The Effects of Dietary β-Hydroxy-β-Methylbutyrate + β-Alanine and Physical Activity on Muscle Morphology and Function in Aged Rats

A thesis presented to
the faculty of
the College of Health Sciences and Professions of Ohio University

In partial fulfillment
of the requirements for the degree
Master of Science

Cara L. Acksel
December 2014

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This thesis titled
The Effects of Dietary β-Hydroxy-β-Methylbutyrate + β-Alanine and Physical Activity
on Muscle Morphology and Function in Aged Rats

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Abstract

ACKSEL, CARA L., M.S., December 2014, Food and Nutrition Sciences

The Effects of Dietary β-Hydroxy-β-Methylbutyrate + β-Alanine and Physical Activity on Muscle Morphology and Function in Aged Rats

Director of Thesis: Robert G. Brannan

Loss of muscle mass and strength is a major health concern for the aging population. Both physical activity and dietary amino acid supplementation are suggested to play a role in increasing muscle mass, though little is known regarding the effect of these interventions on strength and muscle quality. In the present study, forty 22-month Sprague-Dawley rats were placed on a supplemental diet, running regimen, both, or neither (control). Running habits were tracked and analyzed. The medial gastrocnemius was tested to determine muscle mass, force, quality, fatigability, and fiber type. A significant difference was seen in MHC I fiber concentration, MuRF-1 activity, and absolute change in running pace between some groups. The results suggest that that physical activity might have played a larger role then HMB in MHC composition, MuRF-1 activity, muscle quality, and running pace.
Dedication

I would like to dedicate this thesis to my parents, Thomas and Susan Acksel, as they have encouraged me throughout my entire education and time pursuing my master’s degree.

Without your prayers, support, and guidance, I would not be where I am today.
Acknowledgments

I would like to thank the individuals who made this thesis possible. Dr. Russ, thank you for letting me join this research study, for guiding me throughout the entire research process, and challenging me to expand my knowledge.

I would like to thank my thesis committee, Dr. David Russ, Dr. Robert Brannan, and Dr. Anne Loucks, for taking the time to provide all the feedback that went into this document. I would also like to thank everyone who helped me throughout the research process: Allison Wills, Iva Boyd, Jodi Krause, Katie McCorkle, Jack Maynard, Katie McCoy, and the Ohio University staff at the animal care facility. I would like to thank Abbott Laboratories for funding this research study. Finally, I would like to thank all those who offered me encouragement throughout my Master of Science degree.
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Chapter 1: Introduction

The demographics of the United States are changing, because people are living longer than previous generations (Wilson et al., 2012). With this change comes the need to understand the physical changes and challenges that affect the daily lives of those in the geriatric population. Focusing on muscle strength is crucial because muscle weakness can lead to an increased risk of falling, which can lead to other health problems. However, preventing the loss of muscle strength in an aging individual involves more than focusing on building muscle mass (Wilson et al., 2012). Muscle adapts and changes with age. Current thinking about loss of strength is dominated by a focus on loss of muscle mass. However, it has been suggested that aging muscle exhibits a greater decrease in muscle strength compared to the decrease in muscle mass (Clark & Manini, 2008; Goodpaster et al., 2006), such that decreased strength can occur even in elderly individuals who maintain or gain muscle mass (Delmonico et al., 2009; Goodpaster et al., 2006). This does not mean that muscle gain is not helpful in combating some aspects of muscle weakness, but that the quality of the new muscle gained is important too. An important consideration must be whether the increased muscle mass is functional, i.e., performs contraction efficiently and effectively; or dysfunctional, i.e., does not perform contraction efficiently (Amthor et al., 2007; Schirwis et al., 2013).

The most common way that individuals build muscle is through exercise. However, it is becoming more common to combine exercise with supplements in order to maximize effects. Sanchez Oliver and colleagues (2011) found that the percentage of
people participating in fitness activities at a local gym and taking supplements was higher than previous studies (Sánchez Oliver, Miranda León, & Guerra-Hernández, 2011). Lieberman et al. (2010) found that 18.7% of those serving on active-duty service in the U.S. Army used protein and amino acid supplements. In the athletic population, 37.2% report taking creatine supplements and 21.7% take protein powders (Froiland, Koszewski, Hingst, & Kopecky, 2004). While these young, highly active populations are concerned about their protein intake, older individuals might also need to focus on protein intake. In the 1994-95 Continuing Survey of Food Intake by Individuals, it has been found the people over the age of 70 are not getting the needed amount of protein (Wilson et al., 1997). However, the 2011-2012 What We Eat America NHANES report suggests that protein intake has improved compared to the 1994-95 study (U.S. Department of Agriculture, Agricultural Research Service, 2012).

Leucine is a branched chain amino acid that is suggested to simulate skeletal muscle protein synthesis (Anthony et al., 2000). One metabolite of leucine is HMB. While HMB is a precursor of β-hydroxy-β-methylglutaryl (HMG)-CoA and could thus affect de novo synthesis of cholesterol, the present study is primarily concerned with its role in protein breakdown and synthesis (Gropper & Smith, 2013). Supplementation with HMB might be helpful in building muscle in sarcopenic individuals, as several studies have examined the effects of exercise and HMB on muscle mass and strength. However, the results are not consistent among studies (Flakoll et al., 2004; Hao et al., 2011; Pinheiro et al., 2012; Solerte et al., 2008; Wilson, Wilson, & Manninen, 2008; Wilson et al., 2012). Fewer studies have examined the effect of HMB on strength, which is
problematic for assessing its utility in aging populations. While loss of muscle mass plays a role in age-related loss of muscle strength, it is important to recognize that other mechanisms can have an impact on muscle strength (Clark & Manini, 2008; Kamel, 2003; Roubenoff & Hughes, 2000). It is important to note that some studies have looked at muscle quality (Pinheiro et al., 2012), but most studies have looked only at the effect of mass and ability to perform basic tasks, not muscle quality (Flakoll et al., 2004; Hao et al., 2011; Pimentel et al., 2011; Wilson et al., 2012).

β-Alanine is a non-proteogenic amino acid, that is produced in the liver and then transported to skeletal muscle. β-Alanine and histidine combine to form carnosine, which is used as a pH buffer (Artioli, Gualano, Smith, Stout, & Lancha, 2010; Harris, Marlin, Dunnett, Snow, & Hultman, 1990; Harris et al., 2006). Exercising causes the environment around skeletal muscle to become acidic, which might lead to skeletal muscle fatigue (Artioli et al., 2010). Fatigue is known as feeling tired, which is a frequent complaint among older individuals and might lead to reduced or low physical activity and function (Moreh, Jacobs, & Stessman, 2010). The rationale for the present study is to determine whether HMB + β-Alanine supplementation and exercise can be effective ways to promote gains in muscle mass and quality in the geriatric population.

**Research Questions**

In this research study, 22-month old Sprague Dawley rats were placed in a 2 x 2 experimental design (three treatment groups and a control group). One group was fed a standard diet and allowed ad libitum access to running wheels; one group was fed a standard diet plus HMB + β-Alanine and allowed ad libitum access to running wheels;
the control group was fed the standard diet and had no wheel access; and the final group was fed a standard diet plus HMB + β-Alanine but had no wheel access (see Table 1).

Table 1

*Study Treatment Groups*

<table>
<thead>
<tr>
<th>Wheel running</th>
<th>Sedentary</th>
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<tr>
<td>Running Protocol &amp; Control Diet (R)</td>
<td>No Running &amp; Control Diet (C)</td>
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<td>Running Protocol &amp; Nutritional Supplement (RD)</td>
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**Research Questions, Hypotheses, and Statistical Analysis**

<table>
<thead>
<tr>
<th>Research question</th>
<th>Hypothesis</th>
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<tr>
<td>1. What are the effects of physical activity and dietary HMB + β-Alanine, alone or in combo, on muscle size?</td>
<td>Groups receiving dietary HMB + β-Alanine and/or engaging in physical activity will have an increase in muscle size compared to the control group.</td>
<td>2 x 2 (diet x physical activity) fixed effects ANOVA</td>
</tr>
<tr>
<td>2. What are the effects of physical activity and dietary HMB + β-Alanine, alone or in combo, on muscle force production?</td>
<td>Groups receiving dietary HMB + β-Alanine and/or engaging in physical activity will have a larger force production compared to the control group.</td>
<td>2 x 2 x 8 (diet x physical activity x frequency) ANOVA with frequency as the repeated factor</td>
</tr>
<tr>
<td>3. What are the effects of physical activity and dietary HMB + β-Alanine, alone or in combo, on muscle quality?</td>
<td>There will be no change in muscle quality.</td>
<td>2 x 2 x 8 (diet x physical activity x frequency) ANOVA with frequency as the repeated factor</td>
</tr>
<tr>
<td>4. What are the effects of physical activity and dietary HMB + β-Alanine, alone or in combo, on muscle fatigability?</td>
<td>Groups receiving dietary HMB + β-Alanine and/or engaging in physical activity will show less muscle fatigue.</td>
<td>Two separate unpaired t-tests will be used (Differences in fatigue protocols preclude comparison across physical activity groups.)</td>
</tr>
<tr>
<td>5. How does the fiber type differ when considering HMB + β-Alanine supplementation, physical activity, or the interaction between the two?</td>
<td>There will be a shift from Type IIb and IIx fibers to Type IIA or Type I fibers in the control group. The other groups are expected to maintain concentrations of Type IIb and IIx.</td>
<td>2 x 2 (diet x physical activity) MANOVA</td>
</tr>
<tr>
<td>6. What is the effect of dietary HMB + β-Alanine on running?</td>
<td>There will be no difference in wheel running between groups.</td>
<td>Unpaired t-test</td>
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In this study, we expected muscle mass to increase to a greater extent in the rats who were on the HMB + β-Alanine supplement, and that this increase would increase
functional muscle. Functional muscle leads to an increase in strength because functional muscle is capable of producing a contractile force. Some studies have shown that HMB increases body mass (Flakoll et al., 2004; Solerte et al., 2008). HMB has also been studied in relation to strength, and has been found to increase strength and functionality of muscles (Flakoll et al., 2004; Pinheiro et al., 2012) and, by implication, to increase muscle force. Reducing fatigue could depend on two phenomena. First, fatigue will be expected to decrease as a result of β-Alanine supplementation. β-Alanine is a precursor to carnosine, which acts as a buffer to increased pH levels seen during exercise (Artioli et al., 2010). Second, fatigue of the muscle could depend on the muscle fiber shift and metabolic enzyme/metabolic content. Fatigue will be expected to decrease, if there is a shift in older individuals to an oxidative model (Kent-Braun, Ng, Doyle, & Towse, 2002). When looking at myosin heavy chain profiles, it is expected that type II fibers (fast-twitch) will be most prevalent in the medial gastrocnemius. Pellegrino and colleagues (2005) reported that adult male mice on an amino acid diet and allowed voluntary wheel running had a significant increase of 2A and 2X fiber types, while 2B continued to dominate the muscle structure (Pellegrino, Brocca, Dioguardi, Bottinelli, & D’Antona, 2005). However, there might also possibly be a shift toward type I fibers (slow-twitch) located in the muscle, because increased Type I fibers are found in geriatric individuals (Brooks & Faulkner, 1994; Larsson, Grimby, & Karlsson, 1979), and decreased fatigue correlates better with type I fibers (Kent-Braun et al., 2002).
**Purpose and Significance of the Study**

Muscle weakness is a problem for many individuals in the geriatric population, and is now starting to be understood as being caused by more than just a decrease in muscle mass (Delmonico et al., 2009; Young, Stokes, & Crowe, 1984). Because of this, the overall objective of this study is to find the relationship of nutritional supplementation and exercise, and their impact on muscle strength and morphology, with hopes to successfully provide evidence that amino acid supplementation and exercise can be an effective way to promote gains in muscle mass and muscle quality in the geriatric population. The long term impact of this research is to continue to try to determine the mass-independent mechanisms that are contributing to muscle weakness and fatigue related to age and to develop interventions that target these mechanisms.

**Limitations**

This study did not compare rats of older age to rats of a younger age. Thus, this study cannot prove that the aged muscle performs like that of a muscle in a younger individual. Moreover, we cannot determine these affects within an individual animal (i.e., conduct a longitudinal study), as it is not possible to pre-harvest muscles to compare pre- and post-intervention states. Thus, this study can only suggest that an individual muscle, in male rats, will maintain, increase, or decrease from its original state, before starting to take HMB + β-Alanine and exercising, to the completion of the regimen. Also, the *in situ* stimulation used in this study bypasses the central nervous system, and cannot therefore account for any changes in neural drive that might have occurred.
Definition of Terms

**α-ketoisocaproate (KIC).** A molecule formed from leucine through transamination.

**AMP-activated protein kinase (AMPK).** “A downstream component of a protein kinase cascade that acts as a sensor of cellular energy status” (Offermanns & Rosenthal, 2008, p. 69).

**Anabolism.** The process by which organic molecules create larger molecules or structures.

**Appendicular skeletal muscle mass.** The muscle mass (kg) of all of the skeletal muscle of the limbs combined.

**Atrophy.** A decrease in muscle mass.

**Autophagy.** An important catabolic process necessary for degradation of organelles and turnover of protein (Laplante & Sabatini, 2009).

**β-hydroxy-β-methylbutyrate (HMB).** A metabolite of the amino acid leucine.

**Body mass index (BMI).** Measurement used to assess if a person is underweight or overweight. The value is found by taking the weight in kilograms divided by the square of the height in meters.

**Cachexia.** Physical wasting due to loss of muscle and fat during end-stage cancer, infectious diseases, and autoimmune disorders (Offermanns & Rosenthal, 2008).

**Calcaneus.** The large bone of the heel; also considered a tarsal bone.

**Catabolism.** The process by which organic molecules are broken down into smaller molecules or atoms.
**Dynapenia.** The loss of muscle strength due to aging. Also considered, “poverty of strength” (Clark & Manini, 2008; Manini & Clark, 2012).

**Interleukin-6.** A cytokine originating from macrophages, fibroblasts, and tumor cells.

**Isovaleryl-CoA.** An intermediate in the catabolism of leucine.

**Lysosomes.** Cell organelles that contain digestive enzymes.

**Mammalian target of rapamycin pathway.** A key regulator of cell metabolism, translation initiation, overall cell growth and proliferation, protein synthesis; a subunit of two molecular complexes (mTORC1 and mTORC2).

**Maximum isometric force.** The peak force produced from a muscle as it contracts while the muscle or joint angle is held at a constant length or angle.

**Mitochondrial proteases.** Molecules that degrade excess or damaged proteins in the mitochondria.

**Muscle cross-sectional area.** The area of a cross section of a muscle that is perpendicular to its longitudinal orientation, generally at the largest point of the muscle.

**Muscle quality.** The force produced per unit of muscle mass.

**Physical reserve.** The amount of physical ability one is able to draw upon to perform daily activities and functions.

**Sarcopenia.** The loss of muscle mass due to aging; also considered “poverty of flesh”.

**Sepsis.** A bacterial infection in the bloodstream or body tissues.
**Tumor necrosis factor-α.** A hormone-like proinflammatory peptide that belongs to a group of cytokines; released by immune cells and mast cells in response to inflammation, infection, or cell damage (Offermanns & Rosenthal, 2008).

**Type I muscle fibers.** Muscle fibers that are categorized as slow-twitch oxidative fibers; also considered postural muscles.

**Type II muscle fibers.** Muscle fibers that consist of three main sub-classes, a, x, and b. Type IIa are a combination of Type I and II fibers and are categorized as fast-twitch oxidative-glycolytic fibers. Type IIb and IIx are categorized as fast-twitch glycolytic fibers. Humans do not express Type IIb.

**Ubiquitin-proteasome pathway.** The pathway involved in degradation of proteins that have been tagged with ubiquitin.
Chapter 2: Literature Review

Geriatric Population

The demographics of the U.S. population are changing, largely due to the Baby Boomer Generation reaching the age of 65, and increasing life expectancy. According to the Centers for Disease Control and Prevention (CDC) and the U.S. Census Bureau, life expectancy in the year 2011 was 78.7 years in the United States, up from 76.9 years in 2000 (He, Sengupta, Velkoff, & DeBarros, 2005; Hoyert & Xu, 2012). Thus, it is expected that by 2030, the population over the age of 65 will be 72 million, double that reported in 2003. By 2050, the number of individuals over 65 is estimated to climb to 86.7 million people (He et al., 2005).

The growth of the geriatric population has led to an observed rise in health-related problems in this population. The 2012 NCHS Data Brief reported that there is a significant increase in individuals over the age 65 who have two chronic diseases, 45.3%, compared to 21% in those 45-65 years old (Freid, Bernstein, & Bush, 2012). Insulin resistance, a decrease in glomerular filtration rate, and osteoporosis of the femur neck are some possible chronic health conditions that could lead to an increased risk of developing sarcopenia (Foley, Wang, Ishani, Collins, & Murray, 2007; Kim et al., 2014). While chronic diseases can lead to loss of muscle mass, not all loss of muscle mass is considered to be sarcopenia (Ciciliot, Rossi, Dyar, Blaauw, & Schiaffino, 2013). When individuals suffer from chronic disease, healthcare costs can become a concern. In 2000, estimated direct healthcare costs of elderly individuals with sarcopenia was $18.5 billion (Janssen, Shepard, Katzmarzyk, & Roubenoff, 2004).
**Dietary habits and needs.** Dietary intake of protein is one factor that could influence the development of sarcopenia (Clark & Manini, 2008; Roubenoff & Hughes, 2000). The dietary habits of older individuals tend to follow a pattern of a decrease in total energy intake. This decrease can be due to changes in the sensory functions of taste and smell, difficulty chewing and swallowing, medication, sedentary lifestyle habits, decline in socialization or loss of spouse, physical disabilities, or diseases. Sarcopenia can also be considered a factor because, with decreased muscle mass, the body does not need as many calories to sustain it. However, with this decrease in caloric intake comes a decrease in the amount of nutrients consumed, including dietary protein (Blumberg, 1997).

Dietary protein is an important macronutrient, as it is involved with maintaining homeostasis of the muscle (Volpi et al., 2013). However, in the *1994-95 Continuing Survey of Food Intake by Individuals*, it was found that approximately 40% of older adults (those 70 years or older) were not getting the recommended dietary allowance (RDA) of protein (0.8 g • kg\(^{-1}\)•d\(^{-1}\)). There were also approximately 17% that did not consume over 75% of the RDA (Wilson et al., 1997). However, the *2011-2012 What We Eat America NHANES* report suggested that the protein intake improved since 1994-95 study (U.S. Department of Agriculture and Agricultural Research Service, 2012). The exact amount of protein consumed below the RDA that leads to sarcopenia has not been determined. Although, individuals consuming half of the RDA have been found to experience a decrease in lean body mass (Houston et al., 2008; Janssen et al., 2004). It is also possible that the current RDA of protein for adults does not meet the protein needs of
elderly individuals, as some individuals given a 0.8 g • kg\(^{-1}•d\(^{-1}\) diet maintain a negative nitrogen balance (Campbell, Crim, Dallal, Young, & Evans, 1994; Gersovitz, Motil, Munro, Scrimshaw, & Young, 1982). One suggestion is that dietary protein recommendations for elderly individuals should be increased to 1.00-1.25 g • kg\(^{-1}•d\(^{-1}\) (Bauer et al., 2013; Campbell et al., 1994). Another thing to consider is the amount of adequate protein needed for each gender. Gersovitz et al. (1982) found that males tend to reach nitrogen equilibrium, while females are more likely to stay in a negative nitrogen balance, when consuming the RDA standard (Gersovitz et al., 1982). However, Castaneda, Charnley, Evans, and Crim (1995) found that consuming protein at the current RDA level is sufficient for elderly women. The timing of protein intake is also something to consider. Older individuals who consume higher amounts of protein (25-30 g) at each meal are found to encourage anabolism (Bauer et al., 2013). Thus, one suggestion is to spread protein intake throughout the day; the other suggestion is to improve lean body mass using pulse feeding, which involves consuming high amounts of protein at one particular meal (typically the meal consumed at midday) (Bauer et al., 2013; Bouillanne et al., 2013). Alternatively, considering resistance exercise and protein consumption in the elderly population, it has been suggested that consuming protein directly following resistance training provides the best benefit (Esmarck et al., 2001).

**Importance of physical activity.** Physical activity is needed with the geriatric population for multiple beneficial reasons. For individuals older than 60 years old, the primary purpose of exercise is for primary or secondary prevention of chronic diseases. The purpose of physical activity for those older than 80 is to maintain an independent
living lifestyle by improving muscle strength (Vogel et al., 2009). Physical activity is beneficial in providing a longer healthy life, improved blood lipid profiles, possible colon and breast cancer prevention, maintenance of peak oxygen consumption, maintenance or gains in muscular strength and function, a decrease in body fat, a possible decrease of systolic and/or diastolic blood pressure, and a decreased risk of developing coronary heart disease (CHD), type 2 diabetes, stroke, age-related sarcopenia, dementia and/or Alzheimer’s disease (Vogel et al., 2009).

Many individuals over the age of 65 are not engaging in the needed physical activity. The CDC’s 2007 U.S. Physical Activity Statistics report (as cited in Litman, 2010, p. 13) stated that only 39.3% of the geriatric population met the recommended amount of exercise (≥ 30 minutes of moderate-intensity exercise for at least 5 days per week or ≥ 20 minutes of vigorous-intensity exercise for at least 3 days per week). Of the 60.7% not meeting the recommendation, 36.9% had insufficient levels of exercise (≥ 10 minutes of moderate or vigorous-intensity activities per week) and 23.7% were considered inactive (< 10 minutes of moderate or vigorous-intensity activities per week). As a separate statistic, the CDC reported that 32.7% reported no leisure-time physical activity, and did not participate in any physical activities in the month leading up to the survey (CDC, 2007, as cited in Litman, 2010). The type of exercise is also important. Peterson et al. (2009) found that 40% of active individuals are involved with walking, while only 5% are performing strengthening exercises (Peterson et al., 2009).
Skeletal Muscle Physiology—Structure, Function, and Protein Turnover

**Basic skeletal muscle structure.** A muscle is a grouping of muscle fibers. Groupings of muscle fibers form a fasciculus, and multiple fasciculi form a muscle. The bundles of fibers are held together by connective tissue called fascia. Muscle fibers can further be broken down into myofibrils. The functional unit of the myofibril is called the sarcomere. Actin (the thin filament) and myosin (the thick filament) make up a part of a sarcomere’s composition (Housh, Housh, & Devries, 2006).

**Skeletal muscle fiber types.** Muscle fibers are the cells of a muscle. There are multiple ways in which a person can designate muscle fiber types. These include anatomical appearance (i.e., red or white), contractile properties (i.e., slow- or fast-twitch muscles), biochemical properties (aerobic capacity), and histochemical properties (enzyme profile). There are three main fiber types found in human skeletal muscle (I, IIA, and IIX). These are slow-twitch oxidative, fast-twitch oxidative glycolytic, and fast twitch glycolytic, respectively (Housh et al., 2006). In the past, Type IIX fibers were identified as Type IIb fibers in human studies (Bartlett, Gratton, & Rolf, 2012). Type IIb fibers are another fiber type seen in rats. These fibers tend to have the largest fiber CSA compared to the other muscle fibers. Type IIb are stimulated by highly fatigable motor units, which result in a very low fatigue ratio (Larsson, Edström, Lindegren, Gorza, & Schiaffino, 1991; Sieck, Zhan, Prakash, Dao, & Watchko, 1995). Twitch and tetanus forces are found to be higher in Type IIb fibers compared to Type IIA and IIX. Type IIA is the slowest of the fast-twitch fibers; Type IIX is faster than IIA but slower than IIb; and Type IIb is the fastest (Larsson et al., 1991).
Table 3

*Skeletal Muscle Fiber Type and Characteristics*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Type I</th>
<th>Type IIa</th>
<th>Type Ix</th>
<th>Type IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle profile</td>
<td>Slow twitch oxidative</td>
<td>Fast twitch oxidative glycolytic</td>
<td>Intermediate fast twitch glycolytic</td>
<td>Fast twitch glycolytic</td>
</tr>
<tr>
<td>Speed of contraction</td>
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<td>Strength of contraction</td>
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<td>Neuromuscular fatigability</td>
<td>Fatigue resistant</td>
<td>Low fatigability</td>
<td>Moderate fatigability</td>
<td>High fatigability</td>
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<td>Aerobic capacity</td>
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<td>Anaerobic capacity</td>
<td>Low</td>
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**Skeletal muscle contraction.** Muscle contraction is initiated through a neurological signal. When the muscle is stimulated, calcium is released from the sarcoplasmic reticulum. The released calcium then binds to troponin, allowing tropomyosin to change shape. Because of this the myosin is able to bind with actin, forming a cross-bridge. As ATP is broken down, the myosin head swivels and detaches from the actin. This brings the two ends of the sarcomere closer together. This cycle of binding and detaching is repeated until neural stimulation stops (Housh et al., 2006).

**Protein turnover.** Protein is involved in many different processes throughout the body and serves in the role of catalyst, messenger, structural element (contractile and fibrous proteins), buffer, fluid balancer, immunoprotector, transporter, and acute phase
responder. Over 40% of the protein in the body is found within skeletal muscle (Gropper & Smith, 2013). Maintaining skeletal muscle involves a balance between the breakdown and synthesis of protein. Muscle atrophy happens when there is a higher amount of protein breakdown, i.e., negative protein balance. Hypertrophy occurs when there is a higher amount of protein synthesis, i.e., positive protein balance (Fry et al., 2011).

An individual consuming less than the recommended amount of protein can cause a person to have a negative nitrogen balance. This can affect many processes happening within the body. At the cellular level, low protein intake can affect protein synthesis at transcriptional phase, as mRNA of albumin, fibrinogen, transferrin, apolipoprotein E, and transthyretin have been found to be reduced (Young & Marchini, 1990). At the muscular level, when an individual takes in adequate amounts of calories but has low protein intake, it has been found that muscle proteolysis decreases (Mitch & Goldberg, 1996). The Health, Aging and Body Composition (Health ABC) study has also looked at the effect of the amount of protein intake with the response of lean body mass. Individuals with a decreased protein intake presented with a greater loss of lean body mass (LBM) over a period of 3 years (Houston et al., 2008).

**Proteolysis and the ubiquitin-proteasome pathway.** The body is continually breaking down and synthesizing protein. Some proteins have short half-lives and are broken down quickly. Others have longer half-lives and take longer for the body to break them down. The body continually goes through a process of proteolysis and protein synthesis to keep the body functioning properly. Disease happens when this system of degradation and synthesis is not functioning properly. Degradation of protein is an
important aspect as it removes dysfunctional proteins that have formed incorrectly or have oxidative damage. There are three main pathways that affect protein degradation within the body, ubiquitin-proteasome pathway, lysosomes, and mitochondrial proteases. The ubiquitin-proteasome pathway is the main degradation pathway of contractile proteins found within the muscle (Mitch & Goldberg, 1996). One of the E3 muscle specific ubiquitin-protein ligases in the ubiquitin-proteasome pathway is Muscle RING Finger-1 (MuRF-1). There are many mechanisms that can stimulate the activation of this proteasome pathway. Two of the cytokines in this pathway, myostatin and TGF-β, can lead to the triggering of the response of atrogin-1 and MuRF-1 (Gumucio & Mendias, 2013). It has been suggested that the downregulation of MuRF-1 results in the upregulation of atrogin-1 and vice versa (Castillero, Alamdari, Lecker, & Hasselgren, 2013). Another possible upstream regulator is calpains (Hasselgren & Fischer, 2001). However, Fareed et al. (2006) suggested that the calpain and ubiquitin-proteasome systems work parallel and not sequentially.

There are a variety of methods used to stimulate muscle atrophy experimentally. Dexamethasone works through stimulating MuRF-1 and atrogen-1. When both MuRF-1 and atrogan-1 are suppressed, dexamethasone does not have as large of an effect on muscle atrophy compared to when they are present (Castillero et al., 2013). Clarke et al. (2007) found that when MuRF-1 null animals were given a dexamethasone treatment, their myosin heavy chain protein levels increased relative to actin. This suggests that the role of MuRF-1 in the ubiquitin-proteasome pathway is to degrade myosin heavy chains in atrophy conditions (Clarke et al., 2007). Multiple pathways play a role in muscle
atrophy with disuse. Two of many pathways involved are the ubiquitin-proteasome and mTOR pathway. The ubiquitin-proteasome pathway is found to be activated, while the IFG-1/PI3K/Akt pathway is deactivated. Phosphorylated Akt activates the mTOR pathway (Zhang, Chen, & Fan, 2007). Muscle protein breakdown is also seen in individuals with inadequate intake of calories. This is because the body is working to provide essential amino acids needed for maintaining essential life processes. However, during fasting conditions, after a couple of days, the body will move to conserving muscle protein (Mitch & Goldberg, 1996).

**Skeletal muscle protein synthesis.** There are two multiprotein complexes that make up the mTOR pathway (see Figure 1). The pathway that is involved in stimulating cell growth is the mammalian target of rapamycin complex 1 pathway (mTORC1). It is the key regulator of cell metabolism, overall cell growth and proliferation, as well as translation initiation (Fry et al., 2011; Guertin & Sabatini, 2007; Laplante & Sabatini, 2009; Walker et al., 2011). The mTORC1 complex consists of mTOR, raptor, mLST8, and PRAS40 proteins (Guertin & Sabatini, 2007). The anabolic processes of mTORC1 include synthesis of organelles, lipids, and proteins, while also limiting the catabolic process of autophagy, which is an integral part of protein turnover (Laplante & Sabatini, 2009). The pathway that is involved in stimulating growth, proliferation, and survival is the mammalian target of rapamycin complex 2 pathway (mTORC2) (Guertin & Sabatini, 2007; Laplante & Sabatini, 2009). The mTORC2 complex consists of mTOR, mLST8, RICTOR, mSIN1, and PROTOR proteins (Guertin & Sabatini, 2007).
The mTORC1 pathway is activated by multiple mechanisms, essential amino acids; oxygen levels; growth factors, including insulin; and mechanical stimulation. When oxygen or energy levels decrease, AMP-activated protein kinase (AMPK) signals to reduce activation of mTORC1 (Laplante & Sabatini, 2009; Walker et al., 2011). Leucine is one of the main essential amino acids found to activate this pathway (Laplante & Sabatini, 2009), and dietary intake of HMB has been shown to contribute to increasing the mTOR protein expression and activating p70 ribosomal S6 protein kinase (p70S6K) (Pimentel et al., 2011). The p70S6K protein is phosphorylated by mTOR (Chotechuang et al., 2009; Guertin & Sabatini, 2007; Laplante & Sabatini, 2009). Physical activity is also seen to affect the mTOR pathway. Pasini et al. (2012) observed a reduction of mTOR activation in older sedentary rats compared to young rats, but found that exercise performed 3 and 5 days per week induced dose-dependent increases in mTOR activation in older rats. Significance was achieved at 5 days per week (Pasini et al., 2012).

However, signaling proteins in the mTORC1 pathway seem to be depressed in the elderly population in response to resistance exercise, as phosphorylation of mTOR proteins were not upregulated postexercise, as was seen in younger individuals (Fry et al., 2011). It has also been suggested that the mTOR pathway is repressed during endurance exercise (Williamson, Kubica, Kimball, & Jefferson, 2006).
Figure 1. The mTOR pathway. (A) Overall pathway and the physiological mechanisms it stimulates. The right side addresses the mTORC1 complex and the left side addresses the mTORC2 complex. (B) Activation and inactivation of mTORC1. Figure reprinted from “Defining the Role of mTOR in Cancer,” by D. A. Guertin and D. M. Sabatini, 2007, Cancer Cell, 12, p. 10. Copyright 2007 by Elsevier. Reprinted with permission.

Eukaryotic translation initiation factor EIF4E binding protein 1 (4eBP1) and eukaryotic translation initiation factor 4E (EIF4E) are two proteins further downstream in the protein synthesis pathway. These are translation factors for protein synthesis. The 4eBP1-EIF4E complex is considered to be inactive. When 4eBP1 is phosphorylated, there is a decrease in its binding affinity to EIF4E. This allows EIF4E to bind with
EIF4G to form the complex that initiates translation of mRNA (Anthony et al., 2000; Gautsch et al., 1998). Leucine might play a role in increasing the availability of EIF4E and decreasing the inactive 4eBP1•EIF4E complex (Anthony et al., 2000). Exercise has been suggested to lead to the dephosphorylation of 4eBP1 to a greater extent at high versus low exercise intensities (Rose, Bisiani, Vistisen, Kiens, & Richter, 2009; Williamson et al., 2006).

**Sarcopenia**

One current concern for the geriatric population is the loss of muscle mass and strength. Age, cachexia, bed-rest, sepsis, and HIV/AIDS are some factors that can cause an individual to lose muscle mass (Fry et al., 2011). Sarcopenia (GR., “poverty of flesh”) is defined as the loss of muscle mass due to aging (Cruz-Jentoft et al., 2010; Harris, 1997; Morley, Baumgartner, Roubenoff, Mayer, & Nair, 2001). It is assumed to define both the loss of muscle mass and muscle strength as it relates to aging (Houston et al., 2008; Roubenoff & Hughes, 2000). Sarcopenia can be defined two ways, physiologic and pathologic. An individual is considered to be in a pathological state when an individual reaches appendicular skeletal muscle mass values that are two standard deviations below the mean of young adults (18-40 yrs). The individual will also experience a decrease in function and quality of life. Those in a physiological state of sarcopenia are individuals who have not reached the standards of pathological sarcopenia and are not seeing a decrease in function and quality of life (Baumgartner et al., 1998; Kamel, 2003).

Changes in the composition of elderly individual bodies are also important to consider. This is seen in increases of intramuscular fat infiltration and changes in
subcutaneous adipose tissue (Delmonico et al., 2009). Sarcopenia does not just affect individuals with a normal BMI, but it also can affect obese individuals. Obese individuals who are frail are considered “sarcopenic obese” individuals. The healthcare burdens of these obese, older adults are the greatest (Heuberger, 2011). Larsson and Edström (1986) found that the total body weight of an older rat was greater compared to an adult rat, while the weights of some muscles decreased in the older rats compared to the younger adult ones. They suggested that the increased body weight in older individuals is related to an increase in subcutaneous adipose tissue (Larsson & Edström, 1986).

Dynapenia is a new term that is emerging in this area of study. Dynapenia means “poverty of strength.” One of the reasons for this designation is that research has started to focus on the fact that the decline of strength is greater than expected compared with the decline in muscle mass (see Figure 2) (Clark & Manini, 2008; Manini & Clark, 2012).
Figure 2. Age related decrease in muscle mass and strength comparison. Longitudinal change in muscle size and strength, demonstrating the disconnect between the two. Figure was originally adapted from Delmonico and colleagues (2009) and based on data gathered from the Health ABC Study. Reprinted from “Dynapenia and Aging: An Update,” by T. M. Manini and B. C. Clark, 2012, Journal of Gerontology: Series A, Biological Sciences and Medical Sciences, 67, p. 29. Copyright 2012 by the Oxford University Press. Reprinted with permission.

When looking at maximum isometric force and total muscle fiber cross sectional area in aged rodents, muscle force/gram of wet weight and force/CSA are reduced (Russ, Grandy, Toma, & Ward, 2011; Russ, Wills, Boyd, & Krause, 2014). Atrophy happens in a sacropenic state, but it has been suggested that approximately 20% of the decrease in isometric force cannot be explained by atrophy alone (Brooks & Faulkner, 1988, 1994). Thus, one cannot explain the sarcopenic problem in the geriatric population as the combined loss of muscle mass and loss of muscle strength.

**Muscle mass changes with aging.** The amount of muscle mass decreases as an individual ages (Bouchard, Héroux, & Janssen, 2011), with 40% occurring between the ages of 20 and 80 (Lexell, Taylor, & Sjöström, 1988). By the age of 80, over 40% of individuals can be categorized as having sarcopenia (Baumgartner et al., 1998). A number of explanations for the decrease in muscle mass have been suggested. The first
explanation includes the loss of muscle fibers. In the rat models, the number of fibers has been found to decrease by 13%, particularly Type IIA fibers (Larsson & Edström, 1986). In humans, by age 80, the number of muscle fibers in a muscle has decreased approximately 39% (Lexell et al., 1988). There also tends to be a decrease in size of fiber types. Lexell et al. (1988) suggested that there is approximately a 26% decrease in fiber size between the ages of 20 and 80. The mean cross-sectional area for the quad was noted to be decreased around 33% in older women compared to younger women (Young et al., 1984). In the rat model, all the fiber types were found to be smaller in size in the older rats compared to the adult rats (Larsson & Edström, 1986). The second explanation deals with the changes in fiber types. There are two main types of muscle fibers, Type I and Type II. Type II fibers tend to have larger cross-sectional areas compared to Type I fibers. If a muscle increases in Type I fibers, the muscle mass could display a smaller size (Brooks & Faulkner, 1994). Larsson, Grimby, and Karlsson (1979) found that there is a shift from Type II to Type I fibers in the aging process, and a decreased percentage of Type II fibers with an increased percentage in Type I fibers. They also found a decrease in the ratio of Type II to Type I fibers (Larsson et al., 1979). A predominately Type I muscle is considered an endurance muscle. A predominately Type II muscle is seen as a strength muscle (Boirie, 2009). A shift of a muscle from Type II to Type I leads to a shift from a state of muscle strength to muscle endurance (Boirie, 2009).

Muscle strength changes with aging. Muscle strength, quality, and physical function have been shown to decrease as a person ages (Bouchard et al., 2011; Larsson et al., 1979). It has been suggested that muscle strength between the ages of 30 and 80
years decreases 30-40%, which is similar to the decrease in muscle size found in other studies (Brooks & Faulkner, 1994; Grimby & Saltin, 1983; Lexell et al., 1988).

Similarly, it has been reported that 71- to 81-year-old women are on average 35% weaker than 20- to 29-year-old women (Young et al., 1984). Larsson and colleagues (1979) related the loss in muscle strength to the atrophy of muscle fibers, specifically type II fibers. However, atrophy is not the only attribute that might be contributing to muscle weakness (Larsson et al., 1979). In more recent studies, muscle strength was shown to decrease to a greater extent than muscle mass (Delmonico et al., 2009; Goodpaster et al., 2006; Hughes et al., 2001). Delmonico et al. (2009) found that muscle strength decreased at a rate of 2-5 times that of the decrease in muscle size (see Figure 2). Loss of strength has also been found to occur in older individuals who see an increase in weight and muscle size with aging. This suggests that muscle quality is a concern, and that addressing mass might not completely help overcome the problem of muscle weakness (Delmonico et al., 2009; Goodpaster et al., 2006; Hughes et al., 2001).

**Frailty in the elderly population.** Frailty is a medical condition that can be experienced by many elderly individuals. Frailty is defined as an individual having three or more of the following criteria: unintentional weight loss, low muscle strength, feeling of exhaustion, reduced physical activity, and poor physical performance (Bartali et al., 2006; Fried et al., 2001). A criterion that is not listed is nutrition. However, it is suggested that individuals that are defined as frail have poor nutritional scores. They are more likely to have low energy intake and a low intake of protein, folate, and vitamins C, D, and E (Bartali et al., 2006).
Loss of muscle mass and bone density are two physical changes that lead to frailty. Frailty is characterized by symptoms of a declining ability to perform activities of daily living, loss of mobility, falling, poor nutritional status and/or intake, sensory decline, fatigue, loss of strength, cognitive decline, and having one or more chronic diseases. An individual might not display all of these symptoms, but every individual considered frail has diminished physical reserve. When the physical reserve is depleted, functional limitations become evident (Fried, Ferrucci, Darer, Williamson, & Anderson, 2004; Pel-Littel, Schuurmans, Emmelot-Vonk, & Verhaar, 2009).

Frailty is also a precursor to disability, but it is not the same thing. Factors that could lead to frailty and disability are physiological changes with age, chronic diseases, poor nutritional intake, environmental conditions, genetics, and lifestyle choices. Sarcopenia is considered a pathological condition associated with aging that has an influence on frailty (Fried et al., 2001; Heuberger, 2011). Developing sarcopenia could lead to an increased rate of disability. Disability is manifest as increased use of canes or walkers, decreased walking speed, and impaired balance. A history of falling can also be noted in individuals with sarcopenia (Kamel, 2003; Roubenoff & Hughes, 2000).

There are many mechanisms that can cause the loss of muscle mass and strength in individuals with sarcopenia. Issues influencing sarcopenia can include dysfunctions with the nervous system, changes in hormones (low growth hormone, insulin like growth factor-1, estrogen, and testosterone), decreased protein in the diet, inflammatory markers (interleukin-6 and tumor necrosis factor-α), immunologic changes, decreased physical activity (Clark & Manini, 2008; Roubenoff & Hughes, 2000), myocellular changes,
muscle architecture changes, transformation of fiber type, excitation-contraction uncoupling (Clark & Manini, 2008), and increased oxidative stress (Kamel, 2003). This study focuses on how the mechanisms of diet and physical activity work together or separately to promote changes in skeletal muscle mass and quality.

**β-Hydroxy-β-Methylbutyrate**

Protein structure consists of a series of connected amino acids. The order of the amino acids designates the function of the protein. HMB is a metabolite of the amino acid leucine (Gropper & Smith, 2013). The first step in the metabolic breakdown process of leucine happens in the cytosol and mitochondria of the muscle through reversible transamination of leucine to alpha-ketoisocaprate (KIC) (Nissen & Abumrad, 1997). KIC is transported to the liver where it can follow two different routes, conversion to isovaleryl-CoA or HMB. Most of KIC follows the pathway to create isovaleryl-CoA. Approximately 5-10% of KIC is used in the pathway that creates HMB (Kovarik, Muthny, Sispera, & Holecek, 2010; Nissen & Abumrad, 1997). The conversion to HMB happens in the cytosol of the cell through the enzyme dioxygenase (Nissen & Abumrad, 1997). HMB then continues to react with enzymes to create HMG-CoA, which plays a role in cholesterol synthesis (see Figure 3) (Kovarik et al., 2010; Nissen & Abumrad, 1997). The chemical formula of HMB is C$_5$H$_{10}$O$_3$ (Abbott Laboratories, 2010).
Some foods that HMB can be found in are avocado, cauliflower, alfalfa, citrus fruits, and catfish (Abbott Laboratories, 2010). Human studies typically give HMB supplementation in doses ranging from 1.5g to 3.0g (Flakoll et al., 2004; Knitter, Panton, Rathmacher, Petersen, & Sharp, 2000; Nissen et al., 1996). Animal studies with rats...
typically give HMB supplementation in doses that are at least six times the human dose and some report not seeing effects unless it was at least eight times the human dose (Hao et al., 2011; Wilson et al., 2012).

**Effects of HMB on muscle mass and strength.** HMB has been studied to determine its ability to increase muscle mass. In a few studies, HMB has been found to increase lean body mass in humans (Flakoll et al., 2004; Solerte et al., 2008). Others found that there were no changes between HMB supplementation and no supplementation in rats (Pinheiro et al., 2012). Another suggestion is that HMB maintains the mass that already exists, because a continued loss of muscle mass is not seen in either humans or rats (Castaneda et al., 1995; Wilson et al., 2012). However, HMB supplementation appears to function differently in disuse models. When the muscles are not used for a period of time, Hao et al. (2011) found that HMB did not provide protection against the loss of muscle mass in both the plantaris and soleus in rats. However, HMB helped reduce loss of muscle fiber cross-sectional area (CSA) during reloading of the plantaris (Hao et al., 2011). It is possible that reloading has a damaging effect on skeletal muscle (Widrick et al., 2008). Thus, HMB might be working to minimize the damage of the muscle instead of building muscle strength.

HMB supplementation has also been to increase the strength of muscles (Flakoll et al., 2004; Pinheiro et al., 2012; Wilson et al., 2012), suggesting that there is an intrinsic factor that affects the strength of the muscle (Pinheiro et al., 2012). However, consuming HMB during a period of disuse has not shown exactly the same effect on strength. Hao et al. (2011) suggested that the intake of HMB is not able to prevent the decline of muscle
strength seen during the period of muscle unloading, but suggested that HMB might play a role in preventing continued loss of muscle strength during the recovery period (Hao et al., 2011).

Effects of HMB on protein turnover. HMB has multiple roles within the body. One of those roles includes protein turnover. Protein turnover is considered to be happening when the body is involved with proteolysis and protein synthesis. HMB is responsible for inhibiting muscle proteolysis and stimulating synthesis of muscle protein (Aversa et al., 2012; Gropper & Smith, 2013; Nissen et al., 1996; Wilkinson et al., 2013). HMB plays a bigger role in inhibiting proteolysis compared to protein synthesis, at a rate of 80% to 20% respectively (Nissen & Abumrad, 1997). Holecek, Muthny, Kovarik, and Sispeira (2009) found that with HMB supplementation there was a decrease in whole body proteolysis (Holecek, Muthny, Kovarik, & Sispera, 2009). Flakoll (2004) reported that there was an increase of about 20% in the rate at which whole body protein synthesis occurred (Flakoll et al., 2004). Holeck et al. (2009) looked specifically at different areas within the body to measure the changes in protein synthesis. They found that HMB did not have a significant effect on protein synthesis in the skeletal muscle. Holecek et al. (2009) found an increase in protein synthesis in the liver, but the mechanism leading to increased protein synthesis in the hepatic tissue was not clear.

Exercise and HMB. HMB supplementation combined with exercise has different effects than then exercise alone. Nissen et al. (1996) found that exercised-induced muscle damage (as determined by urinary of 3-methylhistidine) was reduced in young, healthy adults when taking a HMB supplement (Nissen et al., 1996), a finding that
held true with both resistance exercise and endurance exercise. Knitter, Panton, Rathmacher, Peterson, and Sharp (2000) found that the combination of endurance exercise and 3.0 g HMB supplementation in humans led to lower markers of muscle damage (Knitter et al., 2000). This suggests that HMB has a protective effect against muscle damage during exercise activities (Knitter et al., 2000; Nissen et al., 1996). However, Wilson et al. (2009) found that timing of acute supplementation had no clear effects on muscle damage. They suggested that a loading period might be needed to attenuate muscle damage (Wilson et al., 2009).

**β-Alanine**

β-Alanine is a non-proteogenic amino acid that is produced in the liver and then transported to other tissues, including skeletal muscle. Once in the muscle, β-Alanine and histidine combine with the help of carnosine synthetase to form carnosine (Artioli et al., 2010). Carnosine is used as a pH buffer (Harris et al., 2006, 1990) and is thought to play a role as an antioxidant (Babizhayev, 1989; Boldyrev, Koldobski, Kurella, Maltseva, & Stvolinski, 1993). Carnosine is also involved with the regulation of calcium sensitivity in excitation-contraction coupling (Batrukova & Rubtsov, 1997; Dutka, Lamboley, McKenna, Murphy, & Lamb, 2012). The role of β-Alanine, in relation to carnosine synthesis, is that it acts as the rate limiting amino acid (Harris et al., 2006).

**Exercise and β-alanine.** One consequence of exercise is a reduction in skeletal muscle pH. This might lead to skeletal muscle fatigue (Artioli et al., 2010). Acidosis might lead to fatigue by decreasing calcium sensitivity of contractile proteins (Chin & Allen, 1998). Carnosine acts to buffer muscle pH. Without supplementation, Tallon,
Harris, Maffulli, and Tarnopolsky (2007) observed that carnosine levels were lower in Type II fibers of older individuals and, overall, there was a decreased concentration of carnosine in elderly skeletal muscle compared to the younger subjects. Tallon et al. (2007) suggested that this leads to a decline in intracellular physiological-chemical buffering capacity and a decrease in anaerobic exercise capacity, since these are some of the roles of carnosine. Del Favero et al. (2012) reported an increase in carnosine content in skeletal muscle with β-Alanine supplementation in elderly individuals, which was associated with increased time to exhaustion. However, they did not see a change in muscle function. The improved time to exhaustion could suggest an improved capacity in physical exercise (del Favero et al., 2012).

**Physical Activity**

**Importance of physical activity on muscle strength.** To be physically active, one must possess the needed strength to perform exercise. Hasegawa et al. (2008) looked at the amount of strength needed to complete basic activities of daily living (ADLs), and determined 2.3 N/kg for hip flexors, 1.7 N/kg for hip extensors, 0.7 N/kg for knee flexors, 2.8 N/kg for knee extensors, and 2.8 N/kg for ankle dorsiflexors were critical values. These values are set points for when older individuals start seeing major repercussions of loss of muscle strength (Hasegawa et al., 2008).

Physical activity is an important component of muscle strength, because it is found to decrease the amount a frailty in older individuals (Peterson et al., 2009). Being physically active at an early age can be beneficial later in life. Tikkanen, Nykanen, Lonnroos, Sipila, Sulkava, and Hartikainen (2012) found that those who were physically...
active during early and mid-years in life (ages 20 to 64), presented with better mobility at an older age. Benefits of exercise were also seen by Solberg et al. (2013), who reported a correlation between increase in lean body mass and upper body strength. Tikkanen et al. (2012) also noted that physical activity before age 65 led to better grip strength and walking speed in men. However, women did not see the same benefits as men (Tikkanen et al., 2012).

**Effect of fatigue on physical activity.** Fatigue is considered feeling tired and can be a limiting factor when it comes to performing physical activity. Fatigue is a frequent complaint among older individuals and can lead to reduced or low physical activity and function (Moreh et al., 2010). Tennant et al. (2012) gave a fatigue survey to rural elderly individuals. They discovered that increased fatigue could be related to impaired physical function (Tennant, Takacs, Gau, Clark, & Russ, 2012). Some functional areas that might decrease with fatigue are handgrip strength and walking speed. Fatigued elderly individuals also might not be able to walk 400 meters (Vestergaard et al., 2009).

Skeletal muscle fatigue is also something to consider with the elderly population. Kent-Braun and colleagues (2002) noticed that less fatigue was measured in older individuals compared with younger individuals when performing incremental isometric exercises. The metabolic response to the exercise protocol suggests that younger individuals rely to a greater extent on nonoxidative sources of ATP compared to older individuals. Gender also plays a role. Men compared to women rely more on nonoxidative sources of ATP. This shift in older individuals to an oxidative source of ATP suggests a shift of muscle fibers to Type I fiber (Kent-Braun et al., 2002).
**Strength and endurance exercise.** The type of exercise could influence what types of changes happen within the muscle. Three types of exercise activities that are typically prescribed to older individuals are traditional strength training, functional strength training, and endurance training. Traditional strength training relates to using weight equipment to perform a set movement, like hamstring curls. Functional strength training focuses on gaining strength in movements that are performed in everyday activities. This form of strength training consists of a functional movement, like step-ups, that are performed with or without weights. Endurance training is an activity that focuses on building cardiorespiratory endurance and strength. Strength training, functional training, and endurance training have been shown to increase lean body mass compared to those who do not participate in these types of exercise activities. The difference between these forms of activity is seen in the main effects of the activity. Endurance training decreases fat mass and functional training increases strength. Individuals who start at a lower baseline measurement tend to see the greatest amount of improvement compared to those that start at a high physical capability (Solberg et al., 2013).

**Resistance exercise.** Resistance exercise, particularly high-intensity, has been found to be effective in stimulating muscle protein synthesis and hypertrophy. Notable differences in the effectiveness of resistance exercise in older individuals compared to younger individuals have been suggested. In older individuals, there is a decreased anabolic response to an anabolic stimulus, i.e., they do not build muscle as easily as younger individuals when performing resistance exercise. A possible reason for this is
that older adults are not able to activate the mTORC1 signaling as affectively during the period of 24 hours post-exercise. Because of this, a form of resistance exercise that might prove to be helpful specifically with older adults is blood flow restriction exercise. This form of resistance exercise has been shown to increase muscle protein synthesis by 56% after exercise with older adults while keeping the mTORC1 pathway active (Walker et al., 2011).

**Aerobic exercise.** Aerobic exercise is capable of stimulating muscle protein synthesis (Pikosky et al., 2006; Short, Vittone, Bigelow, Proctor, & Nair, 2004; Walker et al., 2011). Therefore, engaging in chronic aerobic exercise may lead to elevated muscle protein synthesis while at rest, perhaps because mitochondrial protein synthesis is stimulated by aerobic exercise. Another mechanism to consider is the effect of the muscle being sensitized to insulin (Walker et al., 2011). Change does not just happen if one is minimally physically active. For rats that exercised 3 days a week there was no significant difference between their muscle weight, body weight, and muscle to body weight ratio. Those that exercised 5 days a week experienced significant changes compared to the sedentary group in muscle weight and the muscle weight to body weight ratio (Pasini et al., 2012). Short et al. (2004) found that there was a decline in both whole body turnover and mixed muscle protein synthesis in human subjects with normal aging, and, after 4 months of aerobic exercise, whole body protein turnover did not increase (Short et al., 2004). The activation of AMPK leads to an inhibition of mTOR (Inoki et al., 2006). AMPK is activated by low cellular energy (Inoki, Zhu, & Guan, 2003; Reiling
and Sabatini, 2006), and the metabolic stress activation during muscle contraction can lead to the activation of AMPK (Hardie, 2008; Winder & Hardie, 1996).

**Conclusion**

Throughout a lifetime, the body is in a state of continual change. These changes can either promote health or lead to a diseased state. One diseased state seen in the geriatric population is sarcopenia. Sarcopenia is considered the loss of muscle mass due to aging (Cruz-Jentoft et al., 2010; Harris, 1997; Morley et al., 2001). However, it has been suggested that the loss of muscle mass cannot fully explain the loss of muscle strength; the relative losses of muscle mass and muscle strength are not linear (Clark & Manini, 2008; Delmonico et al., 2009). Finding the right balance between protein breakdown and synthesis is important for maintaining muscle mass. Both HMB and physical activity, individually and in combination, have been found to possibly play a role in protein breakdown and synthesis. This study will extend this previous work by examining the effects of HMB and physical activity on muscular force production (strength), as well as, the better-studied mass and markers of muscle protein metabolism in a rat model of aging.
Chapter 3: Methods

Study Population

In this study, 40 \((n = 40)\), 22-month-old male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) were used. However, only 34 rats completed the study. The rats were divided into two groups. The first group of rats \((n = 20)\) had access to water, food, and a running wheel \(ad libitum\). The second group of rats \((n = 20)\) did not have access to the running wheel after the start of the experiment, but had access to water and food \(ad libitum\). All rats were housed in a room on a 12-hour light-dark cycle. This experimental protocol was approved and follows the guidelines of Ohio University’s Institutional Animal Care and Use Committee policy (see Appendix D).

Acclimation Period

The acclimation period consisted of a diet of regular rat chow and continuous access to the running wheels for both groups. All animals spent 2.5-3 weeks on the standard chow. Following this period, the rats in the first group were switched to a purified diet (Harlan Teklad, Indianapolis, IN, USA) for about 4 weeks, while still having continuous access to the running wheels. The second group started the study after being on the regular chow, and the running wheels were removed at this point.

Voluntary Wheel Running

The rats in group 1 had running wheels attached to their cages. Wheel use was monitored by Activity Wheel Counter, Model 86061, interfaced with the Activity Wheel Monitoring System software (Lafayette Instrument Co, Lafayette, IN). The time of day the rat exercised, the distance in meters, and the speed of the wheel in meters per second
were monitored to show the voluntary activity of the rats, as the animals were not forced to exercise. Group 2 only had running wheel access during the acclimation period.

**Dietary Nutrition Supplementation**

The rats were divided into nutritional diet groups, with 20 rats (n = 10 group 1, n = 10 group 2) receiving the purified diet (AIN-93M) (see Appendix C) and 20 rats (n = 10 group 1, n = 10 group 2) receiving the purified diet plus a nutritional supplement of HMB + β-Alanine (Harlan Teklad, Indianapolis, IN, USA). Diets were color coded, so that the investigator performing the contractile testing (DWR) did not know to which diet an animal had been assigned. Thus, there were 4 treatment groups: wheel running + control diet (R), sedentary + control diet (C), wheel running + experimental diet (RD) and sedentary + experimental diet (D) (see Table 1). The animals assigned to treatment groups were matched for running activity in the acclimation period, which prevented all the avid runners from being placed within the same group. The food was weighed before making it available to the rats, and then weighed at the same time, two times a week, to track the amount of food being consumed by the rats on a weekly basis. Food pellets were pulled out of the bedding and weighed when noticed. The rats were weighed weekly as a way to monitor health. Also, beginning and ending weights were taken to show changes in body mass. The first group was on the experimental diet for about 4 weeks, while the second group was on the experimental diet for about 8 weeks. The study was extended to about 8 weeks as it might take longer for HMB + β-Alanine to exhibit changes without the stimulus of physical activity.
Contractile Testing

Force production. At the end of the dietary intervention, rats were anesthetized through an intraperitoneal injection of a Ketamine:Xylazine (40:10 mg kg\(^{-1}\) body mass, respectively), weighed, and prepared for contractile testing. While anesthetized, the skin covering the posterior lower leg and popliteal fossa and the tissue overlying the plantarflexor muscles was pulled back, and the calcaneus was cut with a bone clipper, in a way that left the Achilles tendon intact. The lateral gastrocnemius (LG), soleus, and plantaris were cut away from the Achilles tendon, leaving only the medial gastrocnemius (MG) muscle attached to the calcaneus (Russ et al., 2014). The calcaneus was attached in a custom made metal clamp, which was attached to the force transducer. In preparation, the sciatic nerve was exposed at the hip, and the animal positioned so that the lower limb was stabilized at the ankle, knee, and hip. Optimal length of twitch force (\(L_o\)) production was determined. A pool of mineral oil was formed in the popliteal fossa, and the temperature of the muscle was maintained through radiant heat targeted at the mineral oil pool. The sciatic nerve was stimulated at a supramaximal intensity (120% stimulation intensity that invokes a maximal twitch response) with a hook electrode (Harvard Apparatus, Holliston, MA, USA) at pulses of 200 μs from a Grass S48 stimulator (Astro-Med, Warwick, RI, USA), in order to produce muscle contractions. The MG muscle was activated with 10, 100 ms, 100-Hz trains at 1 train per 10 s, and then followed by 500 ms trains of 1, 5, 10, 25, 50, 75, 100, and 125 Hz at 1 train per 10 s. This protocol was done to reveal the force frequency relationship (Russ, 2011). Muscle quality was determined by taking force / CSA. CSA was calculated as described by Stone et al. (2007) CSA =
mass / [(L₀ x muscle to fiber length ratio) x (fiber density)] (Stone et al., 2007), using the muscle to fiber length ratio for the rat MG (Eng et al., 2008).

**Fatigability.** After testing force production, a period of 2 min of rest proceeded the fatigue testing. In the first group (R and RD groups), simulation was given at a rate of one 40 Hz, 330-ms train per s over a period of 3 min. After the 3 min of the fatigue test, the muscles received two testing trains consisting of 1 and 100 Hz (Russ & Lovering, 2006). In the second group (C and D groups), animals received 6 min of single twitches (1/s) and received testing trains of 40 and 100 Hz at the end of the protocol. The protocols were different for the groups because the 3 min protocol is extremely glycolytic, rapidly decreasing pH. It is possible that this rapid change overwhelmed the buffering capacity of carnosine. Thus, a switch to a less aggressive 6-min protocol was used to try to prevent this issue.

**Muscle Collection**

After testing for muscle force and fatigability, the MG, LG, and soleus muscles were removed from the rat. Results presented here are all related to the MG. The muscle was blotted dry, weighed, and then frozen in liquid nitrogen. After collecting the tissues, the animals were put down, while still anesthetized, by an intracardial injection of anesthesia (Euthasol, 100 mg kg⁻¹).

**Myosin Heavy Chain Analysis**

The MG muscle was used for analysis of muscle fiber types. The muscle tissue was brought to 4 °C. The MG was minced, while sitting on ice, and weighed (~ 20 mg). Extraction buffer (100mM Na₄P₂O₇; 5mM EGTA; 0.3 M KCl; 1 M Dithiothreitol; pH
8.5) was added to the test tube containing the tissue sample, in the amount of 1mL per 20 mg of muscle, and the tissue samples were homogenized in the extraction buffer until the muscle was broken down (PRO 200, PRO Scientific, Oxford, CT). The samples sat on ice for 20 min. Then the samples were centrifuged at 10,000 G at 4°C for 10 min, and the supernatant was collected. Total protein concentration of the samples was determined by using a Bradford Assay, with BSA as the standard. Samples were stored in a -40 °C freezer.

The myosin heavy chain samples were prepared by diluting the sample in sample buffer (1.0% 2-mercaptoethanol, 4.0% SDS, 16.0% 1.0 M Tris (pH 6.8), 20% glycerol, and 0.2% bromophenol blue) at a 1:2 ratio of sample to sample buffer. The diluted samples (20 μg) were separated at 150V and 10 mA for 24hrs on 8% gels containing 30% glycerol. The gels were stained with Coomassie Brilliant Blue R250. After destaining, the gels were scanned on the LiCor Odyssey system, in order to analyze the bands densitometrically (Russ et al., 2011).

**SDS-PAGE & Western Blotting**

The MG muscle was used for analysis of muscle protein MuRF1 levels. The muscle tissue was brought to 4 °C. The MG was minced, while sitting on ice, and weighed (~ 50 mg). Extraction buffer (10 mm sodium phosphate, pH 7.2, 2 mm EDTA, 10 mm sodium azide, 120 mm sodium chloride, 2% NP-40, plus protease inhibitors) was added to the test tube containing the tissue sample, in the amount of 1 mL to 50 g of sample, and the tissue sample was homogenized until the muscle was broken down (PRO 200, PRO Scientific, Oxford, CT). The samples sat on ice for 1 hr. They were
centrifuged at 14,000 G at 4 °C for 30 min, and then the supernatant was collected. Total protein concentration of the samples were determined by using a Bradford Assay (Pierce, Rockford, IL, USA), using a BSA standard.

Protein samples (40 μg) were separated on a SDS-PAGE 10% gel. Afterwards, the gel was transferred overnight at 4 °C to a polyvinylidene fluoride (PVDF) membrane. After the transfer, membranes were blocked for 1 hr at room temperature in blocking buffer (LiCor, Lincoln, NE, USA). After blocking the membrane, primary incubations happened overnight at 4 °C. The primary antibodies for MuRF-1, Total 4eBP1, Phosphorylated Ratio of 4eBP1, and EIF4E were diluted in blocking buffer at a ratio of 1:2000. Following primary incubations, membranes were rinsed 5 x 5 min in tris-buffered saline and Tween20 (TBS-T), and then incubated for 1 hr at room temperature in secondary antibody (IRDye, LiCor) diluted in blocking buffer at a ratio of 1:10,000. Afterwards, the membranes were rinsed again in TBS-T 5 x 5 min and TBS 1 x 5 min. Using the LiCor Odyssey system, membranes were scanned and analyzed via densitometry, and band intensities were normalized to the total protein per lane determined from the stained and scanned membrane (Russ et al., 2011).

**Statistical Analysis**

The analyses of muscle mass, muscle CSA, and MuRF-1 were performed using a two-way (diet x physical activity) fixed effects analysis of variance (ANOVA) with no repeated measures. Force and muscle quality were analyzed using a three-way (diet x physical activity x frequency) ANOVA with frequency as the repeated factor. Body mass was analyzed using a three-way ANOVA with time as the repeated factor. Analysis of
the MHCs was analyzed using a 2 x 2 (diet x physical activity) multivariate analysis of variance (MANOVA). Analysis of fatigue and running behavior was analyzed using unpaired \( t \)-tests. Post hoc testing was performed using Dunnett’s test in the event of significant main effects or interactions with the ANOVA or MANOVA. The post hoc testing compared all the treatment groups with the control group. Significance level for post hoc tests was set at \( p \leq 0.05 \), and \( p \leq 0.10 \) being considered a trend toward significance. All statistical tests were run using SPSS version 15 (SPSS Inc., Chicago, IL, USA), with a significance level set at \( p \leq 0.05 \). To aid in the interpretation of the findings, effect size (partial \( \eta^2 \) or Cohen’s \( d \)) of this study will be presented as an additional statistical parameter. Effect size of Cohen’s \( d \) is considered small at 0.2, medium at 0.5, and large at 0.8 (Hurley, Denegar, & Hertel, 2011; University of Colorado Colorado Springs). Effect size of partial \( \eta^2 \) is considered small at 0.01, medium at 0.06, and large at 0.14 (Nandy, 2012).
Chapter 4: Results

Experimental Animals

Thirty-four rats completed the study. Three rats from the first wave and three from the second wave died before completing the study. Two died in the first wave died during the acclimation period. One from the running and diet (RD) group died during the experiment. Two from the diet group (D) died before contractile and muscle harvesting was completed. One from the control group (C) died during the experiment. All the data for these animals were excluded from the results. This left the following breakdown by treatment group: C = 9, D = 8, R = 9, and RD = 8. The death rate in this study was typical for the age and strain of rat (Harlan Laboratories, 2013). There are some missing data points due to problems during data collection. The C group is missing one data point for each MHC fiber type, force, and muscle quality. The D group is missing two fatigue index data points. The R and RD groups are both missing one data point for CSA, optimal muscle length, and muscle quality.

Effects of Diet on Running

There was no significant difference between the groups in the total distance run ($p = 0.477$), absolute change in distance ($p = 0.077$), and percent change in distance ($p = 0.099$) (see Table 4).
Table 4

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>RD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total running distance (m)</td>
<td>5014.57 ± 3734.69</td>
<td>3802.06 ± 3017.37</td>
</tr>
<tr>
<td>Running distance absolute change (m)</td>
<td>67.87 ± 63.38</td>
<td>14.69 ± 50.14</td>
</tr>
<tr>
<td>Running distance % change</td>
<td>110.95 ± 143.85</td>
<td>18.67 ± 35.49</td>
</tr>
</tbody>
</table>

Note. Mean ± SD; R = Running group; RD = Running and Diet group.

Food Consumption, Body Weight, and Medial Gastrocnemius Muscle Weight

None of the main effects of diet, running, or diet x running interaction were significant in regards to the amount of food consumed and the starting and ending weight during the experimental period. However, the main effect of running had a significant effect ($p < 0.01; \eta^2 = 0.62; 1-\beta = 1.00$) on body weight change during the experiment, but the main effects of diet ($p = 0.879; \eta^2 < 0.01; 1-\beta = 0.05$) and the diet x running interaction ($p = 0.416; \eta^2 = 0.02; 1-\beta = 0.13$) did not. Post hoc testing showed a significant difference in the R ($p < 0.01$) and RD ($p < 0.01$) groups compared to the C group (see Table 5). The main effects of diet ($p = 0.087; \eta^2 = 0.10; 1-\beta = 0.40$), running ($p = 0.362; \eta^2 = 0.03; 1-\beta = 0.15$), and the diet x running interaction ($p = 0.186; \eta^2 = 0.06; 1-\beta = 0.26$) for CSA showed no significant difference (see Table 5). Also, the main effects of diet ($p = 0.191; \eta^2 = 0.06; 1-\beta = 0.25$), running ($p = 0.434; \eta^2 = 0.02; 1-\beta = 0.12$), and the diet x running interaction ($p = 0.432; \eta^2 = 0.02; 1-\beta = 0.12$) did not show significance with MG weight (see Figure 4).
Table 5

*Food Consumption, Body Weight, and CSA*

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>R</th>
<th>RD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average food consumed</td>
<td>19 ± 3</td>
<td>18 ± 1</td>
<td>17 ± 2</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starting body weight</td>
<td>535 ± 54</td>
<td>501 ± 48</td>
<td>543 ± 49</td>
<td>551 ± 48</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ending body weight</td>
<td>583 ± 73</td>
<td>544 ± 35</td>
<td>536 ± 57</td>
<td>551 ± 53</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight change</td>
<td>48 ± 26</td>
<td>44 ± 24</td>
<td>-7 ± 12</td>
<td>-0 ± 17</td>
</tr>
<tr>
<td>(g) #</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSA (cm²)</td>
<td>0.69 ± 0.09</td>
<td>0.71 ± 0.06</td>
<td>0.62 ± 0.10</td>
<td>0.72 ± 0.09</td>
</tr>
</tbody>
</table>

*Note.* Mean ± SD; *p* ≤ 0.05; # = significantly different with running; § = significantly different from C Group (*p* ≤ 0.05) after post hoc testing; C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.

*Figure 4.* Medial gastrocnemius muscle mass weights. Values are Mean ± SE. Main effects showed no statistical differences. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.
Contractile Force Production

There were no significant main effects or interaction effects of frequency x diet ($p = 0.530; \eta^2 = 0.02; 1-\beta = 0.11$), frequency x running ($p = 0.170; \eta^2 = 0.06; 1-\beta = 0.30$), frequency x diet x running ($p = 0.625; \eta^2 = 0.01; 1-\beta = 0.09$), diet ($p = 0.578; \eta^2 = 0.01; 1-\beta = 0.09$), running ($p = 0.775; \eta^2 < 0.01; 1-\beta = 0.06$), or the diet x running interaction ($p = 0.517; \eta^2 = 0.02; 1-\beta = 0.10$) were observed for contractile force (see Figure 5).

Figure 5. Plantarflexor muscle group contractile force measurements obtained during contractile testing. Values are Mean ± SE. Main effects showed no statistical differences. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.
**Muscle Quality**

There was a significant interaction effect for frequency x running \((p = 0.030; \eta^2 = 0.15; 1-\beta = 0.62)\), but not for frequency x diet \((p = 0.807; \eta^2 < 0.01; 1-\beta = 0.06)\) or frequency x diet x running \((p = 0.097; \eta^2 = 0.09; 1-\beta = 0.41)\). There was a significant main effect of the diet x running interaction \((p = 0.048; \eta^2 = 0.14; 1-\beta = 0.52)\), but not for diet \((p = 0.604; \eta^2 = 0.01; 1-\beta = 0.08)\) or running \((p = 0.757; \eta^2 < 0.01; 1-\beta = 0.06)\). Post hoc testing revealed trends toward differences between the C and R groups at higher frequencies \((75 \text{ Hz } [p = 0.070], 100 \text{ Hz } [p = 0.053], \text{ and } 125 \text{ Hz } [p = 0.062])\) (see Figure 6).

*Figure 6.* Calculated muscle quality values. Values are Mean ± SE. The main effect of frequency x running was significant (not shown). ‡ = trend toward significant difference between C and R Groups \((p < 0.1)\) with post-hoc testing. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.
Effects of Diet on Fatigue

Fatigue was not affected by the main effect of diet. No significant difference in the fatigue index was seen between the R and RD groups at 1 Hz ($p = 0.608$; Cohen’s $d = 0.3$), 40 Hz ($p = 0.686$; Cohen’s $d = 2.1$), and 100 Hz ($p = 0.284$; Cohen’s $d = 0.6$). Also, no significant difference in the fatigue index of the C and D groups at 1 Hz ($p = 0.778$; Cohen’s $d = 0.2$), 40 Hz ($p = 0.192$; Cohen’s $d = 0.8$), and 100 Hz ($p = 0.526$; Cohen’s $d = 0.4$) was observed (see Table 6).

Table 6

| Muscle Fatigue Values Obtained During Contractile Testing |
|-----------------------------|-------------|-------------|-------------|-------------|
|                             | C           | D           | R           | RD          |
| Fatigue index (1 Hz)        | $0.65 \pm 0.17$ | $0.61 \pm 0.30$ | $0.42 \pm 0.20$ | $0.48 \pm 0.18$ |
| Fatigue index (40 Hz)       | $0.31 \pm 0.03$ | $0.28 \pm 0.05$ | $0.25 \pm 0.10$ | $0.27 \pm 0.10$ |
| Fatigue index (100 Hz)      | $0.37 \pm 0.07$ | $0.35 \pm 0.07$ | $0.26 \pm 0.10$ | $0.31 \pm 0.11$ |

*Note.* Mean $\pm$ SD; $p \leq 0.05$. Fatigue could not be compared across all four groups. Different protocols used for Group 1 and Group 2. Group 1 consists of R and RD. Group 2 consists of C and D. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.

Protein Analysis

**Myosin heavy chain.** A significant main effect of running was present for MHC IIx ($p = 0.015$; $\eta^2 = 0.19$; $1-\beta = 0.70$) and MHC I ($p = 0.006$; $\eta^2 = 0.24$; $1-\beta = 0.83$). Post hoc testing revealed that there was a significant difference between C and RD groups for
MHC I fibers \((p = 0.034)\), and trends for differences between C and R for MHC I fibers \((p = 0.088)\) and C and RD for MHC IIx fibers \((p = 0.056)\) (see Figure 7).

**Figure 7.** MG MHC relative protein abundance obtained from MHC gels. Values are Mean ± SE. The main effect of running was significant for MHC IIx and I (not shown). § = significantly different from C Group \((p \leq 0.05)\); † = trend toward significantly difference from C Group \((p < 0.1)\) with post hoc testing. Inset in the graph is a sample of MHC bands obtained during the study. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.

**MuRF-1.** The main effects of diet \((p = 0.010; \eta^2 = 0.20; 1-\beta = 0.76)\) and running \((p = 0.028; \eta^2 = 0.15; 1-\beta = 0.61)\) were significant with MuRF-1 relative protein abundance, but the diet x running interaction \((p = 0.311; \eta^2 = 0.03; 1-\beta = 0.17)\) was not. Post hoc testing revealed that there was a significant difference between C and D \((p = 0.033)\) and C and RD \((p = 0.003)\), and that there was a trend toward a difference between C and R \((p = 0.055)\) (see Figure 8).
Figure 8. MuRF-1 relative protein abundance obtained from SDS-PAGE gels. Values are Mean ± SE. The main effects of diet and running were significant (not shown). § = significantly different from C Group \((p \leq 0.05)\); † = trend toward significantly difference from C Group \((p < 0.1)\) with post hoc testing. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.

**EIF4E and 4eBP1.** There was a significant main effect of running \((p = 0.029; \eta^2 = 0.15; 1-\beta = 0.60)\) on EIF4E relative protein abundance, but not diet \((p = 0.816; \eta^2 < 0.01; 1-\beta = 0.06)\) or the diet x running interaction \((p = 0.397; \eta^2 = 0.02; 1-\beta = 0.13)\). Post hoc tests indicated a trend towards a difference between the R and C groups \((p = 0.075)\) with EIF4E.

No significant main effects or interactions (diet \([p = 0.843; \eta^2 < 0.01; 1-\beta = 0.05]\); running \([p = 0.946; \eta^2 < 0.01; 1-\beta = 0.05]\); and diet x running \([p = 0.515; \eta^2 = 0.02; 1-\beta = 0.10]\)) were present for total 4eBP1 relative protein abundance. The same was found for the phosphorylated ratio of 4eBP1 (diet \([p = 0.568; \eta^2 = 0.01; 1-\beta = 0.09]\); running \([p =
0.411; $\eta^2 = 0.02; 1-\beta = 0.13$]; diet x running [$p = 0.726; \eta^2 < 0.01; 1-\beta = 0.06$]) (see Figure 9).

Figure 9. (A) MG EIF4E relative protein abundance. (B) MG phosphorylated ratio 4eBP1 relative protein abundance. (C) MG 4eBP1 relative protein abundance obtained from SDS-PAGE gels. Values are Mean ± SE. (A) The main effect of running was significant (not shown). ‡ = trend toward significantly difference from C Group ($p < 0.1$) with post hoc testing. (B) and (C) Main effects showed no statistical differences. C = Control group, D = Diet group, R = Running group, RD = Running and Diet group.
Chapter 5: Discussion

The purpose of this study was to find the relationship of nutritional supplementation and exercise, and their impact on muscle strength and morphology, with the intent of providing evidence that amino acid supplementation and exercise can be an effective way to promote gains in muscle mass and muscle quality in the geriatric population. This study showed a significant main effect of diet on MuRF-1 expression, but nothing else. In contrast, there were main effects of running on body weight change, MHC IIx and I composition, and expression of MuRF-1, and EIF4E. A number of interactions were present for muscle quality, with the overall pattern suggesting that animals that ran while on the control diet exhibited the highest muscle quality. Thus, our results suggest that that physical activity might have played a larger role then HMB + β-Alanine supplementation in affecting skeletal muscle.

Running Habits

In the groups that ran, it was noticed that the animals on the experimental diet ran less than those on the control diet (see Table 4). This was especially surprising since we matched animals for running activity prior to the switch to the experimental diets. It is possible that the RD group suffered from paresthesia, a side effect of β-alanine (Décombaz, Beaumont, Vuichoud, Bouisset, & Stellingwerff, 2012; Harris et al., 2006). As the concentration of β-Alanine in the bloodstream increases, some symptoms of paresthesia might become present. The reported sensations are the feeling of pins and needles, tickling, itching, and becoming flush (Décombaz et al., 2012; Harris et al., 2006). The majority of these symptoms seemed to happen in the trunk and arms in
humans (Décombaz et al., 2012). Paresthesia could have caused the rats to have feelings of pins and needles in their extremities, causing them to be more cautious when running or decrease the amount of running done. Using a slow release form of β-Alanine could have been one way to attempt to prevent these symptoms (Décombaz et al., 2012). These findings support the hypothesis that increased protein intake does not affect the amount of physical activity performed.

**Muscle Mass**

The mass of the MG did not differ among the groups. We found that dietary HMB + β-alanine supplementation had little effect on muscle mass, regardless of physical activity, and that physical activity, if anything, tended to lower muscle mass (see Figure 4). Pinheiro et al. (2012) also found that gastrocnemius muscle mass did not increase in rats supplemented with HMB compared to a control group (Pinheiro et al., 2012). Kovarik et al. (2010) saw similar results of no increase in muscle wet weight in the soleus and extensor digitorum longus muscles with HMB supplementation. However, other studies report results contrary to our findings, because they did see an increase in muscle or lean body mass (Baier et al., 2009; Flakoll et al., 2004; Pimentel et al., 2011).

The total body weight change of the rats did differ across groups. The C and D groups saw an increase in their average ending weight compared to their average starting weight. The R and RD groups saw a decrease in their average ending weight compared to their average starting weight (see Table 5). There are a number of potential explanations for these findings. First, there was an increase in overall muscle mass, as other muscles in the body could have seen an increase. This would not show in the
results, because we only looked at the mass of the MG. Second, there was an increase in body fat depots. The increase in weight was seen in the sedentary groups not the physically active groups. Kyle and colleagues (2004) found that fat-free mass index increased slightly in sedentary individuals. However, body fat was significantly higher in sedentary compared to physically active individuals, and body fat was also seen to be higher in the older compared to the younger individuals (Kyle, Genton, Gremion, Slosman, & Pichard, 2004). Further study on the effect of HMB and physical activity on body fat in aged rats could be done to determine whether this effect is replicable, or not.

**CSA and Muscle Quality**

Muscle quality is found by taking the force divided by the CSA. Significant diet x running and frequency x running interactions were observed for muscle quality. Although post hoc testing revealed only trends (see Figure 6), the interactions indicated that the highest muscle quality was observed in the R group (at tetanic frequencies). Given the great similarity among the absolute forces generated across all 4 groups (see Figure 5), differences in muscle CSA likely accounted for the differences in muscle quality. Although there was only a trend ($p = 0.077$) for group differences in CSA, the R group had the lowest mean CSA, consistent with the muscle quality data.

There are two ways that muscle quality can decrease. First, if one decreases the force and increases the CSA, the resulting muscle quality value goes down. The CSA of the C group was greater than the R group and the C group produced less force compared to the R group. This could mean that the fibers of the R were functioning at a higher level than the C group. Second, if one keeps the force constant but increases the CSA,
the resulting value goes down. This is seen between the R group and the D and RD groups. The smaller CSA of the R group, along with similar forces to the D and RD groups, caused the muscle quality value to be higher in the R group compared to the D and RD groups. The reason for this higher value in the R group is that less muscle was producing the same amount of force. This could mean that the fibers of the R were also functioning at a higher level than the C, D, and RD groups. This suggests that just because a muscle is larger does not mean that it is stronger. This is similar to what is seen in studies done with Myostatin deficiency, as the muscle is bigger in these subjects but produces less force (Amthor et al., 2007; Mendias, Kayupov, Bradley, Brooks, & Claflin, 2011).

**Skeletal Muscle Fatigability**

There was no effect of diet observed with fatigue (see Table 6). This suggests that β-Alanine did not have an effect on the MG during the fatigue protocol. This is opposite of what was observed in other studies (del Favero et al., 2012; McCormack et al., 2013; Stout et al., 2008). However, it is possible that the carnosine levels in the Type II fibers were low, which led to a decrease in buffering capacity (Tallon et al., 2007).

**Myosin Heavy Chain**

The fiber composition within a muscle tends to dictate the function of the muscle. A muscle comprised of mainly type I fibers can be a postural muscle and/or one that functions better in endurance activities. A muscle comprised mainly of type II fibers is one that functions in strength activities (Boirie, 2009). Elderly individuals’ muscle fibers
tend to convert to over to predominately type I fibers (Kent-Braun et al., 2002; Larsson et al., 1979).

There were no main effects observed for MHC IIa and IIb fibers. However, the results suggest that the percentage of Type IIa was relatively the same across the fibers. Results also suggested that there was a nonsignificant increase in the percentage of MHC IIb fibers. The control group had the smallest value and IIb fibers increased in the following order: D, R, and RD. However, the main effect of running was seen to have an effect on MHC IIx and I fibers. Overall, there was a decrease in the percentage of MHC IIx fibers compared to the C group. The control group had the highest value and it decreased in the following order: D, R, and RD. None of these changes were significant with post hoc testing, but there was a trend toward significance seen in the RD group compared to the C group. An increase was seen in the percentage of MHC I fibers compared to the C group. In post hoc testing, the D group showed a nonsignificant increase; the R group displayed a trend toward a significant increase; and there was a significant increase in the RD group compared to the C group (see Figure 7).

The percentage increase of Type I fibers in the RD group means that the muscle contains a higher proportion relative to the other fibers in the muscle compared to the C group. It is possible that the increase in the relative content of MHC I fibers occurred in this group because the rats were involved in physical activity. The physical activity could have emulated endurance exercise, and endurance exercise has been found to lead to a shift in muscle fibers from Type II, fast twitch, to Type I fibers, slow twitch (Larsson et al., 1979; Short et al., 2005). Even though the muscle is increasing in the percentage of
Type I fibers compared to the control, it does not necessarily mean that skeletal muscle contractile properties will slow. Instead, one has to consider how all the fiber types are shifting and adapting to the stimulus (Seene, Alev, Kaasik, Pehme, & Parring, 2005). There was also a trend toward a decrease in Type IIx fibers in the RD group compared to the C group. However, in this study, the RD muscle was still predominately Type IIb fibers, and Type IIa did not see a change. Exercise has also shown to shift from a faster Type II fiber to a slower Type II fiber (Sullivan et al., 1995). Coggan and colleagues (1992) found a decrease in the percentage of Type IIb fibers (Type IIx because it is a human study) and an increase in the percentage of Type IIa, but did not see a change in percentage of Type I fibers (Coggan et al., 1992). Seene and colleagues (2007) suggested that with endurance exercise there is a decrease in Type IIb fibers in the extensor digitorum longus and plantaris (Seene, Alev, Kaasik, & Pehme, 2007). In some ways the MG adapted similar to endurance exercise, the shift toward Type I fibers. But in other ways, it did not, since changes and decreases in slower type II fibers were not seen.

It could still be likely that the MG will behave like a fast-twitch glycolytic fiber and show fatigue and a greater production of force. Because the study was unable to look at fatigue between the R and RD groups and the C group, it is unknown how the R and RD muscles behaved compared to the C group. The C group displayed lower force production at the higher frequencies, but it was not significantly different than any of the groups. Because Type IIx fibers saw a shift down, Type I fibers saw a shift up, and there was a small shift up of Type IIb fibers in relation to the C group, this could possibly be canceling out the effects of each other creating no significant difference in force.
Protein Markers

MuRF-1. One protein marker highlighting protein breakdown is MuRF-1. A decrease in MuRF-1 activity levels suggests that protein breakdown is being inhibited (Bodine & Baehr, 2014; Castillero et al., 2013). An increase in MuRF-1 levels in skeletal muscle samples suggest that protein breakdown is occurring at a faster rate (Bodine & Baehr, 2014).

The effect of diet and physical activity were mutually exclusive in their roles of altering MuRF-1 activity levels because there was no significant difference seen with the interaction factor. However, a decrease in MuRF-1 activity was observed in the MG in the D, RD, and R groups compared to the C group (see Figure 8). This decrease in MuRF-1 levels suggests that the breakdown of skeletal muscle was less in the treatment groups compared to the control group, and that HMB and physical activity played a role in reducing the activity level of ubiquitin-proteasome pathway. The results are similar to many studies that found that HMB plays a role mainly in inhibiting protein breakdown (80%) and slightly increasing protein synthesis (20%) (Aversa et al., 2012; Gropper & Smith, 2013; Nissen et al., 1996; Nissen & Abumrad, 1997; Wilkinson et al., 2013).

Similar to our findings, Fernandez-Gonzalo, Lundberg, and Tesch (2013) found lower levels of MuRF-1 in a trained state compared to an untrained state in muscle after completing an single bout exercise protocol (Fernandez-Gonzalo, Lundberg, & Tesch, 2013). However, contrary to our findings, Stefanetti et al. (2014) saw an increase in MuRF-1 mRNA after endurance exercise training and single endurance exercise sessions,
but they did not see any changes in the MuRF-1 protein level with either of these types of training.

The inhibition of proteolysis could potentially play a role with muscle mass and muscle quality. Even with the inhibition of proteolysis, MG muscle mass was not significantly different between any of the treatment groups compared to the control (see Figure 4). The groups that saw a significant decrease in MuRF-1 (D and RD) had similar muscle quality to the control group. The R group showed only a trend toward a decrease in MuRF-1, but also trended toward significance compared to the C group in muscle quality. This could mean that the old dysfunctional muscle is not being broken down in the D and RD groups as much as the R group. An increase in dysfunctional contractile proteins could lead to decreased force production compared to functional contractile muscle. This would impair muscle quality (Marzetti, Lees, Wohlgemuth, & Leeuwenburgh, 2009; Masiero & Sandri, 2010; Russ, Gregg-Cornell, Conaway, & Clark, 2012). The decrease of protein turnover can also increase the possibility of posttranslational modification of oxidation and glycation (Degens, 2007).

**EIF4E and 4eBP1.** The main effect of running showed significance with EIF4E. With post hoc testing, a trend in EIF4E concentrations was seen in the R group compared to the C group (see Figure 9). This could suggest that HMB did not have a major effect on the protein synthesis pathway. However, contrary to this, Eley and colleagues (2007) noted an increase in 4ePB1 phosphorylation and an increase in EIF4E•EIF4G complex with HMB supplementation (Eley, Russell, Baxter, Mukerji, & Tisdale, 2007). Because the R groups seemed to be affected to a greater extent, this study suggests that running
might cause some phosphorylation of 4eBP1 and release of EIF4E. However, during exercise, it is suggested that protein synthesis happens through regulation of a different pathway than mTOR, as endurance exercise activates AMPK, which inhibits mTOR signaling (Serino et al., 2011; Williamson et al., 2006). It is possible that phosphorylation of EIF4E happens through activating ERK1/2 instead of mTOR (Williamson et al., 2006). An alternate pathway is suggested because no increase was seen in 4eBP1 phosphorylation in these other studies (Serino et al., 2011).

**Summary**

As expected, HMB + β-Alanine inhibited MuRF-1 expression. However, dietary supplementation did not show significant effects on the other interventions. In contrast, physical activity showed a greater variety of physiological effects, including affecting MHC IIx and I fiber relative protein abundance, reduction of body weight, improved muscle quality, enhanced EIF-4e expression, and reduced MURF-1 expression. Further study needs to be done to determine whether a more structured exercise protocol would result in similar changes and whether endurance versus resistance exercise would have differential effects. Further study could examine the effect of fatigue across all groups and not just the variable of diet. Exploring other mechanisms along with diet and physical activity might be the next step in determining the relationships among the mechanisms that cause sarcopenia.
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Appendix A: Myosin Extraction

Extraction Buffer Solution

100 mM Na$_4$P$_2$O$_7$ (decahydrate)
5 mM EGTA
0.3 M KCl
1 mM Dithiothreitol (DTT)
pH the solution to 8.50

Extraction Procedure

1. Using the liquid nitrogen cooled mortar and pestle, pulverize muscle, scrape the powder out, and weigh out approximately 20 mg.
2. Add to that 1 mL extraction buffer per 20 mg of muscle
3. Homogenize the muscle to mix with the buffer solution.
4. Rest on ice for 20 minutes
5. Centrifuge at 10,000G at 4°C for 10 minutes
6. Collect and keep the supernatant (contains the myosin fraction)
Appendix B: Myosin Heavy Chain Gel Protocol

Sample Buffer

1. Combine the following:
   a. 1.0% 2-mercaptoethanol
   b. 4.0% SDS
   c. 16.0% 1.0 M Tris (pH 6.8)
   d. 20% glycerol
   e. 0.2% bromophenol blue

6x Buffer Solution

1. 6x Buffer Solution Recipe
   a. 0.6 M Tris
   b. 0.9 M Glycine
   c. 0.6% SDS
   d. Distilled H₂O
2. To make 1 Liter of solution, combine 72.7g Tris, 67.6g Glycine, and 6g SDS. Add distilled water to around 700-800 mL and stir. Once the granules are dissolved, bring solution up to 1 L with distilled water.
3. Create Upper Chamber Buffer (UCB) and Lower Chamber Buffer (LCB) Solution
   a. Dilute the 6x Buffer Solution to a 3x Solution (UCB).
   b. Dilute the 6x Buffer Solution to a 1x Solution (LCB).

Coomassie Blue Stain

1. Ingredients
   a. 500 mL Methanol
   b. 500 mL Water
   c. 100 mL Acetic Acid
   d. 2.75 g Coomassie Brilliant Blue R250
2. Mix all of the above ingredients, including Coomassie, in a 1000mL container. Place in a stir bar and mix. Set up filter and vacuum flask. Filter through whatman #1 filter paper (cut paper to fit) and hook up to vacuum-to speed filtering. Be sure not to fill above spout! Pour into Coomassie Stain jar. Rinse equipment thoroughly.

Destain 1

1. Combine all of the above ingredients
   a. 500 ml Methanol
   b. 500 ml H₂O
   c. 100 mL Acetic Acid
Destain 2

1. Combine all of the above ingredients
   a. 100 ml Methanol
   b. 1800 ml H2O
   c. 100 ml Acetic Acid

Separating Gels

1. Combine the following ingredients:
   a. 3 mL Glycerol
   b. 1.98 mL 40% Acrylamide
   c. 0.4 mL 2% Bis
   d. 1.333 mL 1.5M Tris
   e. 1 mL 1M Glycine
   f. 0.4 mL 10% SDS
   g. 1.78 mL Distilled Water
   h. 5 uL TEMED

2. Spin 2 minutes until the Glycerol has dissolved in the liquid solution.
3. Degas for 20 minutes.
4. Add 0.1 mL 10% APS.
5. Use a pipette to pour gel into casing. Add 0.1% SDS overlay.
6. Let gel set for approximately 1 hour.
7. Use a small tip to vacuum overlay from the set separating gel, and blot dry with filter paper strips.

Stacking Gel

1. Combine the following ingredients:
   a. 3 mL Glycerol
   b. 1.333 mL 30% Acrylamide-bis
   c. 1.4 mL 0.5M Tris
   d. 0.4 mL 100mM EDTA (pH 7)
   e. 0.4 mL 10% SDS
   f. 3.362 mL Distilled Water
   g. 5 uL TEMED

2. Spin 2 minutes until the Glycerol has dissolved in the liquid solution.
3. Degas for 20 minutes.
4. Place comb in the casting and use pipette to pour gel into casing.
5. Let gel set at least one hour.
6. Remove the combs once the stacker is set.
7. Clean the wells before adding sample. This is done by pipetting UCB over the wells, and then siphoning it off. Repeat that process 2x. However these times also using a syringe to mix the UCB to get the excess film out of the wells.
8. Add UCB to the wells in the casing and part of the cassette.
9. Add samples already diluted 2:1 (buffer:sample) into designated wells
10. Put together the cassette system. Put LCB around the cassette almost to the top of the glass plates. Place in the refrigerator and top off the UCB and LCB if needed.
11. Add 144 uL BME to the UCB on each side.
12. Run gel at 150 Volts and 10 mA for 24 hours.
13. Stain the gel
   a. 45 minutes to 1 hour in the stain
   b. 30 minutes in destain 1
   c. Overnight in destain 2
### Appendix C: Purified Diet Ingredients (AIN-93M)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>140.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>1.8</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>465.692</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>155.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>40.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral Mix, AIN-93M-MX (94049)</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin Mix, AIN-93-VX (94047)</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>TBHQ, antioxidant</td>
<td>0.008</td>
</tr>
</tbody>
</table>

This information can be obtained from the following article: AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet (Reeves, Nielsen, & Fahey, 1993).
Appendix D: IACUC Approval

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The Ohio University Institutional Animal Care and Use Committee (IACUC) has granted approval for the animal research protocol indicated below:

Protocol Number: 12-H-064
New e-protocol number: 12-H-064
Title: Effects of nutritional supplementation on in situ muscle strength and fatigue resistance in aged rats

Approval Date: 1/17/2013 Through 1/16/2016
Primary Investigator: David Russ

This approval is valid only for the personnel, procedures and species reviewed and approved by the IACUC. Any changes must be submitted as an addendum and approval granted prior to initiating the change.

Ohio University is fully accredited by AAALAC, Int. The NIH Assurance number is A3610-01 and the USDA license number is 31-R-082

Jo Ellen Sherow, Director, Office of Research Compliance
Date

cc: Scott Carpenter, Associate Director, Laboratory Animal Resources