± 0.6
	White	$\text{CO}_2:\text{ASM}$	0.36 ± 0.0	0.36	0.31 ± 0.1	0.31 ± 0.0	0.34 ± 0.0
Assay	Portion of QUAD	Parameter	Cohort 2 (23 Weeks)				
			CA (n= 2)	FWR (n= 3)	Low-Work (n= 3)	Med-Work (n= 3)	High-Work (n= 3)
Glucose Oxidation	Red	CO_2 Produced	19.2	21.1 ± 4.7	15.7 ± 5.4	22.3 ± 5.1	29.1 ± 6.3
	White	CO_2 Produced	7.9	7.1 ± 1.3	6.1 ± 0.3	4.9 ± 1.0	6.6 ± 0.7
Fatty Acid Oxidation	Red	CO_2 Produced	19.8	20.3 ± 3.1	19.8 ± 0.8	28.3 ± 6.2	28.2 ± 4.9
	Red	ASM Produced	31.4	28.0 ± 4.4	27.0 ± 0.8	29.5 ± 2.6	32.3 ± 3.4
	Red	$\text{CO}_2 + \text{ASM}$	51.2	48.3 ± 7.5	48.1 ± 0.6	57.8 ± 3.7	60.6 ± 8.3
	Red	$\text{CO}_2:\text{ASM} \uparrow$	0.63	0.73 ± 0.0	0.73 ± 0.0	1.01 ± 0.3	0.86 ± 0.1
	White	CO_2 Produced	3.1	1.4 ± 0.6	$0.8 \pm 0.1^*$	1.4 ± 0.3	$1.0 \pm 0.2^*$
	White	ASM Produced	7.3	7.4 ± 1.1	6.0 ± 1.5	4.0 ± 0.3	5.4 ± 1.6
	White	$\text{CO}_2 + \text{ASM}$	10.4	8.8 ± 1.4	6.8 ± 1.4	5.5 ± 0.2	6.4 ± 1.6
	White	$\text{CO}_2:\text{ASM}$	0.43	0.19 ± 0.1	0.16 ± 0.1	0.37 ± 0.1	0.23 ± 0.1

Data expressed as mean $\pm$ SEM; mean was reported when $n < 3$, but not SEM; units for all parameters were $\mu\text{mol}/\text{mg}$ protein/min, except for $\text{CO}_2:\text{ASM}$ ratio (unitless). ASM= acid soluble metabolites; CA= cage-activity controls; FWR= free-wheel runners; QUAD= quadriceps muscle; * Indicates $<$ CA-control sub-group in cohort 2 ($p < 0.05$). $\uparrow$ Indicates all runners $>$ CA in cohort 2 ($p < 0.05$).

4.6.3.2 Oxidative Enzyme Activities in Red and White Portions of Quadriceps

Muscles

BHAD and CS enzyme activities were assayed in red and white portions of QUAD muscles to further assess SkM oxidative capacity. BHAD activities were no different between cohorts in QUAD muscles (red and white; both $p > 0.05$), but CS activity was higher in white QUAD portions of cohort 2 compared to cohort 1 ($167 \pm 8 \mu\text{mol/mg protein/min}$ vs. $142 \pm 8 \mu\text{mol/mg protein/min}$; $p = 0.03$). In cohort 1, when all runners ($n = 13$) were compared to CA control ($n = 3$), BHAD and CS enzyme activities were higher in red muscle (both $p < 0.01$), but not white muscle (both $p > 0.05$). In cohort 2, when all runners ($n = 12$) were compared to CA control ($n = 2$), BHAD and CS enzyme activities were no different in red and white muscle (all $p > 0.05$).

Comparisons within cohort 1 showed that the High-Work sub-group exhibited greater BHAD activity than the CA sub-group in red-QUAD ($p < 0.05$; all others $p > 0.05$) (Figure 34), but there were no sub-group differences in CS activity (all $p > 0.05$) (Figure 35). Interestingly, comparisons within cohort 2 showed CS activities in red-QUAD were only different between the Low-Work and Med-Work sub-groups ($p < 0.05$; all others $p > 0.05$) (Figure 35); there were no differences in BHAD activities between cohort 2 sub-groups (all $p > 0.05$).

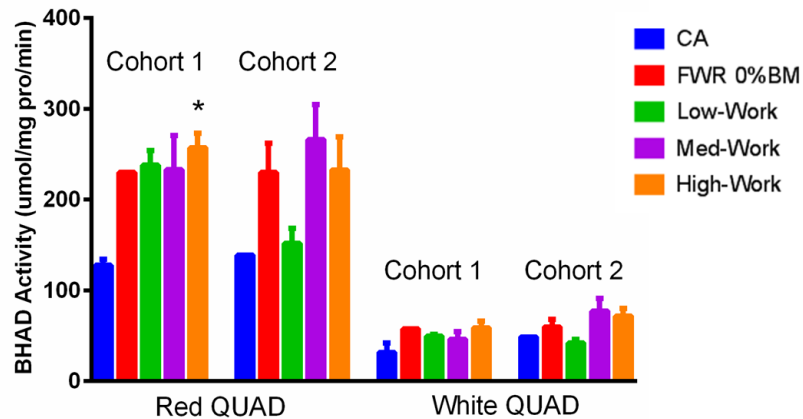


Figure 34. Beta-hydroxyacyl-CoA Dehydrogenase Enzyme Activities in Red and White Portions of Quadriceps.

There were no differences in beta-hydroxyacyl-CoA dehydrogenase (BHAD) activities of red or white quadriceps (QUAD) muscles between cohorts. Red-QUAD BHAD activities were higher for all runners compared to CA-controls in cohort 1 ($p < 0.01$), but not in cohort 2. Comparisons within cohort 1 showed that, in red-QUAD, BHAD activity was only greater in the High-Work sub-group compared to the CA-controls ($p > 0.05$). There were no sub-group differences in red or white QUAD of mice from cohort 2. CA= cage-activity controls; FWR= free-wheel runners; * Indicates $>$ CA sub-group in cohort 1 ($p < 0.05$).

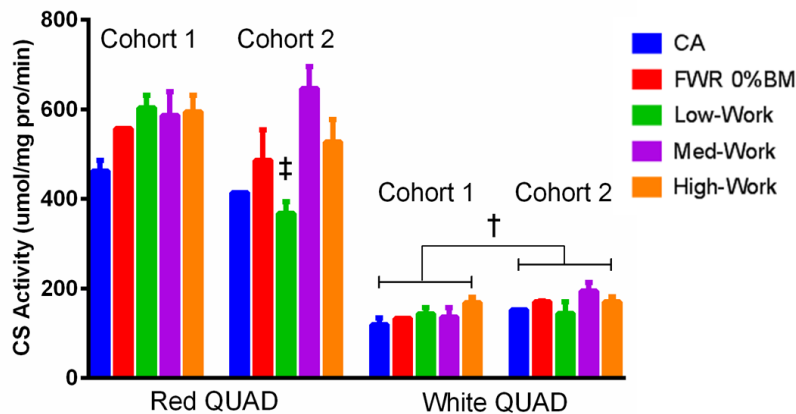


Figure 35. Citrate Synthase Enzyme Activities in Red and White Portions of Quadriceps.

There were no differences in citrate synthase (CS) activities of red-QUAD muscles between cohorts. However, white-QUAD muscles of cohort 2 exhibited greater CS activity than cohort 1 ($p < 0.05$). Red-QUAD CS activities were higher for all runners compared to CA-controls in cohort 1 ($p < 0.01$), but not in cohort 2. There were no differences in CS activity between sub-groups in cohort 1 (all $p > 0.05$); however, in cohort 2, red-QUAD muscles from the Med-Work sub-group had greater CS activity than the Low-Work sub-group ($p < 0.05$). CA= cage-activity controls; FWR= free-wheel runners; QUAD= quadriceps muscle; † Indicates cohort 2 $>$ cohort 1; ‡ Indicates Low-Work sub-group $<$ Med-Work sub-group in cohort 2 ($p < 0.05$).

4.6.4 Satellite Cell Proliferation and Differentiation of Pooled Skeletal Muscles

After skeletal muscles were removed from the hindlimbs and forelimbs during sacrifice, remaining skeletal muscles were harvested from mouse carcasses and pooled together for satellite cell analyses. There were no differences in proliferative or differentiative capacity between cohorts (both $p > 0.05$), and we were only able to obtain usable data from a portion of mice (proliferation, $n = 18$ of 30; differentiation, $n = 24$ of 30), so sub-groups in each cohort were pooled together for comparisons. There were no differences in differentiative or proliferative capacity between pooled sub-groups (all $p > 0.05$) (Figure 36).

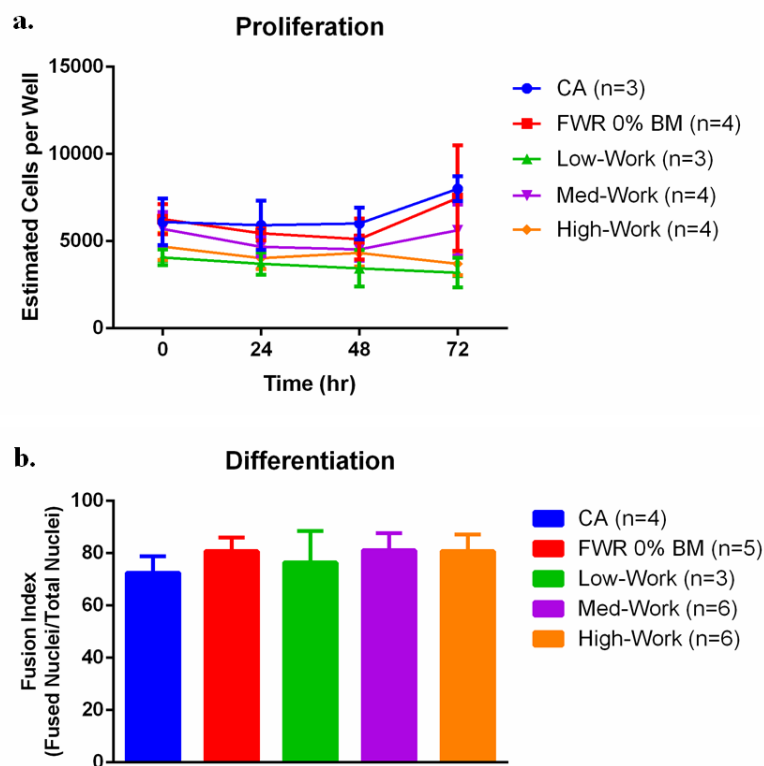


Figure 36. Satellite Cell Properties of Skeletal Muscles: Data Pooled Between Cohorts by Sub-groups.

After prime running muscles were removed from mice, remaining skeletal muscles were pooled together and their satellite cells were isolated. Satellite cell proliferation and differentiation data were pooled between cohorts by sub-group. There were no differences in satellite cell **a)** proliferative capacity, or **b)** differentiative capacity between pooled sub-groups (all $p > 0.05$). CA= cage-activity controls; FWR= free-wheel runners.

4.6.5 Markers of Muscle Fiber Damage and Regeneration

4.6.5.1 Plasma Creatine Kinase

To assess a marker of muscle damage, plasma was successfully collected from most animals during sacrifice (n= 26 of 30) for analysis of CK activity. There were no differences in plasma CK activity between the first cohort and the second cohort (104 ± 15 IU/L vs. 113 ± 21 IU/L, respectively; $p = 0.716$). There were also no differences between sub-groups within cohorts (all $p > 0.05$) (Figure 37a,b). However, when all runners were pooled (n= 22) and compared to pooled CA-controls (n= 4), runners had ~54% lower CK activity than CA (91 ± 8 IU/L vs. 196 ± 51 IU/L, respectively; $p < 0.001$). Further, when running sub-groups were pooled between cohorts, most running sub-groups, except for the Med-Work group, exhibited markedly lower plasma CK activity than pooled CA-controls ($p < 0.05$) (Figure 37c).

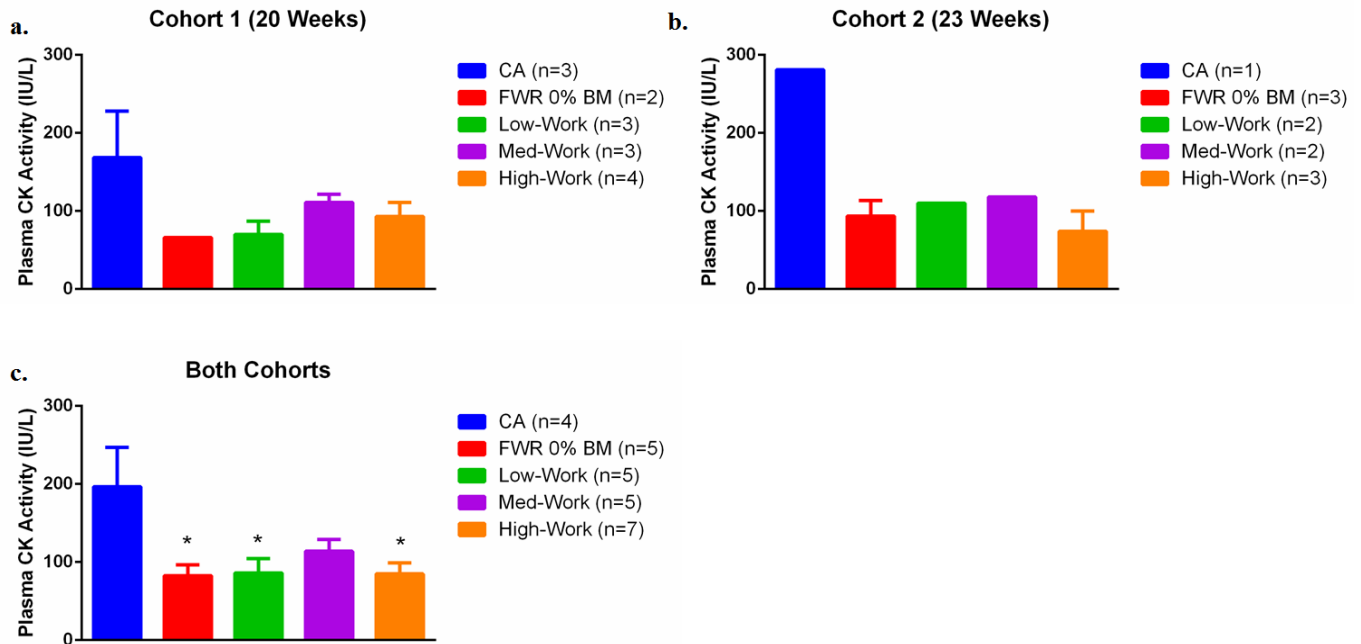


Figure 37. Plasma Creatine Kinase Activity of Sub-groups in Both Cohorts and of Pooled Sub-groups.

Plasma creatine kinase (CK) activity is a marker of muscle damage. There were no differences in plasma CK activity between sub-groups within the first cohort (**panel a.**) or within the second cohort (**panel b.**). However, sample sizes were smaller because some blood samples were not successfully collected during sacrifice. When plasma CK data were pooled between cohorts by sub-group, most running sub-groups exhibited lower plasma CK activity than pooled CA-controls (all $p < 0.05$; except for Med-Work) (**panel c.**). CA= cage-activity controls; FWR= free-wheel runners; * Indicates $< \text{pooled CA-controls}$ ($p < 0.05$).

4.6.5.2 Centrally-Located Nuclei in Muscle Cross-sections

To assess fiber damage/regeneration in running muscles, centralized nuclei were quantified in muscle cross-sections (GAS, SOL, TA, and Tri). There were no differences in the percentage of centrally nucleated fibers (CNF) between cohorts (all $p > 0.05$). However, we observed that within both cohorts, SOL muscles had more CNF than GAS muscles: cohort 1 ($4.4 \pm 1.4\%$ vs. $1.4 \pm 0.2\%$, respectively; $p < 0.001$); cohort 2 ($3.5 \pm 0.7\%$ vs. $1.6 \pm 0.4\%$, respectively; $p = 0.01$). In cohort 1, SOL muscles also had higher CNF than TA muscles ($2.2 \pm 0.9\%$; $p = 0.049$); but in cohort 2, SOL muscles were no different than TA muscles ($3.3 \pm 1.2\%$; $p = 0.26$).

Further, we observed SOL muscle CNF differences between sub-groups in both cohorts. Surprisingly, in cohort 1, the Low-Work and Med-Work mice had higher CNF than the CA mice ($p < 0.05$). The Low-Work sub-group (cohort 1) also had higher SOL CNF than the FWR 0% BM and High-Work sub-groups (both $p < 0.05$) (Table 21).

Also, in cohort 1, Low-Work had higher CNF in Tri muscles than High-Work ($p < 0.05$). However, in contrast to cohort 1, Tri muscles from High-Work mice in cohort 2 had higher CNF than the CA, FWR 0% BM, and Med-Work sub-groups (all $p < 0.05$) (Table 21).

Table 21. Centrally-Located Nuclei in Selected Running Muscles					
Muscle	Cohort 1 (20 Weeks)				
	CA (n= 3)	FWR 0% BM (n= 1-2)	Low-Work (n= 3)	Med-Work (n= 3-4)	High-Work (n= 4)
GAS (%)	1.2 ± 0.1	1.9	1.8 ± 0.2	1.1 ± 0.5	1.2 ± 0.3
SOL (%)	2.1 ± 0.5*	2.9*	7.6 ± 0.6†	6.0 ± 1.0†	3.3 ± 0.6*
TA (%)	0.6 ± 0.4	0 #	5.3 ± 3.7	1.6 ± 0.6	2.5 ± 1.9
Tri (%)	2.5 ± 0.4	1.4	7.9 ± 3.3	3.6 ± 0.6	0.6 ± 0.2*
Muscle	Cohort 2 (23 Weeks)				
	CA (n= 2)	FWR 0% BM (n= 3)	Low-Work (n= 3)	Med-Work (n= 2-3)	High-Work (n= 2-3)
GAS (%)	1.4	1.0 ± 0.5	2.4 ± 0.4	2.2 ± 1.7	1.2 ± 0.5
SOL (%)	4.4	0.6 ± 0.3	5.0 ± 1.3	3.0 ± 0.8	5.0 ± 1.7
TA (%)	0.8	6.2 ± 4.0	2.9 ± 0.9	1.7 ± 0.3	4.7 ± 4.1
Tri (%)	1.3‡	1.2 ± 0.3‡	2.2 ± 0.1	1.1 ± 0.3‡	5.8 ± 1.6

Data expressed as mean ± SEM; when $n < 3$ only sub-group mean was reported. CA= cage-activity controls; FWR= free-wheel runners; GAS= gastrocnemius; SOL= soleus; TA= tibialis anterior; Tri= triceps; * Indicates < Low-Work sub-group in cohort 1 ($p < 0.05$); † Indicates > CA-control in cohort 1 ($p < 0.05$); # Excluded from analysis ($n= 1$); ‡ Indicates < High-Work sub-group in cohort 2 ($p < 0.05$).

Section 4.7: Selected Analyses with Pooled CA-control Data

As noted above, CA-control sub-groups in each cohort were small ($n= 2-3$). To overcome the statistical limitations of a small n , CA-control data were pooled during and after the dynamic training phase. Pooled CA data were only used when t-tests showed there were no differences between CA-control groups in each cohort (all $p > 0.05$). For example, CA-controls in cohort 1

were not different from CA-controls in cohort 2 with respect to the following parameters (all $p > 0.05$): body mass, tetanic torque, maximal running capacity, FAO capacity and efficiency, BHAD and CS enzyme activities. Several important outcomes which show differences between runners and pooled CA-controls are reported in the figures below (specifically tetanic torque, maximal running capacity, and metabolism). In addition, because there were no differences in outcomes between running sub-groups within cohorts (one-way ANOVAs showed $p > 0.05$), runners within each cohort were pooled (cohort 1, $n = 13$; cohort 2, $n = 12$) and compared to pooled CA-controls ($n = 5$). Other outcomes are briefly discussed at the end of section 4G.

Analyses performed for each specific variable:

- Cohort 1 CA-controls vs. cohort 2 CA-controls
- Pooled CA-controls vs. running sub-groups in each cohort
- Pooled CA-controls vs. pooled running sub-groups in each cohort (i.e., all runners within each cohort)

4.7.1 Pooled CA-controls vs. Runners in Each Cohort: Plantar Flexor Tetanic Torque

- **Rationale for pooling CA mice:** there were no differences in absolute or normalized tetanic torque between CA controls in each cohort ($p = 0.68$ and 0.18 , respectively).
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohorts 1 and 2, there were no differences in absolute or normalized tetanic torque between running sub-groups, according to one-way ANOVAs (all $p > 0.05$).
- **Pooled CA-controls vs. all runners within each cohort:** Cohort 1 runners ($n = 13$) did not increase tetanic torque (normalized to BM) relative to pooled CA-controls ($n = 5$)

(599 ± 19 vs. 554 ± 24 N*mm/kg, respectively; $p = 0.22$). Cohort 2, runners ($n = 12$) did increase tetanic torque (normalized to BM) relative to pooled CA-controls ($n = 5$) (662 ± 20 N*mm/kg vs. 554 ± 24 N*mm/kg, respectively; $p = 0.007$) (Figure 38).

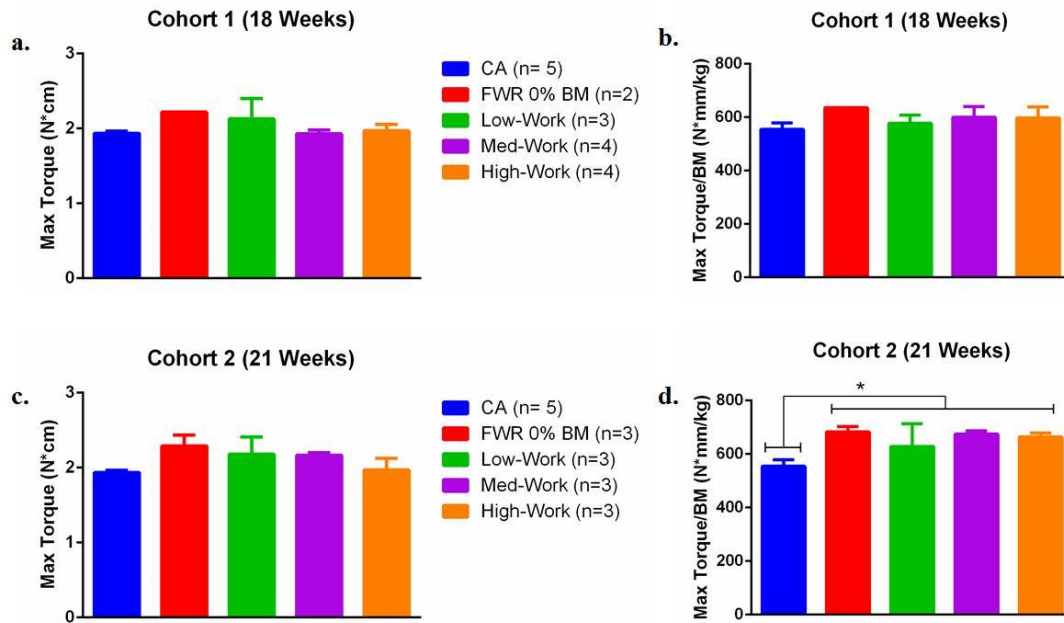


Figure 38. Pooled CA-controls vs. Runners in Each Cohort: Plantar Flexor Tetanic Torque during Dynamic Training.

Absolute and relative (normalized to body mass (BM)) tetanic torque data were pooled for CA-control groups and compared to all runners in each cohort for final PFT measures obtained during dynamic training (cohort 1 at 18 weeks; cohort 2 at 21 weeks). **a)** In cohort 1, running did not affect absolute tetanic torque (in N*cm) ($p > 0.05$). **b)** In cohort 1, running did not affect relative tetanic torque (in N*mm/kg) ($p > 0.05$). **c)** In cohort 2, running did not affect absolute tetanic torque ($p > 0.05$). **d)** However, all runners in cohort 2 ($n = 12$) increased tetanic torque normalized to BM by ~19% relative to pooled CA-controls ($n = 5$) ($p = 0.007$). CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates all runners in cohort 2 > pooled CA-controls ($p < 0.05$).

4.7.2 Pooled CA-controls vs. Runners in Each Cohort: Maximal Running

Capacity

- **Rationale for pooling CA mice:** there were no differences in maximal running capacity between CA-controls in each cohort ($p = 0.557$).
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohorts 1 and 2, there were no differences in maximal running capacity between running sub-groups, according to one-way ANOVAs (both $p > 0.05$).
- **Pooled CA-controls vs. all runners within each cohort:** Cohorts 1 and 2, runners in both cohorts exhibited robust gains in maximal running capacity, compared to pooled CA-controls. Cohort 1, maximal running capacity of runners ($n = 13$) was ~69% higher than pooled CA-controls ($n = 5$) ($1233 \pm 92s$ vs. $728 \pm 130s$, respectively; $p = 0.012$) (Figure 39a). Cohort 2, runners ($n = 12$) exhibited ~115% greater maximal running capacity than pooled CA-controls ($n = 5$) ($1568 \pm 69s$ vs. $728 \pm 130s$, respectively; $p = 0.001$) (Figure 39b).

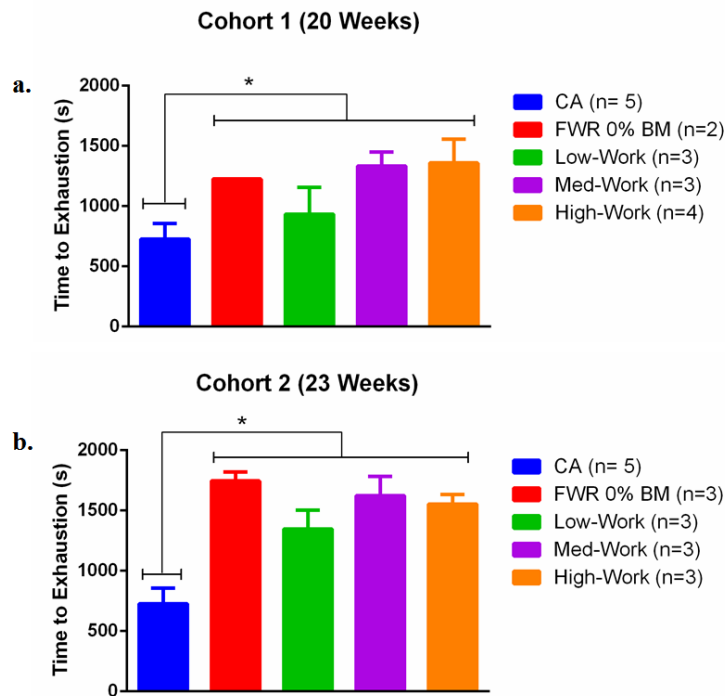


Figure 39. Pooled CA-controls vs. Runners in Each Cohort: Maximal Running Capacity.

Maximal running capacity was assessed one day prior to sacrifice for each cohort by a treadmill assay (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Cohort 1, runners (n= 13) increased their maximal running capacity by ~69% relative to pooled CA-controls (n= 5) (p= 0.012). **b)** Cohort 2, runners (n= 12) increased their maximal running capacity by ~115% relative to pooled CA-controls (n= 5) (p= 0.001). CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates all runners > pooled CA-controls (p < 0.05).

4.7.3 Pooled CA-controls vs. Runners in Each Cohort: Metabolism

4.7.3.1 Fatty Acid Oxidative Capacity and Efficiency in Red-QUAD

- **Rationale for pooling CA mice:** there were no differences in FAO capacity (CO_2 production and ASM production) or FAO efficiency (CO_2 :ASM ratio) of red-QUAD homogenates between CA-control groups each cohort (all p > 0.05).
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohorts 1 and 2, there were no differences in FAO capacity (CO_2 production and ASM production) or FAO

efficiency (CO₂:ASM ratio) of red-QUAD homogenates between running sub-groups, according to one-way ANOVAs (all $p > 0.05$).

- **Pooled CA-controls vs. all runners within each cohort:** Cohort 1, runners ($n= 13$) increased their FAO capacity (CO₂ production) by ~26% compared to pooled CA-controls ($n= 5$) in red-QUAD homogenates ($23.7 \pm 1.2 \mu\text{mol/mg pro/min}$ vs. $18.8 \pm 1.7 \mu\text{mol/mg pro/min}$, respectively; $p= 0.047$). Cohort 2, runners ($n= 12$) did not increase their CO₂ production in red-QUAD homogenates compared to pooled CA-controls ($24.2 \pm 2.2 \mu\text{mol/mg pro/min}$ vs. $18.8 \pm 1.7 \mu\text{mol/mg pro/min}$, respectively; $p= 0.08$) (Figure 40). Cohorts 1 and 2, ASM production (Figure 40) and oxidative efficiency (Figure 41) were unaffected by running in red-QUAD homogenates (all $p > 0.05$).

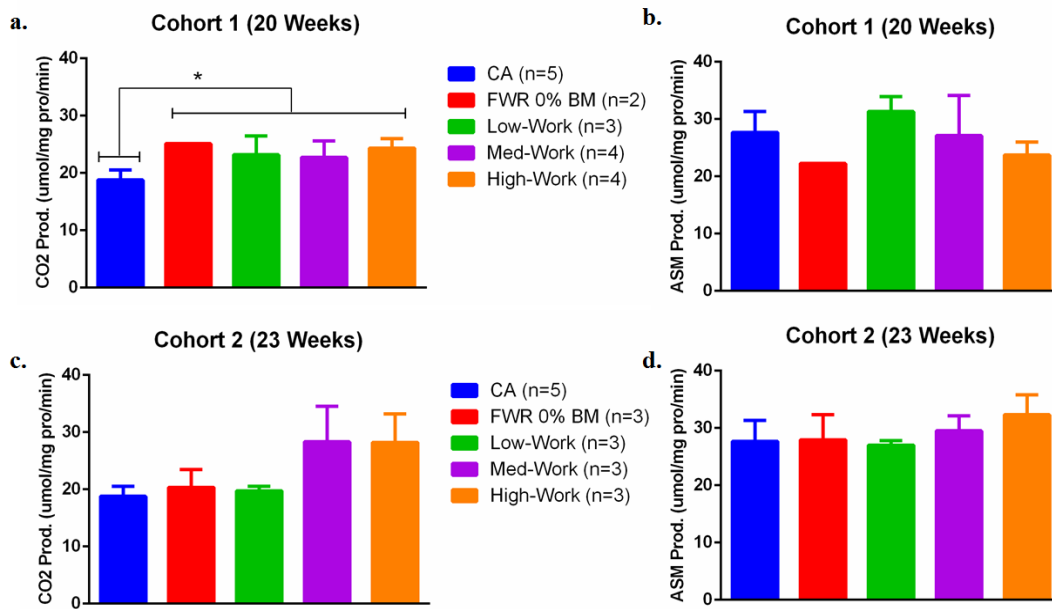


Figure 40. Pooled CA-controls vs. Runners in Each Cohort: Fatty Acid Oxidative Capacity in Red-QUAD

After sacrifice, fatty acid oxidative capacity was assessed when red portions of quadriceps (QUAD) muscle were incubated with radio-labeled palmitate (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Cohort 1, runners (n= 13) increased their oxidative capacity (CO₂ production) by ~26%, compared to pooled CA-controls (n= 5) (p= 0.047). **b)** Cohort 1, runners did not increase ASM production (p > 0.05). **c)** Cohort 2, runners (n= 12) did not increase CO₂ production, compared to pooled CA-controls (p= 0.08). **d)** Cohort 2, runners did not increase ASM production (p > 0.05). ASM= acid soluble metabolites; CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates all runners in cohort 1 > pooled CA-controls (p < 0.05).

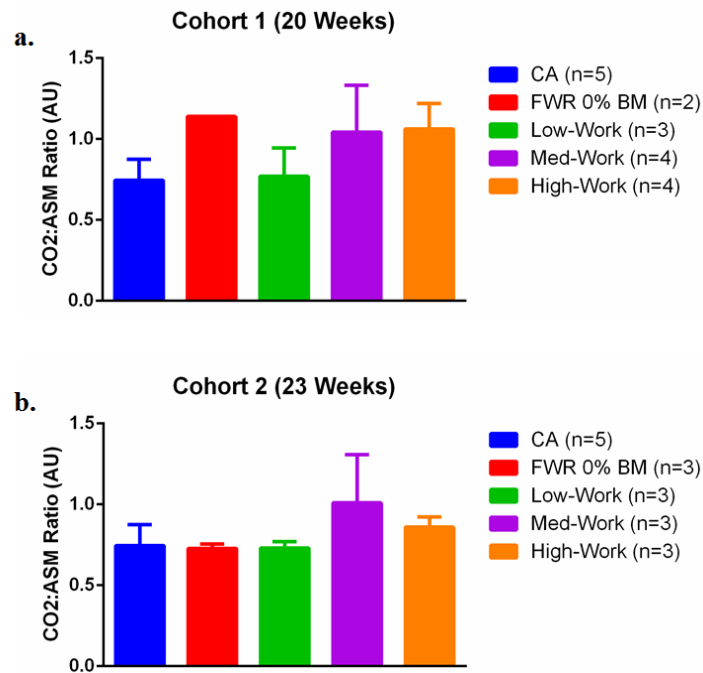


Figure 41. Pooled CA-controls vs. Runners in Each Cohort: Fatty Acid Oxidative Efficiency in Red-QUAD.

After sacrifice, fatty acid oxidative efficiency was assessed by CO₂:ASM ratio when red portions of quadriceps (QUAD) muscle were incubated with radio-labeled palmitate (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Cohort 1, runners (n= 13) did not increase oxidative efficiency in red-QUAD homogenates, compared to pooled CA-controls (n= 5) ($p > 0.05$). **b)** Cohort 2, runners (n= 12) did not increase their oxidative efficiency, relative to pooled CA-controls (n= 5) ($p > 0.05$). ASM= Acid Soluble Metabolites; AU= Arbitrary Units; CA= pooled cage-activity controls; FWR 0% BM= free-wheel runners.

4.7.3.2 Oxidative Enzymes in Red-QUAD

- **Rationale for pooling CA mice:** there were no differences in BHAD or CS activity of red-QUAD homogenates between CA-control groups in each cohort (both $p > 0.05$).
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohort 1, there were no differences in BHAD or CS activity of red-QUAD homogenates between running sub-groups, according to one-way ANOVAs (both $p > 0.05$). Cohort 2, the Med-Work sub-group exhibited greater CS activity than the Low-Work sub-group ($p = 0.006$).

- Pooled CA-controls vs. all runners within each cohort:** Cohorts 1 and 2, runners increased BHAD activity relative to pooled CA-controls (both $p < 0.05$). Cohort 1, runners ($n = 13$) increased CS in red-QUAD homogenates compared to pooled CA-controls ($n = 5$) ($p < 0.001$). Cohort 2, runners ($n = 12$) did not increase CS activity relative to pooled CA-controls ($n = 5$) ($p = 0.157$) (Figure 42).

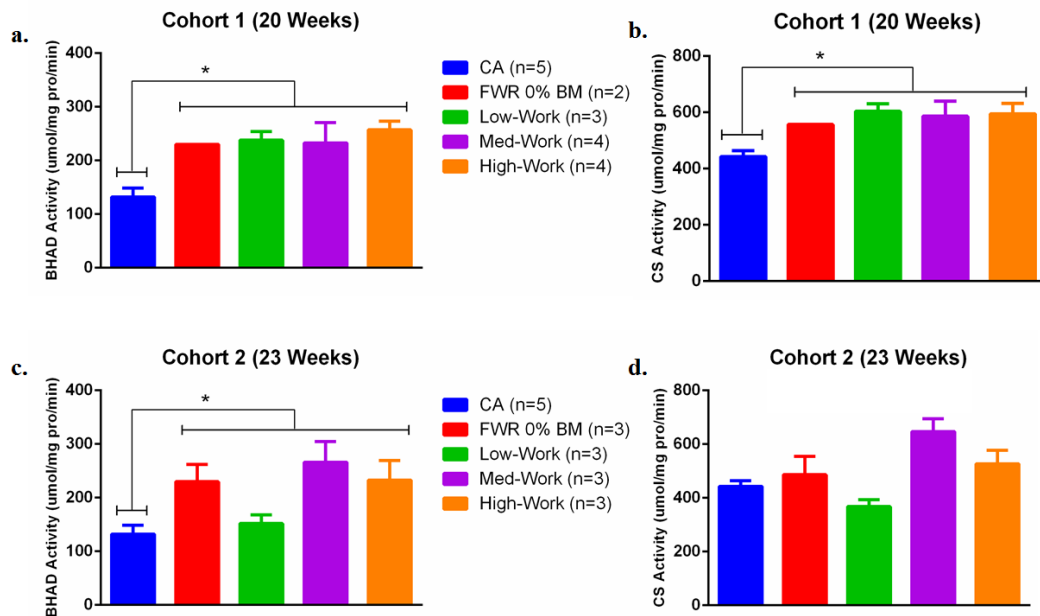


Figure 42. Pooled CA-controls vs. Runners in Each Cohort: Oxidative Enzymes in Red QUAD

After sacrifice, activity of oxidative enzymes (BHAD and CS) was assessed in red portions of quadriceps (QUAD) muscles (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Runners in cohort 1 ($n = 13$) increased BHAD activity by ~83% relative to pooled CA-controls ($n = 5$) ($p < 0.05$). **b)** Runners in cohort 1 also increased CS activity, relative to pooled CA-controls (+33%; $p < 0.05$). **c)** Similarly, runners in cohort 2 ($n = 12$) increased BHAD activity by ~67%, relative to pooled CA-controls ($n = 5$) ($p < 0.05$). **d)** However, runners in cohort 2 did not increase CS activity relative to CA-controls ($p > 0.05$). BHAD= Beta-hydroxyacyl-CoA Dehydrogenase; CS= Citrate Synthase; CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates all runners $>$ pooled CA-controls ($p < 0.05$).

4.7.3.3 Fatty Acid Oxidative Capacity and Efficiency in White-QUAD

- **Rationale for pooling CA mice:** there were no differences in FAO capacity (CO_2 production and ASM production) or FAO efficiency (CO_2 :ASM ratio) of white-QUAD homogenates between CA-control groups each cohort (all $p > 0.05$).
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohorts 1 and 2, there were no differences in FAO capacity (CO_2 production and ASM production) or FAO efficiency (CO_2 :ASM ratio) of white-QUAD homogenates between running sub-groups, according to one-way ANOVAs (all $p > 0.05$).
- **Pooled CA-controls vs. all runners within each cohort:** Cohort 1, white-QUAD homogenates of runners ($n = 13$) did not exhibit differences in CO_2 production, ASM production, or CO_2 :ASM ratio compared to pooled CA-controls ($n = 5$) (all $p > 0.05$). Cohort 2, runners ($n = 12$) exhibited ~108% lower CO_2 production compared to pooled CA-controls ($1.2 \pm 0.2 \mu\text{mol/mg pro/min}$ vs. $2.5 \pm 0.4 \mu\text{mol/mg pro/min}$, respectively; $p = 0.02$) but ASM production was unchanged ($p > 0.05$) (Figure 43). Cohort 2, runners had a lower CO_2 :ASM ratio (decreased ~70%) than pooled CA-controls (0.23 ± 0.04 vs. 0.39 ± 0.02 , respectively; $p = 0.01$) (Figure 44).

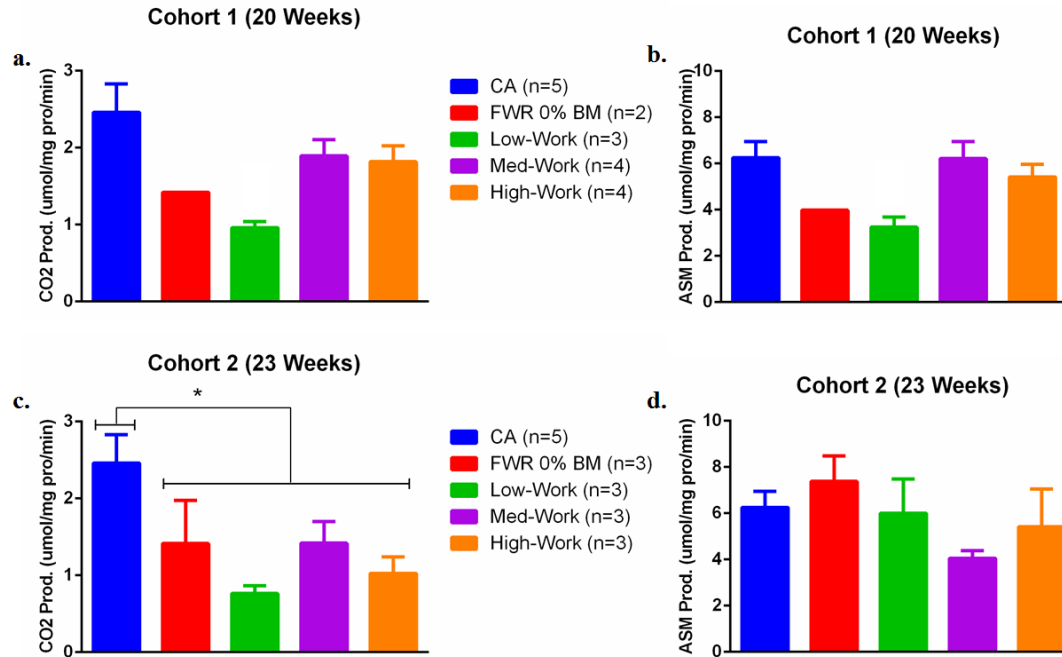


Figure 43. Pooled CA-controls vs. Runners in Each Cohort: Fatty Acid Oxidative Capacity in White-QUAD.

After sacrifice, fatty acid oxidative capacity was assessed when white portions of quadriceps (QUAD) muscle were incubated with radio-labeled palmitate (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Cohort 1, runners (n= 13) did not increase CO₂ production, compared to pooled CA-controls (n= 5) (p > 0.05). **b)** Cohort 1, runners (n= 13) did not increase ASM production (p > 0.05). **c)** Cohort 2, runners (n= 12) exhibited ~108% lower CO₂ production compared to pooled CA-controls (n= 5) (p= 0.02). **d)** Cohort 2, ASM production of runners (n= 12) was not different from pooled CA-controls (n= 5) (p > 0.05). ASM= Acid Soluble Metabolites; CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates all runners in cohort 2 < pooled CA-controls (p < 0.05).

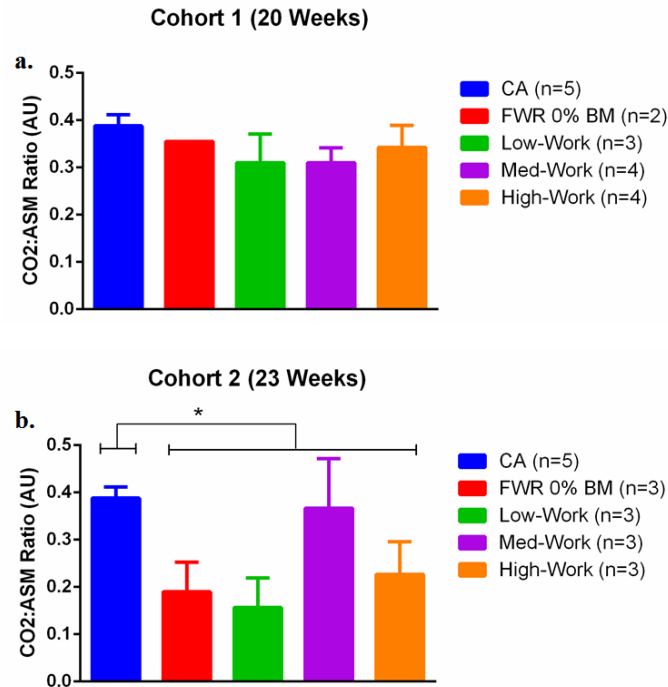


Figure 44. Pooled CA-controls vs. Runners in Each Cohort: Fatty Acid Oxidative Efficiency in White-QUAD.

After sacrifice, fatty acid oxidative efficiency was assessed by CO₂:ASM ratio when white portions of quadriceps (QUAD) were incubated with radio-labeled palmitate (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Cohort 1, oxidative efficiency of runners ($n = 13$) was not different from pooled CA-controls ($n = 5$) ($p > 0.05$). **b)** Cohort 2, runners ($n = 12$) had ~70% lower oxidative efficiency than pooled CA-controls ($n = 5$) ($p = 0.01$). ASM= Acid Soluble Metabolites; AU= Arbitrary Units; CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates pooled CA-controls > all runners in cohort 2 ($p < 0.05$).

4.7.3.4 Oxidative Enzymes in White-QUAD

- **Rationale for pooling CA mice:** there were no differences in BHAD or CS activity of white-QUAD homogenates between CA-control groups in each cohort (both $p > 0.05$).
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohorts 1 and 2, there were no differences in BHAD or CS activity of white-QUAD homogenates between running sub-groups, according to one-way ANOVAs (all $p > 0.05$).
- **Pooled CA-controls vs. all runners within each cohort:** Cohort 1, BHAD and CS activities of white-QUAD homogenates were not different between runners ($n = 13$) and

pooled CA-controls (both $p > 0.05$). Cohort 2, runners ($n= 12$) exhibited ~66% higher BHAD activity than pooled CA-controls ($63 \pm 6\mu\text{mol/mg pro/min}$ vs. $38 \pm 8\mu\text{mol/mg pro/min}$, respectively; $p= 0.045$). Also, runners in cohort 2 exhibited ~29% higher CS activity than pooled CA-controls ($170 \pm 9\mu\text{mol/mg pro/min}$ vs. $132 \pm 12\mu\text{mol/mg pro/min}$, respectively; $p= 0.034$) (Figure 45).

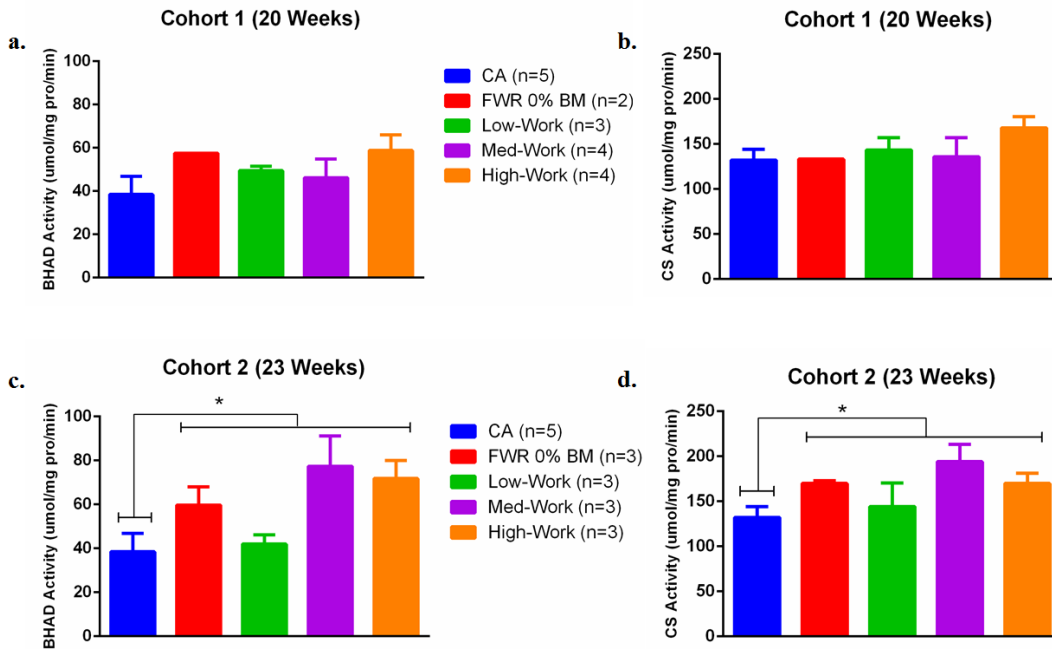


Figure 45. Pooled CA-controls vs. Runners in Each Cohort: Oxidative Enzymes in White-QUAD.

After sacrifice, activity of oxidative enzymes (BHAD and CS) was assessed in white portions of quadriceps (QUAD) muscle (cohort 1 at 20 weeks; cohort 2 at 23 weeks). **a)** Cohort 1, runners ($n= 13$) did not increase BHAD activity compared to pooled CA-controls ($n= 5$) ($p > 0.05$). **b)** Cohort 1, runners ($n= 13$) did not increase CS activity compared to pooled CA-controls ($n= 5$) ($p > 0.05$). **c)** Cohort 2, runners ($n= 12$) increased BHAD activity by ~66%, relative to pooled CA-controls ($n= 5$) ($p= 0.045$). **d)** Cohort 2, runners increased CS activity by ~29%, relative to pooled CA-controls ($p= 0.034$). BHAD= Beta-hydroxyacyl-CoA Dehydrogenase; CS= Citrate Synthase; CA= pooled cage-activity controls; FWR= free-wheel runners; * Indicates all runners $>$ pooled CA-controls ($p < 0.05$).

4.7.4 Pooled CA-controls vs. Runners in Each Cohort: Other Results

- **Rationale for pooling CA mice:** there were no differences between CA-controls in each cohort for the following parameters (all $p > 0.05$): twitch torque properties, twitch and tetanic temporal properties, body mass and composition, muscle masses and CSA, food consumption, glucose oxidative capacity, and centralized nuclei.
- **Pooled CA-controls vs. running sub-groups in each cohort:** Cohorts 1 and 2, there were no differences between running sub-groups for the following parameters (all $p > 0.05$): twitch torque properties, twitch and tetanic temporal properties, body mass and composition, muscle masses and CSA, food consumption, glucose oxidative capacity, and centralized nuclei.
- **Pooled CA-controls vs. all runners within each cohort:** Cohorts 1 and 2, data collected from runners generally were not different from pooled CA-controls ($n=5$) for the following parameters ($p > 0.05$): twitch torque properties, twitch and tetanic temporal properties, body mass and composition, muscle masses and CSA, food consumption, glucose oxidative capacity, and centralized nuclei. However, there were some differences observed between pooled CA-controls and runners: cohort 1, pooled CA-controls ($n=5$) had greater GAS fiber CSA than runners ($n=13$) ($p=0.002$); cohort 2, pooled CA-controls ($n=5$) had shorter twitch TPT than runners ($n=12$) ($p=0.04$); cohort 2, TA muscles of pooled CA-controls ($n=5$) had fewer centralized nuclei than runners ($n=12$) ($p=0.04$).

Chapter 5: Discussion

Section 5.1: Interpretation of Results

5.1.1 Important Findings

This study investigated a prototype RWR system as a model of resistance and endurance exercise training in mice. The primary ideas were to modulate resistance as a function of body mass to increase hindlimb plantar flexor torque, and simultaneously induce endurance training adaptations. The most important findings of this study included: (1) voluntary wheel running varied considerably between mice; (2) relative to CA-control, long-term (i.e., 20-23 weeks) FWR and RWR had no appreciable effects on skeletal muscle mass, CSA, or fiber type; (3) relative to CA-control, wheel running independent of added-resistance ($\pm$ added-resistance) enhanced plantar flexor torque normalized to body mass in cohort 2 but not cohort 1, likely as a function of dynamic training duration; and, (4) wheel running ($\pm$ added-resistance) promoted mixed effects on SkM metabolism. These findings provide important insight into RWR as an exercise model.

5.1.1.1 Variability in Wheel Running

Although the mice in this study were an in-bred strain, considerable variability in running responses between mice was evident. For example, table 12 illustrated that there was high running variability within cohorts (i.e., high standard error) and between cohorts for each stage of training. When cumulative distances were compared between cohorts for the same training duration (i.e., from 0 to 20 weeks), mice in the second cohort ran $\sim 2X$ more (on average) than mice in the first cohort ($435 \pm 54\text{km}$ vs. $221 \pm 25\text{km}$, respectively; $p = 0.001$). These differences occurred despite our attempts to limit running variability with incremental pre-training,

resistance stratification, and with maximum work limits. However, sample sizes were small, and the work limit settings may have been too high, because mice rarely reached their work limits.

An error was discovered with the wheel resistance settings during dynamic training which may have affected wheel work calculations for 5.5 of 7 training weeks for cohort 1, but only 3.5 of 10 training weeks for cohort 2. However, as a counterargument to incorrect wheel loads, RWR mice in both cohorts ran similar distances before and after the stepper motor correction during dynamic training. Resistance runners in cohort 1 ($n = 11$) ran $1340 \pm 319\text{m/day}$ before the correction, and $1131 \pm 230\text{m/day}$ after (before vs. after, $p = 0.60$); resistance runners in cohort 2 ($n = 9$) ran $2276 \pm 491\text{m/day}$ before the correction, and $1468 \pm 374\text{m/day}$ after (before vs. after, $p = 0.21$). These results could suggest that wheel resistances were similar, on average, before and after the correction. After wheel resistances were corrected, mice on the dynamic program (in both cohorts) voluntarily ran against relatively low resistances, ranging from ~ 1.5 - 4.5g (~ 5 - 15% BM). When wheel loads exceeded $\sim 4.5\text{g}$ resistance during exercise bouts, RWR mice from both cohorts stopped running.

RWR mice in the present study ran considerably less (at lower loads) than RWR mice in other studies. For example, Konhilas et al. (2005) reported that male C57BL/6 mice ran ~ 4.0 - 5.0km/day against 4.0 - 7.0g (~ 16 - 25% BM) (153), and similar results were reported by others (186). Even in a study by Call et al. (2010), which used *mdx* (dystrophic) mice, the RWR group ran $\sim 2.0\text{km/day}$ against their highest resistance (7.0g ; $\sim 23\%$ BM) (11). In contrast, Soffe et al. (2015) showed that after progressive RWR training, one group of male C57BL/6J mice ran $1.61 \pm 0.32\text{km/day}$ at 4.0g and another group ran $0.87 \pm 0.52\text{km/day}$ against 6.0g ($\sim 19\%$ BM) (185). In each of these studies, there was high variability reported in the running data (i.e., high standard error). Together, these results and our results highlight a challenge of studying

voluntary exercise in animals: intrinsic motivation for physical activity can vary greatly, even between inbred strains. It is possible that, even when animals are randomized (or selected based on screening), some will not exercise enough to achieve SkM overload.

5.1.1.2 Effects on Muscle Phenotype and Function

To account for variability in resistance running performance among mice and between cohorts during dynamic training, resistance runners were grouped by work tertiles (i.e., Low-, Med- and High-Work) for subsequent analyses. Nevertheless, there were still no appreciable effects of wheel running ($\pm$ added-resistance) on SkM phenotype (i.e., mass, CSA, fiber type) and there were mixed effects on muscle function. These results were surprising considering that during dynamic training, there were stark differences in wheel work patterns between running sub-groups in each cohort. Figure 28 illustrated that after the stepper-motor correction, the Med-Work and High-Work sub-groups in both cohorts performed more work than their respective Low-Work and FWR 0% BM counterparts (all $p < 0.05$). Also, in cohort 1, the High-Work sub-group performed more work than Med-Work ($p < 0.05$), but in cohort 2, the difference between High-Work and Med-Work was non-significant ($p = 0.073$). One explanation for the absence of differences in muscle phenotype characteristics is that sub-group sample sizes were small for each cohort ($n = 2-4$), so comparisons were underpowered.

Some researchers have reported that RWR increased size of prime running muscles (e.g., SOL, PLN, GAS, Tri) (10, 185, 186), while others have shown mixed results (11-13, 153, 182). Ishihara et al. (1998) showed that RWR (but not FWR) increased PLN muscle mass and size of SO, FOG, and FG fibers in rats, but had no effect on PLN fiber type distribution (10). In a follow-up study of RWR-only vs. controls (in mice), Ishihara et al. (2002) reported that RWR promoted small gains in fiber size and SDH activity of TA fibers (IIA, IIX, and IIB) with no

change in TA mass (13). Similarly, Konhilas et al. (2005) showed that RWR (but not FWR) promoted SOL muscle mass gains, along with increased type IIA, IID/X, and IIB fiber sizes in whole-calf muscle cross-sections (153). To summarize, RWR has been shown to promote muscle hypertrophy, compared to both sedentary controls and FWR, which suggests that RWR can promote different adaptations than FWR. However, in the present study, wheel running ($\pm$ added-resistance) did not affect muscle mass, fiber type, or CSA.

Although wheel running had no effects on muscle phenotypic traits, we observed some effects of wheel running on muscle function. In cohort 1, there were no effects of wheel running on tetanic PFT. However, in cohort 2, all runners ($n=12$) exhibited greater tetanic PFT than pooled CA-controls during their final PFT measures ($+19\%$ when normalized to BM; $p=0.007$), but added-resistance did not enhance the effect.

Of note, final tetanic PFT was different between cohorts: with 3 additional weeks of training, mice in cohort 2 ($n=12$ runners; $n=2$ CA-controls; $652 \pm 18 \text{ N}\cdot\text{mm/kg}$) exhibited greater tetanic PFT than mice in cohort 1 ($n=13$ runners; $n=3$ CA-controls; $585 \pm 18 \text{ N}\cdot\text{mm/kg}$) ($+11\%$ when normalized to BM; $p=0.02$). Further, runners in cohort 2 ($662 \pm 20 \text{ N}\cdot\text{mm/kg}$) exhibited 11% greater PFT than runners in cohort 1 ($600 \pm 20 \text{ N}\cdot\text{mm/kg}$; $p=0.035$), but there were no differences in PFT between CA-control groups from each cohort ($p=0.16$). Because mice in cohort 1 ran considerably less than mice in cohort 2 (on average and overall), these results suggest that runners in cohort 1 ($\pm$ added-resistance) did not achieve SkM overload during training, whereas runners in cohort 2 did. Although there were no strength differences between work tertiles in cohort 2, the sample sizes were small, and wheel resistances were relatively low (only up to $\sim 15\%$ BM at the end of the study), so the distinction between FWR and RWR conditions may have been too small to detect/promote divergent functional adaptations.

Only one other study has investigated if RWR promotes functional adaptations in mouse SkM. Call et al. (2010) showed that, following 12 weeks of wheel running (added-resistance up to ~20% BM), *mdx* mice exhibited ~26% greater SOL tetanic strength than sedentary *mdx* controls. However, when SOL tetanic strength was compared between FWR and RWR groups, no differences were observed (11). The findings of Call et al. suggest that wheel running ($\pm$ added-resistance) can promote functional adaptations similar to resistance training (i.e., increased muscle strength compared to sedentary controls), but wheel loads may need to exceed 20% BM to distinguish RWR from FWR (i.e., to facilitate more resistance-like training than endurance-like training). Although in the present study, mice were incrementally pre-trained to reach higher resistances, only a portion of the mice in this study ($n=7/30$) were able to tolerate higher wheel loads (up to ~25% BM). This %BM may represent a threshold for mice, above which they will not run. However, others have shown that mice can run against ~41% BM resistance (153), or even up to ~88% BM (186).

5.1.1.3 Effects on Muscle Metabolism

A novel aspect of the present study is that it assessed the effects of RWR on both SkM strength and markers of endurance training adaptation. By the end of this study, runners in cohorts 1 ($n=13$) and 2 ($n=12$) clearly had greater maximal running capacity than pooled CA-controls ($n=5$) (+69% and +115%, respectively; both $p < 0.05$), which indicates that running groups ($\pm$ added-resistance) were better-conditioned than CA-controls. We also made two important observations regarding metabolic adaptations to wheel running: (1) added-resistance did not inhibit metabolic adaptations (i.e., resistance runners were not different from free-wheel runners); (2) generally, muscle metabolism results (i.e., oxidative enzymes and oxidative

capacity) were consistent in red-QUAD homogenates, but results were less clear in white-QUAD homogenates.

Runners in cohort 1 (n= 13) increased BHAD activity (+83%; $p < 0.001$) and CS activity (+33%; $p < 0.001$) in red-QUAD, relative to pooled CA-controls (n= 5). Runners in cohort 2 (n= 12) only increased BHAD activity, relative to pooled CA-controls (BHAD +67%; $p < 0.05$).

When red-QUAD muscle homogenates were incubated with radio-labeled palmitate, runners in cohort 1 increased oxidative capacity (i.e., production of CO_2) by 26%, compared to pooled CA-controls ($p = 0.047$), but runners in cohort 2 did not significantly increase oxidative capacity, relative to pooled CA-controls (+29%; $p = 0.08$). It's important to note that there were no differences in oxidative capacity between running sub-groups in either cohort (all $p > 0.05$), which suggests that added-resistance did not inhibit metabolic adaptations to wheel running.

Results of white-QUAD homogenates were less clear. In cohort 1, white-QUAD metabolism results (i.e., oxidative enzymes and oxidative capacity) of runners (n= 13) were not different from pooled CA-controls (n= 5) (all $p > 0.05$). However, in cohort 2, the oxidative enzyme data appeared to contradict the oxidative capacity data. BHAD and CS activities were higher in cohort 2's runners (n= 12) compared to pooled CA-controls (+66% and +29%, respectively; both $p < 0.05$). In contrast, runners in cohort 2 exhibited ~108% less oxidative capacity than pooled CA-controls ($p = 0.02$), and oxidative efficiency of runners was lower than pooled CA-controls ($p < 0.05$).

BHAD and CS results in this study were similar to those reported by others: slower, more oxidative muscles clearly adapted to wheel running by increasing oxidative enzymes, while faster, more glycolytic muscles adapted less clearly (156, 165). For example, Davidson et al. (2006) showed that FWR increased CS activity in SOL, red-GAS, and PLN muscles, but not in

white-GAS (156). Yet, their results also showed that FWR increased SDH activity in SOL, red-GAS, PLN, and white-GAS. Interestingly, Davidson et al. reported that FWR increased PFK activity (marker of glycolytic capacity) only in white-GAS muscle; together, these results suggest that FWR promoted clear metabolic training adaptations in red muscles, but mixed results in white muscles (156). However, Davidson et al. did not assay FAO or GO capacity directly (i.e., with fatty acid or glucose). In the present study, wheel running increased oxidative enzymes and FAO capacity (but not GO capacity) in red-QUAD; however, in white-QUAD, wheel running seemed to promote contradictory results (i.e., increased oxidative enzymes and decreased FAO capacity). However, sample sizes were small in the present study, so statistical power was limited, as well as the generalizability of the results.

It is also possible that some effects of training were not observed relative to CA-controls, because mice in the CA groups underwent 8 weeks of pre-training. However, Olenich et al. (2014) showed that, within 4 weeks of detraining, male C57BL/6 mice no longer demonstrated SkM training effects compared to untrained controls (i.e., angiogenic markers, capillarity of running muscles, and maximal running speed) (195). Similar effects have been observed with two-weeks of detraining (i.e., decreased capillarity, oxidative enzymes, and maximal running capacity) (196). In the present study, CA-control mice detrained for much longer (12-15 weeks), so it is reasonable to expect that the training adaptations CA mice may have accrued during pre-training were no longer present at sacrifice.

5.1.1.4 Characterizing Resistance Wheel Running as an Exercise Model

This was the first study to show that RWR can simultaneously promote muscle strength gains and endurance training adaptations in mice. However, the effects of RWR were not clearly distinguishable from the effects of FWR; this may be due to the relatively low wheel resistances

tolerated by mice. As noted above, other studies have shown that RWR can promote greater SkM growth than FWR, but maximal wheel resistances were higher in those studies than the present study (~41-73% BM vs. ~25% BM) (10, 153). Suijo et al. (2013) showed that when mice ran against no load or very high load (up to ~88% BM), only RWR (not FWR) increased phosphorylation of mTORC1 and p70^{S6K}, relative to sedentary controls, indicating that RWR at high load promotes hypertrophic signaling (186).

If RWR can promote endurance training adaptations (present study and Ishihara et al. (2002)), strength gains (present study and Call et al. (2010)), muscle mass gains (Konhilas et al. (2005), Ishihara et al. (1998)), and hypertrophic signaling (Suijo et al. (2013)), then RWR may be useful for future studies that seek to elicit both resistance and endurance adaptations. Resistance and endurance adaptations have been reported for concurrent training in humans (123-126). In concurrent training, individuals perform both resistance exercises and endurance exercises in separate work bouts separated by a short period of time (typically on the same day) (129). At present there isn't a comparable model of concurrent training in mice, which raises the question, is RWR training in mice an example of concurrent training in humans? Because RWR does not clearly fit the definition of concurrent training, it may be better to consider RWR as a model of simultaneous training (i.e., simultaneous endurance and resistance activity), rather than concurrent training. Because adaptations in skeletal muscles occur at the molecular level, it is intriguing to consider the interactions in the adaptive resistance and endurance signaling pathways during simultaneous training, which are thought to conflict.

The results from the present study suggest that wheel loads $\leq 25\%$ BM promoted more endurance-like training adaptations than resistance-like training adaptations. This could be explained by adaptive signaling interference: activation of endurance training adaptive mediators

(e.g., AMPK, CaMKII, HIF, SIRT1) may have inhibited mediators of resistance training adaptations (e.g., Akt, mTOR) (Figure 46). AMPK signaling is preferentially activated by endurance training, whereas mTOR is preferentially activated by resistance training (129, 197). Activation of AMPK has been shown to inhibit mTOR activation (and protein synthesis) by phosphorylating mTORC1 regulators, such as tuberous sclerosis complex 2 (TSC2) and raptor (104, 129, 197, 198). Additionally, AMPK activation enhances SkM proteolytic pathways via activation of FOXOs (107, 129, 132, 134), which may prevent muscle hypertrophy under loading conditions. In contrast, resistance training has been shown to activate mTOR (and protein synthesis) by inhibiting TSC2 (via Akt and ERK1/2 (137)) (Figure 46). Since wheel loads were relatively low in the present study, it's possible that added-resistance did not activate muscles to the degree needed to shift the signaling balance from primarily endurance (AMPK) to primarily resistance (mTOR) activation; hence there were no differences in muscle mass or CSA, but there were some improvements in muscle function and aerobic exercise capacity, and added resistance did not modulate SkM adaptations.

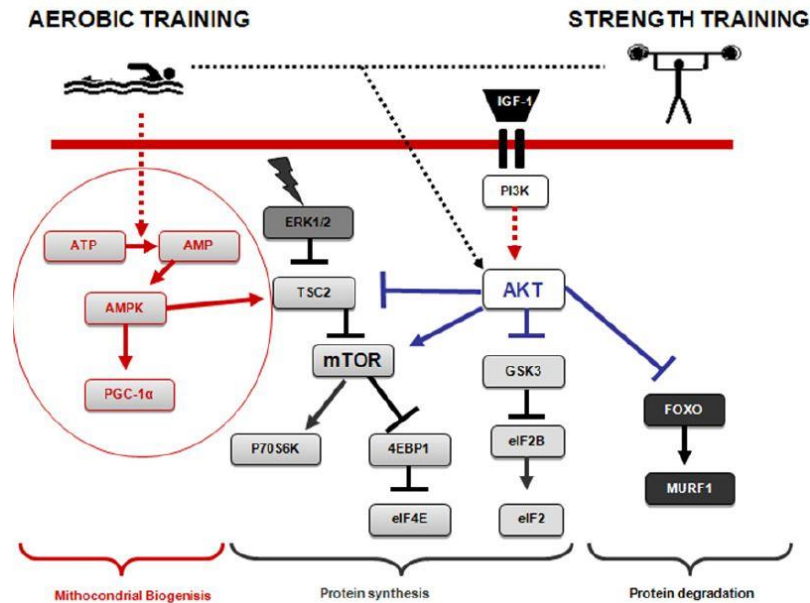


Figure 46. Molecular Model of Adaptive Signaling Interference.

The signaling pathways above are the outcome of mouse and rat training studies, but similar pathways are thought to operate in humans. Results from the present study primarily showed that RWR promoted endurance training adaptations (instead of resistance training adaptations), which could suggest that the adaptive signaling that mediates resistance training was inhibited by endurance training signals, similar to concurrent training interference. Potential mechanisms include AMPK-mediated inhibition of mTOR signaling (and protein synthesis) and the upregulation of protein catabolic pathways by AMPK (e.g., the ubiquitin-proteasome system via FOXOs). Interference signaling from aerobic training mediators may have promoted endurance training adaptations (e.g., mitochondrial biogenesis via PGC-1 α) and prevented fiber hypertrophy. This graphic was adapted from original work by Tiago Fernandes, Úrsula P.R. Soci, Stéphan F.S. Melo, et al. (2012). Signaling Pathways that Mediate Skeletal Muscle Hypertrophy: Effects of Exercise Training, Skeletal Muscle - From Myogenesis to Clinical Relations, Dr. Julianna Cseri (Ed.), ISBN: 978-953-51-0712-5, InTech, DOI: 10.5772/51087. Available from: <http://www.intechopen.com/books/skeletal-muscle-from-myogenesis-to-clinical-relations/signaling->

5.1.2 Conclusions

This study primarily served as a pilot for a new RWR system. During the study, several problems were overcome and a new dynamic RWR training program was developed, which may be useful for future studies. Additionally, important observations were made about variability in wheel running among inbred mice, and the results suggest that wheel running ($\pm$ added-

resistance) may promote both SkM endurance and strength adaptations; however, it is still unclear whether RWR promotes characteristically different SkM adaptations than FWR. Data from this study suggest that RWR (as employed in this study) may be a model of simultaneous training that more closely represents endurance training than resistance training. Future studies of RWR should seek to maximize wheel resistance (ideally above 25% BM) to create a clear distinction between RWR and FWR training regimens.

Section 5.2: Hypotheses Conclusions

1. **Hypothesis:** Relative to cage-activity controls and free-wheel runners, RWR mice will develop stronger plantar flexor muscles.
 - **Conclusion:** Reject- in cohort 2, runners ($\pm$ added-resistance) developed stronger plantar flexor muscles than pooled CA-controls (+19% tetanic PFT; $p = 0.007$); added resistance did not enhance strength gains. Additionally, runners in cohort 1 did not significantly increase PFT, relative to pooled CA-controls ($p > 0.05$).
2. **Hypothesis:** Relative to cage-activity controls, RWR and free-wheel runners will similarly increase whole-body lean mass and decrease fat mass over time.
 - **Conclusion:** Reject- Running ($\pm$ added-resistance) had no effect on lean mass or fat mass over time (all $p > 0.05$).

3. **Hypothesis:** Relative to cage-activity controls, RWR and free-wheel running will similarly increase treadmill running fitness to exhaustion, but RWR will exhibit better hanging wire endurance than CA-controls and FWR.
- **Conclusion:** Partially accept- In both cohorts, RWR and FWR similarly improved treadmill performance compared to pooled CA-controls ($p < 0.05$). Hanging wire performance was not improved by running in cohort 1 ($p > 0.05$). However, in cohort 2, the High-Work sub-group improved hanging wire test performance compared to pooled CA-controls, FWR and Low-Work (all $p < 0.05$), but not compared to Med-Work ($p > 0.05$).
4. **Hypothesis:** Relative to cage-activity controls, RWR and free-wheel runners will have lower plasma CK activity at sacrifice.
- **Conclusion:** Accept- runners ($\pm$ added-resistance) had markedly lower plasma CK than pooled CA-controls ($\sim 54\%$ lower; $p < 0.001$).
5. **Hypothesis:** Relative to cage-activity controls and free-wheel runners, RWR will increase mass and cross-sectional area of gastrocnemius, soleus, tibialis anterior, and triceps muscles.
- **Conclusion:** Reject- There were no differences in absolute or relative muscle masses between running sub-groups and pooled CA-controls in both cohorts (all $p > 0.05$). There were also no differences in muscle cross-sectional area between running groups and pooled CA-controls, except in GAS muscles of cohort 2,

which were smaller in runners (n= 12) compared to pooled CA-controls (p= 0.005).

6. **Hypothesis:** Relative to cage-activity controls, free-wheel running and RWR will similarly increase glucose and fatty acid oxidative capacities, as well as beta-hydroxyacyl CoA dehydrogenase and citrate synthase activities in quadriceps muscles.

- **Conclusion:** Partially Accept- In cohort 1, runners ($\pm$ added-resistance) increased FAO capacity by 26% in red-QUAD homogenates relative to pooled CA-controls (p= 0.047), but runners in cohort 2 did not significantly increase FAO capacity (p= 0.08). Runners in both cohorts increased BHAD activity relative to pooled CA-controls (both p < 0.05), but only runners in cohort 1 increased CS activity (p < 0.001). Mixed effects were observed in white-QUAD homogenates. In both cohorts, running ($\pm$ added-resistance) had no effect on GO capacity (both p > 0.05).

7. **Hypothesis:** Relative to cage-activity controls, free-wheel running and RWR will similarly decrease central nucleation in muscle fibers.

- **Conclusion:** Reject- Wheel running ($\pm$ added-resistance) did not reduce central nucleation of muscle fibers. However, central nucleation was higher in SOL muscles than in other muscles (GAS and TA; both p < 0.05).

8. **Hypothesis:** Relative to cage-activity controls, free-wheel running and RWR will increase the differentiative and proliferative capacities of satellite cells in skeletal muscles.
- **Conclusion:** Reject- There were no differences in satellite cell differentiative or proliferative capacity between pooled running sub-groups and pooled CA-controls (all $p > 0.05$). However, satellite cells were not obtained from running muscles.
9. **Hypothesis:** RWR will increase type IIA-positive fibers relative to CA-controls and free-wheel runners, but free-wheel runners will express more type I-positive fibers (in gastrocnemius, soleus, tibialis anterior, and triceps muscles).
- **Conclusion:** Reject- A subset of samples was analyzed for myosin heavy chain expression. Wheel running ($\pm$ added-resistance) had no effect on type I or type IIA MHC expression, compared to pooled CA-controls ($n = 4$) (all $p > 0.05$).
10. **Hypothesis:** RWR will promote SkM adaptations similar to both endurance and resistance training, whereas FWR will only promote endurance training adaptations.
- **Conclusion:** Reject- wheel running ($\pm$ added-resistance) improved both aerobic fitness (both cohorts) and plantar flexor strength (cohort 2), compared to pooled CA-controls (all $p < 0.05$), but the effects of RWR were not clearly distinguishable from those of FWR (i.e., no differences were observed between resistance runners and free-wheel runners).

Section 5.3: Future Directions

Although there were interesting adaptive responses to wheel running in this study, additional assays could be conducted on the tissues that were harvested. For example, hypothalami were collected; to further investigate the wheel running variability that we observed, these tissues could be analyzed for mRNA and protein expression of biomarkers associated with intrinsic motivation for physical activity (e.g., dopaminergic receptors (199, 200)).

More important, although FWR and RWR promoted robust changes in maximal aerobic exercise capacity, there were inconsistent metabolic adaptations (i.e., mitochondrial enzyme data did not clearly match up with the FAO data); so it would be beneficial to confirm an aerobic training effect in harvested muscles. This could be achieved by determining protein content of mitochondrial enzymes (e.g., COXIV, CPT1, etc.) and transcription factors that mediate aerobic adaptations (e.g., NRFs, MEF2, PGC-1 α , etc.). Additionally, although there were no changes in muscle mass, wheel running promoted changes in muscle strength so it may be beneficial to determine myofibrillar protein content in muscle samples.

If these assays demonstrate aerobic training effects and increased myofibrillar content, then it may be worthwhile to probe endurance training and resistance training adaptive signaling (e.g., phosphorylation status of targets in the AMPK and mTORC1 signaling pathways, respectively), as well as regulators of protein turnover (e.g., activation of calpains and ubiquitin proteases). Investigating the interaction between these adaptive signaling pathways could determine whether our RWR model promoted interference signaling seen in concurrent training. However, the activities of these adaptive signaling pathways may have been altered by fasting and the treadmill fitness assays prior to sacrifice (within 24 hours) (201); so it may be more

prudent to perform a follow-up study for the purpose of probing adaptive signaling, which would allow more time for maximal exercise recovery (2-3 days instead of 18-20 hours), and would shorten fasting and wheel inactivity times before sacrifice (<5 hours instead of 14-15 hours (201)).

A surprising result of this study was that wheel running ($\pm$ added-resistance) did not significantly affect animal body masses, even though the running groups did not eat more than CA-controls. These results could be investigated by comparing energy intake (food consumption) to energy expenditure (wheel work). These results could also be investigated by watching in-cage video footage that was obtained from several runners and CA-control mice during this study. Videos could, at least qualitatively, reveal if wheel running affected foraging behavior, non-wheel play, and sedentary time. Video data could also help to determine if added-resistance affected running wheel behaviors (e.g., running gait, running speed, and natural positioning on the wheel).

The main limitations of this study were the unforeseen issues with wheel resistances, study-design changes, and the lack of truly sedentary controls. While wheel resistance problems were overcome with a small fix, a better-designed/tested RWR system may be needed to avoid problems in future studies. One improvement to the current system design could be a built-in resistance feedback mechanism. This kind of upgrade could be realized by fixing a small, very sensitive strain gage to the running wheel actuator arm (e.g., model MMF325246; Micro-Measurements, Inc.); the strain gage could sense small deviations in force applied to the axle and trigger the computer to adjust resistance in real-time. It would be prudent to pilot the new system first in a small group of mice to troubleshoot problems and to optimize training regimens, before employing it in a larger-scale study. This system would be ideal for both static resistance

RWR and dynamic resistance RWR. One final note, regardless of the RWR training regimen used, a randomized sedentary control group should be used for comparisons (instead of, or in addition to, a detrained CA-control group).

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Appendices

Appendix A: Power Analysis

Prior to the study, we performed a power analysis using JMP10 software (SAS Institute, Inc.) to determine requisite sample sizes for 80% power ($\beta = 0.80$). JMP software determines study power for different sample sizes based on typical data distributions, usually derived from preliminary data. We used plantar flexor torque data (normalized to BM) from a preliminary/pilot study for this analysis (Figure 47).

The screenshot shows the 'Sample Size' dialog box in JMP10 software. The 'k Means' section is active, indicating a test for differences among k means. The 'Alpha' is set to 0.05, 'Std Dev' is 90.14306, and 'Extra Parameters' is 0. A list of 10 prospective means is provided: 680.4, 637.1, 596.7, 378.1, 462.9, 615.7, 484.7, 497.7, 687.2, and 598.7. The 'Sample Size' is set to 24, and the 'Power' is 0.8. A note at the bottom states: 'Sample Size is the total sample size; per group would be n/k'.

Sample Size	
k Means	
Testing if there are differences among k means.	
Alpha	0.05
Std Dev	90.14306
Extra Parameters	0
Enter up to 10 Prospective Means showing separation across groups	
	680.4
	637.1
	596.7
	378.1
	462.9
	615.7
	484.7
	497.7
	687.2
	598.7
Enter Power or Sample Size to get the other.	
Enter neither to get a plot of Power vs. Sample Size	
Sample Size	24
Power	0.8
Sample Size is the total sample size; per group would be n/k	

Figure 47. Power Analysis Performed Prior to the Study.

Prior to the study, we only expected to have 4 groups (k), and the power analysis indicated that based on a typical distribution of PFT data (tetanus normalized to BM; data from preliminary work), we would need a total sample size of at least 24 (i.e., n= 6 animals per group) to achieve 80% power ($\beta = 0.80$). Screenshot taken while using JMP10 software (SAS Institute, Inc.).

Appendix B: Plantar Flexor Torque Supplemental Information

B.1 Knee Flexion Angles

Plantar flexor torque assays were performed to determine muscle function throughout this study. When we performed plantar flexor torque assays, we sought to position the knee as close to 90° (flexion) as possible to be consistent with methods reported by others (166). However, the size of the U-bracket on the 809B *in-situ* apparatus (Aurora Scientific) prevented the knee from reaching 90°; when knee angles were measured, we could only get the knee to ~110-130° (flexion).

Mice were shaved and prepared for PFT measures as described in section 3.B.1; then the location of the hip was palpated for and marked with a pen. After the mouse was positioned in the apparatus, we measured the horizontal distance from the hip to the knee (~5-7mm) and the vertical distance from the hip to the knee (~12-14mm). The tibia was parallel to the platform, so if we assumed that the femur was a straight bone, then the U-bracket provided a right angle from which the knee flexion angle could be determined trigonometrically (based on the horizontal and vertical distances between the hip and the knee).

B.2 Current Optimization

Muscle torque that is produced during our *in-vivo* PFT protocols partly depends on the current (amps) delivered during stimulations. Current optimization was not performed on mice the present study, but was performed in a previous study (Doering Masters Thesis, 2013, Virginia Tech, unpublished), which served as the basis for the procedures used in the present study. To determine the optimum current with which to stimulate the plantar flexors, twitch (1Hz

stimulation) and tetanic (150Hz stimulation) PFT properties were tested over a range of currents with C57BL10/SnJ and *mdx* mice (age ~4 weeks). Currents ranged from 10-50mA in 5mA increments (1 minute rest between stimulations). The optimization process revealed that, for both twitches and tetany, torque responses plateaued at 35mA.

B.3 Problems with Work Loop Analyses

As described in section 3.B.1, plantar flexor contractile properties were obtained while the ankle was rhythmically moved in a sinusoidal arc about the neutral position by the stepper motor (± 0.0743 cm amplitude; two consecutive sine waves completed; 0.143s per cycle). Protocols were based on work previously described (187). During the first sine wave, the plantar flexors produced passive torque. At the start of the second sine wave, the plantar flexors were stimulated (1, 20, or 40Hz; ~30 seconds between each). Torque and displacement data from the sine wave stimulations were analyzed for passive and stimulated work-loops. Passive work-loops were subtracted from active work-loops to account for passive torque. The 1Hz stimulations worked properly and were analyzed, but there were no differences between groups over time (all $p > 0.05$; data not shown). However, contractions (i.e., both muscle activation & relaxation) elicited by the 20Hz and 40Hz stimulations were not fully completed within the time window of the second sine-wave, so these files could not be analyzed in DMA (Aurora Scientific) (Figure 48). This issue was not discovered until after the study when we began analyses.

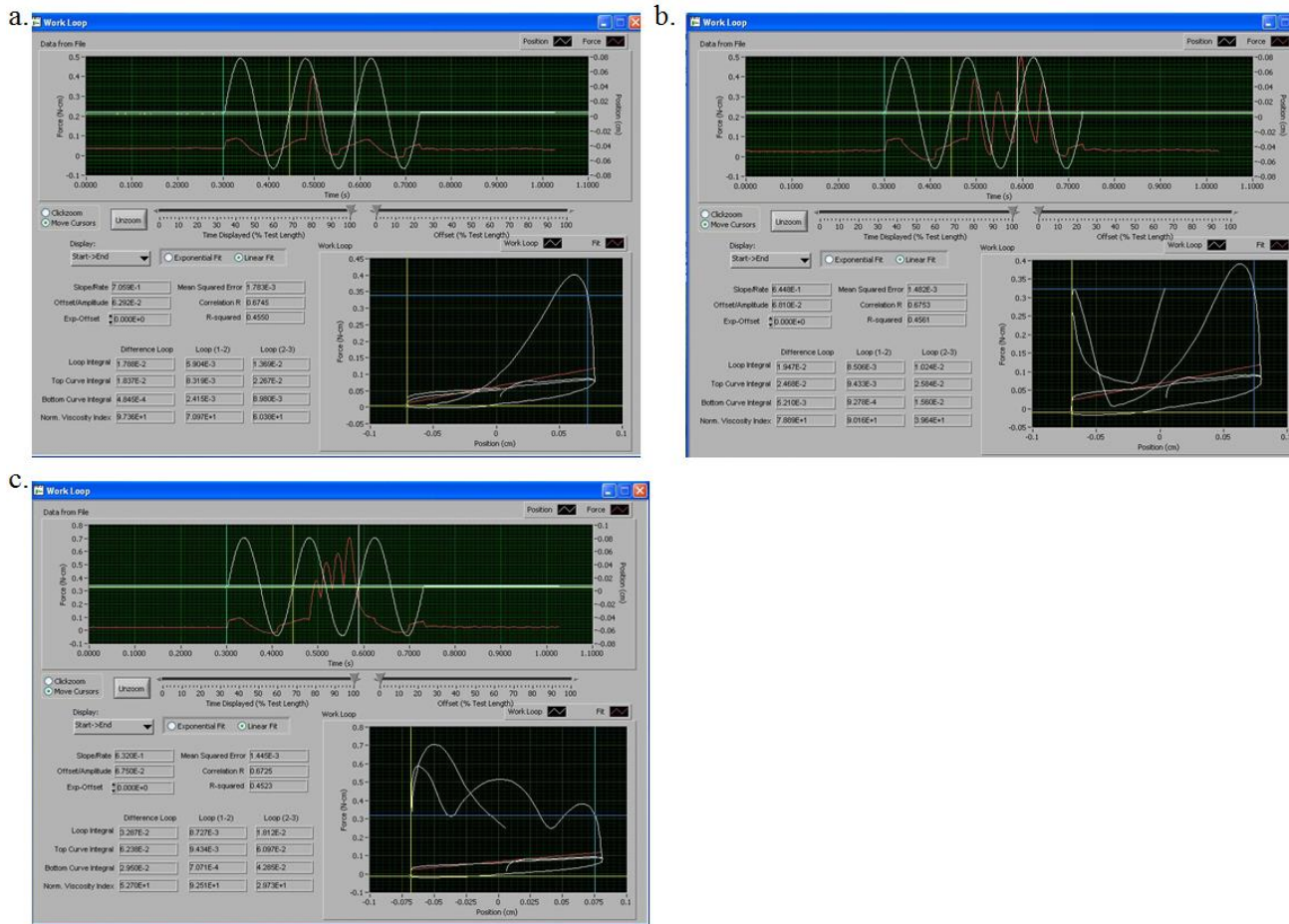


Figure 48. Problems with Work Loop Analyses.

In DMA, the work loop applet analyzes up to two work loops and can determine the difference between work loops (i.e., difference between passive and active work loops). The problem we encountered can be seen in the top graph shown in **panels a, b, and c**; these graphs plot force (torque) produced by plantar flexors (N*cm; in red) and foot position (cm; in white) over time (s). Each sine wave that the foot underwent is visually divided into sections by cross-hair cursors (in light blue, yellow, and white). **a)** Contraction was completed before the ankle finished its second sine wave when the plantar flexors were stimulated at 1Hz. **b)** However, contractions were not completed before the end of the second sine wave when the plantar flexors were stimulated at 20Hz and **c)** 40Hz. Screenshots taken while using DMA 5.211 software (Aurora Scientific).

Appendix C: Wheel Resistance Calibration

To recalibrate the wheels, wheel resistances were decreased to 0g by the controller program (VT Metabolic Phenotyping Core), and then the wheel was removed from the axle and replaced by a light metal bar (Figure 49a). Next, a 2g mass was hung from the light metal bar 5.75cm from the running wheel axle (same radius as the running wheel) and resistance was manually increased up to 2g (i.e., threaded rod was turned until the 2g mass no longer exceeded the axle's inertia (Figure 49b)). Once wheel resistance was in balance with the 2g mass, the threaded rod was loosened by 3 full turns. The control software was programmed to set resistance based on this calibration (i.e., when the program started, if we programmed the resistance setting to 2g, then the threaded rod would be tightened by 3 full turns). Linearity between the stepper-motor/threaded-rod turns and wheel resistances was established before the study.

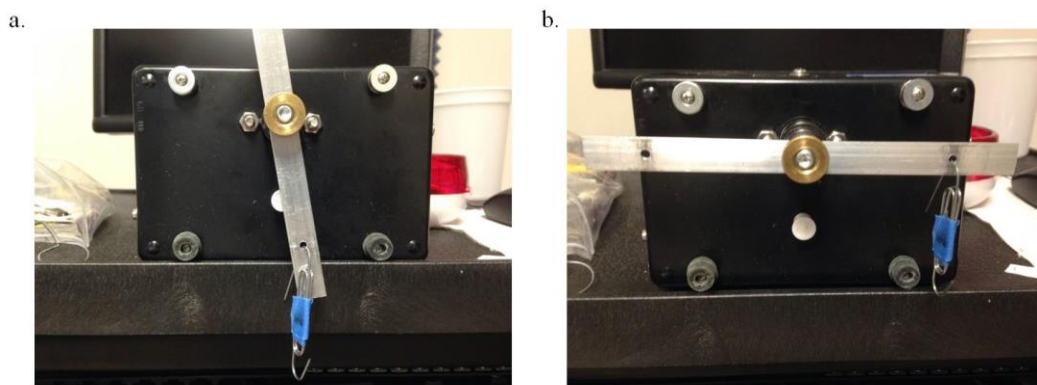


Figure 49. Wheel Calibration Procedure.

a) Wheels resistances were reset to zero by the controller program (VT Metabolic Phenotyping Core), and then the wheel was replaced from the axle by a thin metal bar. The paper-clip with blue tape served as a 2g hanging mass. The mass was hung 5.75cm from the axle (same radius as the running wheels). **b)** Wheel resistance was manually increased to 2g; the resistance setting was confirmed when the 2g mass was balanced by the inertia of the axle. After the 2g mass was balanced, the threaded rod was manually loosened by 3 full turns to complete the calibration process.