AJUGA TURKESTANICA AS A COUNTERMEASURE AGAINST SARCOPENIA AND DYNAPENIA

A Thesis
by
MARCUS MICHAEL LAWRENCE

Submitted to the Graduate School
Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2012
Department of Health, Leisure and Exercise Science
FOREWORD

The research detailed in this thesis will be submitted as a research article to *Muscle & Nerve*, an internationally peer-reviewed journal owned by the American Association of Neuromuscular and Electrodiagnostic Medicine and published by John Wiley & Sons. The thesis has been prepared according to the guidelines set forth by the Graduate School of Appalachian State University.
ABSTRACT

AJUGA TURKESTANICA AS A COUNTERMEASURE AGAINST SARCOPENIA AND DYNAPENIA. (August 2012)

Marcus Michael Lawrence, B.S., California State University Monterey Bay

M.S., Appalachian State University

Chairperson: Kevin A. Zwetsloot

Protein synthesis signaling through the PI3k-Akt pathway and mitochondrial biogenesis signaling through the AMPK and p38-MAPK pathways are reduced in aged skeletal muscle, leading to the loss of muscle mass and strength. Phytoecdysteroids, in particular 20-hydroxyecdysone (20E), from the plant Ajuga turkestanica increases protein synthesis in C2C12 skeletal muscle cells and muscle strength in young rats. The objective of this study was to determine if an extract from A. turkestanica (ATE), enriched in phytoecdysteroids, affects muscle fiber size, weight, and strength and PI3k-Akt, AMPK, and p38-MAPK signaling in skeletal muscle of aged mice. Aged male C57BL/6 mice (20-months) received ATE, 20E, or control (CT; vehicle only of 100% non-denatured ethanol) for 28 days. No significant differences (NSD) were observed between treatments in body, muscle, or organ mass, as well as during the in vivo contractile function (i.e., strength and endurance) of the gastrocnemius-plantaris-soleus complex. Hemotoxylin and eosin staining revealed 41% and 30% larger fiber cross-sectional area (CSA) in the triceps brachii ($p = 0.014$) and plantaris ($p = 0.018$) muscles of 20E-treated sedentary mice, respectively, compared to CT. ATE treatment resulted in larger, but NSD in CSA of the triceps brachii
(26%; $p = 0.088$) and plantaris (8%; $p = 0.497$) muscles, compared to CT. Western blotting performed on gastrocnemius muscles demonstrated NSD between treatments in non-contracted and contracted phosphorylation of protein synthesis signaling markers (i.e., Akt$^{\text{Ser473}}$ and p70S6k$^{\text{Thr389}}$). NSD were observed between treatments in non-contracted phosphorylation of upstream mitochondrial biogenesis signaling markers (i.e., p38-MAPK$^{\text{Thr180/Tyr182}}$ and AMPK$^{\text{Thr172}}$) and contraction-induced phosphorylation of p38-MAPK$^{\text{Thr180/Tyr182}}$. Additionally, contraction-induced phosphorylation of AMPK$^{\text{Thr172}}$ was significantly greater in 20E-treated mice when compared to CT and ATE, respectively. In conclusion, these data suggest that 20E rescues the loss of muscle fiber size and increases activation of contraction-induced AMPK pathway signaling in sedentary aged skeletal muscle.
DEDICATION

To my thesis committee chairperson, Dr. Kevin A. Zwetsloot, thank you for all of your guidance and assistance throughout my graduate career at Appalachian State University. You have been and will continue to be an amazing teacher, mentor, and friend. You are truly an inspiration, and I hope to be half the mentor, teacher, and friend to my future students that you were to me. You have established the foundational framework for me to excel in not only any aspect of research and teaching, but in my life as well.

To the members of my thesis committee, Dr. R. Andrew Shanely and Dr. Susan Tsivitse Arthur, thank you for all of your excellent guidance and review throughout this entire process. Dr. Shanely, I am grateful for your time, expertise, attention to detail, and critical analysis. You have given me the necessary tools to advance into my doctoral work and eventually my career. I will forever strive to be as attentive to detail as “overkill Andy.” Dr. Arthur, I am appreciative of your expertise, love for research, and kindheartedness. I look forward to beginning my doctoral work knowing that I will have your support and guidance.

To my other half, Marissa, thank you for showing me true happiness and being my constant support. I am very enthusiastic to start my doctoral work and eventually my career knowing that I will have you by my side. Finally, to my family, Mom, Dad, and Katie, thank you for all of your unconditional love and support. You are the reason I am where I am today, and I will forever be in your debt for your endless past, present, and future love and support!
ACKNOWLEDGMENTS

I would like to extend my gratitude to Dr. Mary Ann Lila and Dr. Mary Grace for their assistance with the *Ajuga turkestanica* extract, Ian Cooley and Dr. Susan Arthur for their assistance with the cryoSTAT sectioning, Dr. David Nieman and Dr. Amy Knab for their financial assistance and guidance throughout the project, Casey John, P. Colton Tessener, B.A. Ray, Justin Via, Prescott Lederer, Kelsey Hargrove, Colin Romoda, and Dustin Westbrook for their time and assistance with fiber CSA analysis, Josh Saunders and Josh Samuels for their time and assistance with the extraction and supplementation protocol, and P.L. Thomas, Inc. for graciously donating the 6% *Ajuga turkestanica* plant material. I would also like to thank the Office of Student Research, Cratis D. Williams Graduate School, and the Graduate Student Academic Senate for partial financial assistance in conducting this research project. In addition, I am grateful for the support provided to me through a fellowship from the Agriculture and Food Research Initiative Grant number 2010-65200-20354 from the USDA National Institute of Food and Agriculture.
# TABLE OF CONTENTS

Abstract ........................................................................................................................................ iv
Dedication ....................................................................................................................................... vi
Acknowledgments ......................................................................................................................... vii
List of Tables ................................................................................................................................ ix
List of Figures .............................................................................................................................. x
Chapter 1: Introduction and Literature Review .......................................................................... 1
Chapter 2: Experiment .................................................................................................................. 26
  Materials and Methods ............................................................................................................. 29
Chapter 3: Results ........................................................................................................................ 39
Chapter 4: Discussion ................................................................................................................... 43
References ..................................................................................................................................... 49
Appendix A: Institutional Animal Use and Care Committee Documents ................................. 83
Vita .................................................................................................................................................. 86
LIST OF TABLES

Table 1. Animal mass data........................................................................................................ 69
Table 2. Muscle wet weight data of non-contracted hindlimb............................................... 70
Table 3. Organ wet weight data .............................................................................................. 71
Table 4. In vivo contractile function data of the gastrocnemius-plantaris-soleus (GPS) complex..................................................................................................................... 72
LIST OF FIGURES

Figure 1. *Ajuga turkestanica* 1st batch flow chart ................................................................. 73

Figure 2. *Ajuga turkestanica* 2nd batch flow chart ................................................................. 74

Figure 3. Experimental design schematic .................................................................................. 75

Figure 4. HPLC chromatogram of the final *Ajuga turkestanica* extract (ATE) ..................... 76

Figure 5. ATE and 20E phytoecdysteroid contents ................................................................. 77

Figure 6. Plantaris and Triceps Brachii fiber cross-sectional area (CSA) ............................... 78

Figure 7. Akt and p70S6k resting and contracted phosphorylation ........................................ 79

Figure 8. Akt and p70S6k resting and contracted total protein content ................................... 80

Figure 9. p38-MAPK and AMPK resting and contracted phosphorylation ............................ 81

Figure 10. p38-MAPK and AMPK resting and contracted total protein content .................... 82
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

With the growing number of “baby-boomers” (those born between 1946 and 1960) getting older in the United States of America (USA), there is an ever increasing number of older adults living longer. Although this speaks positively for modern-day health care, a large percentage of this population is unable to perform independent activities of daily living (ADLs). The inability to perform ADLs independently increases the burden on the country’s health care system to care for these individuals. The large majority (1 in 5) of this population suffers from chronic conditions, such as sarcopenia and dynapenia, both of which can contribute to reductions in ADL.

The term sarcopenia (from the Greek words sарx [flesh] and penia [loss], translating to the “poverty of flesh”) is defined as the age-associated loss of skeletal muscle mass. The term dynapenia (from the Greek words дynami [strength] and penia [loss], translating to the “poverty of strength”) is defined as the age-associated loss of skeletal muscle strength. These two chronic conditions often arise from or lead to a decrease in physical activity which leads to an increased susceptibility to other chronic disorders such as insulin resistance, type 2 diabetes, cardiovascular disease, and even death. With the loss of muscle mass and strength with age, there is typically a reduction in physical activity which then accelerates the loss of muscle mass and strength and creates a vicious cycle that continuously builds on one another and can eventually lead to death. Therefore, there is a critical need to understand
the mechanisms of sarcopenia and dynapenia in order to create viable countermeasures that can attenuate the negative outcomes associated with the two conditions.

Older adults have been found to have a blunted ability to promote muscle protein synthesis (MPS) as well as mitochondrial biogenesis.\textsuperscript{6-9} Both of these factors have been found to have a large effect on the age-associated loss of muscle mass and strength. Blunted MPS in older adults is due, in large part, to reductions in signaling pathways that regulate MPS, amino acid and protein intake, hormone levels, and physical activity.\textsuperscript{8,9} Whereas, blunted mitochondrial biogenesis in older adults is due, in large part, to reductions in signaling pathways directly involved in mitochondrial biogenesis, upregulation of reactive oxygen and nitrogen species (RONS), as well as reductions in antioxidant enzyme content and activity.\textsuperscript{6,7} Further research is needed to devise optimal countermeasures for improving MPS and mitochondrial biogenesis with aging.

\textit{Ajuga turkestanica} is a plant known to have adaptogenic properties (i.e., enhances mental and physical performance capabilities by promoting vitality and increasing the organism’s resistance to stress and aging).\textsuperscript{10} \textit{A. turkestanica} contains bioactive components that have the potential for being a countermeasure against sarcopenia and dynapenia. One possible mechanism of investigation for \textit{A. turkestanica} as a countermeasure against sarcopenia and dynapenia is through measuring the effects of \textit{A. turkestanica} on MPS and mitochondrial biogenesis signaling with aging. In summary, sarcopenia and dynapenia are two chronic conditions that affect the global economy and are caused, in part, by reductions in MPS and mitochondrial biogenesis signaling. \textit{A. turkestanica} presents itself as a proposed countermeasure against sarcopenia and dynapenia and will be examined in that capacity by
determining *A. turkestanica*’s effects on MPS and mitochondrial biogenesis signaling with aging.

The goal of this literature review is to provide background information on sarcopenia and dynapenia, to examine the role of MPS and mitochondrial biogenesis signaling in sarcopenia and dynapenia, and to review current countermeasures used to prevent or reverse the effects of sarcopenia and dynapenia. Finally, *A. turkestanica* will be reviewed as a proposed countermeasure against sarcopenia and dynapenia.

**LITERATURE REVIEW**

**Sarcopenia and Aging.** Estimates of the prevalence of sarcopenia in the USA range from 13% to 24% in adults over 60 years of age to more than 50% in persons aged 80 and older.\(^{11}\) The estimated direct healthcare costs attributable to sarcopenia in the USA in 2000 were $18.5 billion ($10.5 billion in men, $7.7 billion in women), which represented about 1.5% of total healthcare expenditures for that year.\(^{12}\) From the age of 20 to 80 years old, there is approximately a 40% reduction in muscle mass and a 20% decline in muscle cross-sectional area.\(^{13}\) These losses can be attributed to a loss in both muscle fiber size and number.\(^{14}\) The most commonly used method for determining sarcopenia in an individual is by establishing a skeletal muscle mass index obtained by dividing appendicular skeletal muscle mass (ASM), evaluated by dual-energy X-ray absorptiometry (DEXA), by body height squared (i.e., ASM/ht\(^2\)).\(^{15}\) Older adults presenting a skeletal muscle mass index of 1-2 standard deviations below the mean of young control adults have class I sarcopenia (moderate); whereas, older adults with a skeletal muscle mass index of 2 or more standard deviations below the mean of young control adults have class II sarcopenia (severe).\(^{15}\) Sarcopenia affects 100% of the population, as everyone loses muscle mass over time.
However, the rate of decline in muscle mass is determined by the individual, as some individuals lose muscle mass faster than others.\textsuperscript{16}

Sarcopenia is a multifactorial process associated with genetic heritability,\textsuperscript{17} protein intake,\textsuperscript{17} energy intake,\textsuperscript{17} apoptosis,\textsuperscript{17} hormonal changes,\textsuperscript{17} insulin resistance,\textsuperscript{17} changes in proinflammatory cytokines,\textsuperscript{17} decreases in mitochondrial density,\textsuperscript{18} function and volume,\textsuperscript{18} and increases in RONS production.\textsuperscript{18} Sarcopenia typically correlates to a decrease in physical activity and a concomitant increase in physical frailty and even death.\textsuperscript{19} Baumgartner et al.\textsuperscript{20} demonstrated that the likelihood of having a physical disability was 4 times greater in sarcopenic older men and women than in older persons with a normal muscle mass.

**Dynapenia and Aging.** Dynapenia is a term coined\textsuperscript{4} to dissociate the loss of both muscle mass (i.e., sarcopenia) from the loss of muscle strength (i.e., maximal voluntary force output) alone. Since the original definition of sarcopenia was introduced in 1989 by Dr. Irwin Rosenberg\textsuperscript{3} (i.e., the age-associated loss of muscle mass and size) there has been an exponential increase in research pertaining to the mechanisms involved in the age-associated loss of muscle mass. In the past decade, the definition of sarcopenia has grown to include the age-associated loss of muscle mass as well as strength. Early epidemiological data on sarcopenic older adults linked the loss of muscle mass with the loss of muscle strength\textsuperscript{21}. However, recent research is demonstrating that this may not be the case.\textsuperscript{4,22-25} There is a definite overlap between the two conditions, therefore both conditions need to be examined together and not just solely examined independently.

Dynapenia is a multifactorial process that can be largely attributed to reductions in the neuromuscular system as well as reductions within the skeletal muscle system (i.e.,
sarcopenia). However, the latter system seems to play a smaller role in dynapenia than previously thought. There is currently no universal method for determining dynapenia, but the most recent method utilized determining muscle strength (kg) via dynamometer and dividing that value by appendicular skeletal muscle mass (kg) as determined by DEXA. The same authors also defined type I dynapenia, the only type of dynapenia accepted throughout the literature, as muscle strength (via hand dynamometer) as less than 1.53 kg per kg skeletal muscle mass. Unfortunately, there is not a definitive method for determining dynapenia, therefore, there is lack of epidemiological data to determine the prevalence of this condition.

The neuromuscular system, as opposed to the skeletal muscle system, is the greatest contributor to strength output, and this can be seen during the initial phase of adaptation to resistance training (RT). The initial increases in maximal voluntary force (i.e., strength) observed during the early adaptation period of RT (the first 2 to 6 weeks) are evident, independent of an increase in muscle mass, suggesting an increase in the synchronization and recruitment of neuromuscular factors. Stevens et al. compared the neuromuscular system’s ability to fully activate knee extensor muscles (i.e., maximal voluntary force) compared to maximal electrical stimulation of the same muscles in 46 younger and 46 older adults to determine if there was a difference in “added force” with the electrical stimulation above what the subjects were able to recruit voluntarily. The authors reported that there was an 11% reduction in central nervous system activation in older adults when compared to young controls indicating that older adults have a reduced ability to voluntarily activate muscle strength. Changes in the neuromuscular system with aging can be characterized by a loss of motor neurons (due to apoptosis), a decrease in motor unit number (a motor unit is
an α-motor neuron and all the muscle fibers that it innervates), and fiber type transformation (i.e., a selective loss of fast-twitch fibers with a concomitant increase in slower “hybrid” fibers). All of these factors contribute to dynapenia to a greater extent than just the loss of muscle mass alone.

The original longitudinal studies used skeletal muscle cross-sections and strength output to track the changes between the two measurements and to determine if there was a link between the two. The original data suggest that there was a link between the loss of muscle mass and strength, however, recent research is reporting that this may not be the case. For example, Goodpaster et al. measured appendicular skeletal muscle lean mass (via DEXA and computer tomography) and knee extensor strength (via isokinetic dynamometry) three years after an initial baseline measurement of the same variables in a group of 1880 older adults (age 70-79 years) and reported that the loss of lean mass was associated with the loss of strength; however, the rate of loss of strength was three times greater than the rate of loss of lean mass. The authors also demonstrated that a gain in lean mass was not accompanied by concomitant increases or maintenance of strength. In other words, greater precedence should be placed on the loss of muscle strength, than just the loss of muscle mass. Visser et al. demonstrated in the Health, Aging, and Body Composition study of 70-79 year old adults that the loss in muscle mass was associated with physical function decline; however, the degree of reduction in those variables (i.e., muscle mass and physical function) were both reliant on the level of strength of the individual; indicating that strength level was the key factor leading to reductions in muscle mass and physical function. To further dissociate sarcopenia and dynapenia, Newman et al. demonstrated that older adults with dynapenia had a 50% increase in all-cause mortality risk independent of
sarcopenia, but that older adults with sarcopenia did not have a significant increased all-cause mortality risk independent of dynapenia.

Sarcopenic factors, beyond just the loss of muscle mass, also play a role in reducing maximal voluntary force. These factors include changes in skeletal muscle architecture (i.e., infiltration of intramuscular lipids making the muscle intrinsically weak, and increases in rigidity of muscle proteins), fiber type transformations, and a reduction in excitation-contraction coupling (i.e., the process of muscle contractions from an action potential in the central nervous system to the actual contraction of the muscle myofilaments). This age-related decline in both the neuromuscular and skeletal muscle systems leads to impairments in maximal muscle strength, power, and rate of force development that translates into a reduction in physical function. The loss of strength and power is greater in the lower body than upper body and this is directly attributable to a decline in ADLs. Since the two chronic conditions (i.e., sarcopenia and dynapenia) can overlap and, in most cases, influence one another (although dynapenia may have a greater influence than sarcopenia), understanding the mechanisms by which the two chronic conditions act upon each other is of great relevance to overcome the burden that these two conditions place on our society.

**Regulation of Skeletal Muscle Mass and Aging.** It has been hypothesized that a contributing factor to the age-associated loss of skeletal muscle mass and strength is due to impairments in skeletal muscle protein homeostasis (i.e., the balance between MPS and muscle protein breakdown; MPB). The ability to maintain skeletal muscle protein homeostasis and, thus, overall lean mass relies on the pathways involved in both MPS and MPB to function properly. With age there appears to be reductions in MPS pathways and upregulation of MPB pathways leading to the loss of skeletal muscle mass and strength.
The major pathways involved in MPS and MPB that are associated with sarcopenia and dynapenia will be briefly reviewed including the interaction between the two pathways that contribute in large part to sarcopenia and dynapenia.

**PI3k-Akt Pathway and Aging.** The largest contributor to the loss of muscle mass (and in small part the loss of muscle strength) due to aging is a blunted response to promote MPS. MPS is controlled in large part by the phosphoinositide 3-kinase (PI3k)-Akt (or protein kinase B)-mammalian target of rapamycin (mTOR; or PI3k-Akt-mTOR) pathway. MPS through the PI3k-Akt-mTOR pathway can be stimulated via growth factors (i.e., insulin and insulin-like growth factor 1; IGF-1), nutrition (i.e., amino acids), and mechanical stimuli (i.e., resistance training; RT). The PI3k-Akt-mTOR pathway can also be activated by second messenger signaling via the Ca^{2+}-dependent G-protein coupled-receptor pathway (discussed below).

PI3k causes increases in phosphatidylinositol 3,4,5-triphosphate (PIP3), leading to recruitment of Akt from the cytosol to the sarcolemma and phosphorylation of Akt. Akt activation is a crucial step involved in MPS as well as reduction in MPB (discussed below). Activated Akt phosphorylates tuberous sclerosis protein 1/hamartin (TSC1) and tuberous sclerosis protein 2/tuberin (TSC2), or the TSC1/TSC2 complex that releases their inhibition on mTOR activation. Thus, Akt’s phosphorylation of the TSC1/TSC2 complex allows mTOR to induce its effects on MPS. Akt also phosphorylates glycogen synthase kinase-3β (GSK-3β), leading to its inhibition and, thus, upregulation of protein synthesis. GSK-3β inhibits eukaryotic translation initiation factor 2E (eIF-2E) which is involved in protein translation. Thus, inhibition of GSK-3β leads to increased MPS. Additional details of this pathway are beyond the scope of this review.
mTOR is a highly conserved serine-threonine kinase that is a key regulatory protein for processes such as cell proliferation, cell growth, energy homeostasis, substrate metabolism, and protein synthesis.\(^4\) The mTOR signaling cascade is a key control point for MPS and, thus, muscle growth (i.e., hypertrophy).\(^3\) Administration of the pharmaceutical mTOR inhibitor rapamycin has been shown to completely block MPS in response to resistance training (i.e., anabolic stimuli).\(^4\)

mTOR exists in two structurally and functionally different complexes that include mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).\(^4\) mTORC1 is a critical control point in MPS;\(^3\) whereas, mTORC2 is a regulator of insulin-stimulated glucose uptake in skeletal muscle.\(^4\) mTOR’s effects on MPS are directly linked to the phosphorylation of its downstream targets 70-kilodalton ribosomal S6 protein kinase (p70S6k) and eukaryotic translation initiation factor 4E-binding protein (4E-BP1; also known as PHAS-1).\(^4\) mTORC1 phosphorylates 4E-BP1, thereby enhancing translation. Typically, 4E-BP1 inhibits translation when it is bound to the eukaryotic translation initiation factor-4E (eIF-4E) and, thus, mTORC1 releases 4E-BP1 from its binding site allowing translation to occur.\(^4\) p70S6k is a crucial regulator of initiation of mRNA translational machinery of protein synthesis.\(^4\) Furthermore, in insulin stimulated skeletal muscle cells, p70S6k activation is inhibited by expression of the TSC1/TSC2 complex.\(^3\) The inhibition of p70S6k by the TSC1/TSC2 complex expression indicates that Akt must phosphorylate and, thereby, release the TSC1/TSC2 complex’s inhibition of mTOR in order for mTOR to phosphorylate p70S6k and initiate MPS translation. Thus, the PI3k-Akt-mTOR-p70S6k (PI3k-Akt) pathway is a crucial regulator of MPS.
Since the ability to gain or even maintain lean muscle mass (and strength, to a certain extent) is critically regulated by the PI3k-Akt pathway, it is necessary to understand how that pathway is affected by aging. There are conflicting findings in regards to resting MPS in older adults, as some studies have shown no differences in resting MPS\textsuperscript{47,48} when compared to young healthy controls, whereas others have demonstrated a decrease in resting MPS.\textsuperscript{49,50} Therefore, there is no consensus on the effects of resting MPS on aging. However, in response to anabolic stimuli (i.e., RT) there is a clear blunting of MPS with aging.\textsuperscript{8}

No differences have been found between resting PI3k-Akt signaling in older adults when compared to untrained young controls.\textsuperscript{51-53} However, in response to anabolic stimuli (i.e., RT) Fry et al.\textsuperscript{8} demonstrated that aged individuals were found to have a reduction in not only the phosphorylation of specific targets (i.e., mTOR, p70S6k, and 4E-BP1) within the PI3k-Akt pathway, but also in MPS (as measured by the amount of L-[ring-\textsuperscript{13}C\textsubscript{6}]phenylalanine incorporated into muscle biopsy samples) when compared to young controls. Fujita et al.\textsuperscript{52} administered supraphysiological and physiological (i.e., insulin infusion) doses to older adults postprandial (i.e., post-meal) and reported a reduction in the phosphorylation of Akt and p70S6k in the physiological dose experiment. This indicates that there is not only an age-related insulin resistance, but also that there are reductions within the PI3k-Akt pathway in response to anabolic stimuli.\textsuperscript{52} Other studies have also reported a reduced efficiency or blunting of phosphorylation within the PI3k-Akt pathway leading to reduced ability to promote MPS.\textsuperscript{54-56} Therefore, the loss of muscle mass and strength with aging may be due, in part, to a blunted response to proper activation of the PI3k-Akt pathway.
Muscle Protein Breakdown, PI3k-Akt, and Aging. Since the age-associated loss of muscle mass and strength is not only reliant on MPS, but also MPB, it is important to examine the interaction between the MPS and MPB pathways. The loss of muscle mass is determined by the net balance between MPS and MPB. There are at least four major pathways involved in MPB including lysosomal proteases (i.e., cathepsins), the Ca$^{2+}$-dependent calpain system, the caspase-dependent system and the ubiquitin-proteasome pathway (for review see$^{33,57}$). While all four of these pathways are significantly affected by aging$^{57}$ only the most predominant pathway, the ubiquitin-proteasome pathway that has cross talk with the PI3k-Akt pathway, will be reviewed.

The ubiquitin-proteasome pathway works by tagging a protein for degradation with attachment of single protein pieces or chains of the protein called ubiquitin and then transporting the ubiquitinated protein to the proteasome for degradation. Ubiquitin is added to proteins targeted for degradation in the proteasome by a process involving at least three classes of proteins called E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes.$^{33}$ Two E3 ligases have been clearly linked to muscle atrophy in skeletal muscle, Muscle Ring Finger 1 (MuRF1), and Muscle Atrophy F-box (MAFbx).$^{58}$ MuRF1 and MAFbx are activated by the forkhead box (FOXO) family of transcription factors as well as the nuclear factor kappa B (NF-$\kappa$B) pathway (discussed below).$^{59,60}$ Both MuRF1 and MAFbx are upregulated in almost all cases of muscle atrophy, which appears to be the result of an increase in the amount of FOXO family of transcription factors activation.$^{60}$ Bodine et al.$^{58}$ demonstrated that knocking out either MuRF1 or MAFbx spared the loss of muscle mass, indicating that both E3 ligases are crucial for MPB.
There is cross talk between the PI3k-Akt pathway and the ubiquitin-proteasome pathway. Akt not only indirectly activates mTOR, but it also has been shown to play a role in downregulating MPB by phosphorylating and, thus, inhibiting the activation of the FOXO family of transcription factors. Stitt et al. found that overexpression of Akt in skeletal muscle myotubes inhibited both the FOXO family of transcription factors activation and also decreased the expression of the E3 ligase MAFbx. This study indicates the importance of the PI3k-Akt pathway’s, specifically Akt, inhibition of MPB by phosphorylation of the FOXO family of transcription factors. One current theory of increased MPB with aging is that with reduced physical activity (i.e., mechanical stimuli) there is reduced phosphorylation of the FOXO family of transcription factors by Akt leading to upregulation of MuRF1 and MAFbx and thus MPB. Therefore, proper activation (i.e., the normal activation observed in healthy young adults) of the PI3k-Akt pathway is essential for preventing and even reducing the age-associated loss of muscle mass and strength.

Sarcopenia and dynapenia are not only associated with a depressed rate of MPS in response to anabolic stimuli, but have also been linked to a chronic increase in pro-inflammatory cytokines, specifically interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). Visser et al. demonstrated in the Health, Aging, and Body Composition study of 3075 older adults between 70-79 years old, that individuals with the highest levels of circulating pro-inflammatory cytokines, specifically IL-6 and TNFα, had the lowest levels of muscle mass and strength. IL-6 has been linked to the age-associated loss of muscle mass and strength. However, the effects of IL-6 on skeletal muscle has produced conflicting results. IL-6 has been shown to be both a pro- and anti-inflammatory cytokine, with the differing biological effects believed to be due to the site (i.e., adipose and muscle tissue,
respectively) of IL-6 secretion. Thus, there is currently no consensus on IL-6’s effects on sarcopenia and dynapenia.

TNFα has also been linked to the age-associated loss of muscle mass and strength. TNFα has been found to induce RONS production as well as upregulation of MPB via the ubiquitin proteasome pathway. The TNFα induced RONS production leads to activation of nuclear factor kappa B (NF-κB) via degradation of inhibitory kappa B (I-κB) in the proteasome. NF-κB is a transcription factor that regulates cytokine and cytokine receptor gene expression, and is regulated by attachment of I-κB. Hence, when I-κB is degraded in the proteasome via TNFα induced RONS production, NF-κB is then allowed to induce its effects on skeletal muscle. NF-κB activation via TNFα induced RONS production has been found to increase MuRF1 and MAFbx expression leading to MPB. Therefore, a countermeasure that can both activate MPS (by activating the PI3k-Akt pathway) and reduce MPB (by down regulating the ubiquitin-proteasome pathway) is essential in counteracting the age-associated loss of skeletal muscle mass and strength.

**Regulation of Mitochondrial Biogenesis and Aging.** Mitochondrial homeostasis (i.e., the balance of protein turnover during repair or recycling of mitochondrial proteins through protein synthesis and protein degradation) consists of maintaining mitochondrial biogenesis. Mitochondrial biogenesis is defined as the process by which new mitochondrion are formed in the cell. The proteins that form new mitochondrion or repair damaged mitochondrion are produced from the transcription and translation of genes both in the nuclear genome and in the mitochondrial genome. With cellular aging, there is a loss of mitochondrial homeostasis and increased dysfunction.
The mitochondrial theory of aging was proposed in 1956 and states that, with aging, there is increased RONS production from mitochondria that results in mutations of mitochondrial DNA (mtDNA), a reduced efficiency to produce adenosine tri-phosphate (ATP), and ultimately cellular dysfunction. The age-associated loss of muscle mass and strength has, in part, been linked to an increase in RONS production leading to mitochondrial dysfunction and a reduced ability to stimulate mitochondrial biogenesis. RONS refers to both free radical (i.e., one or more unpaired electron in an atom or molecule) and non-free radical species (where the reactive center is either nitrogen or oxygen). The primary free radicals generated in cells are superoxide (O$_2^-$) and nitric oxide (NO), and both molecules can readily react to form a series of other RONS. RONS production increases with age due to altered electron transport chain function (i.e., the greatest producer of cellular high energy phosphates; ATP) and decreases in antioxidant enzyme (i.e., superoxide dismutase, glutathione peroxidase, and catalase) content and function. The increases in RONS production and decreases in antioxidant enzyme content and activity leads to damage of mtDNA and can subsequently lead to an accumulation of cell loss through apoptosis such as in sarcopenia and dynapenia. Therefore, the pathways involved in maintaining mitochondrial homeostasis through promoting mitochondrial biogenesis will be reviewed in the context of their contribution to sarcopenia and dynapenia.

**AMPK, p38-MAPK, and Mitochondrial Biogenesis.** At the molecular level, there are several transcription factors and cofactors that are involved with the activation and regulation of mitochondrial biogenesis. However, the major control point or “master regulator” of mitochondrial transcription (and, thus, mitochondrial biogenesis) has been found to be peroxisome proliferators-activated receptor co-activator 1 (PGC-1α).
has been suggested to provide control of cellular ATP homeostasis via regulation of mitochondrial biogenesis.\textsuperscript{80} Cui et al.\textsuperscript{81} demonstrated PGC-1\(\alpha\)'s physiological importance by reporting that repression of PGC-1\(\alpha\) by a mutant form of the Huntington protein (i.e., Huntington’s disease) leads to mitochondrial dysfunction (and, thus, reduced mitochondrial biogenesis) and neurodegeneration. The same authors also demonstrated that overexpression of PGC-1\(\alpha\) rescues cells from the negative effects of the Huntington protein.\textsuperscript{81} Also, with aging there is reduced expression of PGC-1\(\alpha\),\textsuperscript{82} and its absence is believed to promote the accumulation of damaged mitochondria,\textsuperscript{79} which is thought to be the basis of increased mitochondria dysfunction\textsuperscript{79} and, thus, increases in sarcopenia and dynapenia. PGC-1\(\alpha\) can be activated by numerous factors including silent informational regulator ortholog of mammalian sirtuin (SIRT1), the acetylase transferase (GCN5), nitric oxide (NO), 5’ AMP-activated protein kinase (AMPK), and p38- mitogen-activated protein kinase (p38-MAPK),\textsuperscript{78,83} however, only the AMPK and p38-MAPK pathways will be reviewed.

During endurance training (ET; i.e., low-intensity exercise with a relatively high number of contractions) the high demand and breakdown of ATP generates high levels of adenosine monophosphate (AMP). When AMP to ATP (AMP:ATP) ratios are high in the cell (indicating a low cellular energy level), AMPK is activated in skeletal muscle.\textsuperscript{84} AMPK directly phosphorylates PGC-1\(\alpha\)\textsuperscript{84} and induces post-translational modifications that make PGC-1\(\alpha\) more stable and, thus, active.\textsuperscript{85} In addition, Canto et al.\textsuperscript{86} demonstrated that ET induced PGC-1\(\alpha\) post-translational modifications and activation was blunted in AMPK-knockout mice, indicating AMPK activation is a crucial regulator of PGC-1\(\alpha\) activation and mitochondrial biogenesis initiation.\textsuperscript{87} With the increased AMP:ATP ratio (i.e., low-energy level), AMPK will not only increase the activation of energy producing mechanisms (i.e.,
post-translational modifications of PGC-1α and activation of mitochondrial biogenesis), but there has also been strong evidence indicating that AMPK has cross-talk with the PI3k-Akt pathway. When cellular AMP:ATP ratio is high and AMPK is activated, AMPK will inhibit large energy-consuming pathways involved in anabolic processes such as the PI3k-Akt pathway. AMPK will activate the TSC1/TSC2 complex and, thereby, inhibit mTOR as well as decrease phosphorylation of p70S6k and 4E-BP1, thereby inhibiting MPS. In aging, AMPK’s ability to “sense” the reduced cellular energy level is not only diminished, but total AMPK content and activity are both decreased as well, thereby leading to a decreased activation of PGC-1α and thus mitochondrial biogenesis.

In addition, ET activates stress-activated protein kinases, in particular p38-MAPK. p38-MAPK directly phosphorylates and activates PGC-1α as well as transcription factors that directly bind to the PGC-1α promoter. Administration of the p38-MAPK inhibitor SB-202190 to skeletal muscle cells prevented mitochondrial biogenesis, indicating that p38-MAPK is a crucial regulator of mitochondrial biogenesis. In addition, Pogozelski et al. reported that the p38γ-MAPK isoform, and not the p38α-MAPK or p38β-MAPK isoforms, was required to induce mitochondrial biogenesis in skeletal muscle in response to ET in mice. p38-MAPK content and activity decrease with aging, leading to decreased PGC-1α expression and, thus, mitochondrial biogenesis. Taken collectively, mechanisms that can activate the AMPK and p38-MAPK pathways to stimulate PGC-1α expression and activation and stimulate mitochondrial biogenesis could be a potent stimuli to help reduce or even prevent the age-associated loss of skeletal muscle mass and strength.
Countermeasures against Sarcopenia and Dynapenia. Several countermeasures have been studied to establish feasible and safe interventions to improve muscle mass and strength. Such interventions include RT, increasing food or energy intake, increasing protein or essential amino acid intake, and pharmaceuticals (i.e., hormone-replacement therapy). All of these have the potential for increasing muscle mass and strength in older adults and will be further reviewed below. Also, the effects of *A. turkestanica* on health and performance capabilities as well as the potential of *A. turkestanica* to be a viable countermeasure against sarcopenia and dynapenia will be reviewed.

**Resistance Training.** RT programs have proven to be one of the most effective interventions in counteracting the decreases in muscle mass and strength associated with age. The current recommendations from the American College of Sports Medicine for increasing muscle strength for older adults suggest that novices train 2 to 3 times per week with loads corresponding to 8 to 12 repetitions, while, for more experienced lifters, heavier load protocols and up to 4 to 5 training sessions per week are suggested.\(^{95}\) Numerous studies have shown improvements in both muscle mass and strength utilizing RT in older adults.\(^{96-99}\)

Traditionally, RT programs for older adults have been geared towards developing overall strength in the individuals. However, Latham and colleagues compiled a systematic review on RT in older adults and suggest that increasing muscle strength does not necessarily translate into improvements in ADLs.\(^{100}\) Several other studies have reported that muscle power is more strongly correlated to improvements in ADLs in older adults.\(^{1,2,101-105}\) Muscle strength reflects the ability of the muscle to produce force; whereas, muscle power is defined as the product of force times velocity, or the ability of the muscle to produce force quickly. Therefore, since sarcopenia and dynapenia can contribute, in large part, to reductions in
ADLs the emphasis should be placed on increasing overall power output and not just strength.

However, high volume (i.e., high number of total repetitions, sets, load, and intensity) RT programs are not feasible for many aged people in terms of compliance and ability to perform the exercises (i.e., functional performance). Several studies\textsuperscript{106,107} report that high volume RT programs in older adults impair any positive gains experienced in muscle mass and strength, particularly in women. This may be due, in part, to the blunted anabolic response to RT programs that is seen in older adults when compared to healthy, young controls.\textsuperscript{8,108} Therefore, particular attention needs to be placed on designing RT programs for older adults that take into account appropriate volume, frequency and feasibility of the workouts. Properly designed RT programs for older adults have demonstrated increases in muscle mass and strength, but to a lesser extent than healthy, young controls.\textsuperscript{8,108} In summary, when properly designed RT programs are utilized as a countermeasure in older adults, there is an increase in muscle mass and strength, but to a lesser extent than the gains experienced by healthy, young adults which may be due, in part, to reductions in MPS signaling.

\textit{Energy Intake, Protein Intake, and Essential Amino Acids}. One of the strongest causes of the reduction in MPS and increases in physical frailty is nutritional status. This includes energy intake (i.e., the amount of food and energy consumed), protein intake, and essential amino acid intake. A reduction in energy intake can have drastic impacts on muscle mass and physical function. This reduction can compromise mitochondrial energy metabolism and lead to muscle fatigue, weakness, and debility.\textsuperscript{109} If body weight decreases with a reduced energy intake, not only are fat stores lost, but almost always skeletal muscle
mass is also lost. When older adults experience weight loss, the reduction in muscle mass is
greater than the reduction in young adults with similar weight loss. The optimal body mass
index (BMI) for older adults (i.e., individuals over the age of 51 years) is higher than
younger adults and is estimated to be between 22 and 33 kg/m$^2$. To achieve this BMI range,
an older adult needs to ingest 24-36 kilocalorie per kilogram (kcal/kg) body weight per day
(the equivalent to 1440-2160 kcal/day in a 60kg person). Therefore, maintenance of
energy intake is essential for reducing and slowing the progression of sarcopenia and
dynapenia.

Since MPS requires protein, increasing dietary protein intake can stimulate MPS.
However, the amount and type of dietary protein intake is different for older adults than
young adults. The current recommended dietary allowance (RDA) for protein intake is 0.8 g
protein per kg/day in healthy adults. The mean requirement for older adults is estimated to
be 0.89 g protein/kg/day and up to 1.3 g protein/kg/day during debilitating conditions such
as hospitalization. Houston et al. demonstrated that the loss of appendicular lean mass
in older adults can be attenuated when a higher amount of dietary protein is consumed over a
three year time period.

The type of dietary protein also plays a large role in balancing MPS and MPB.
Animal protein has been found to have a greater effect on promoting MPS and inhibiting
MPB when compared to vegetable protein. Also, the ability to absorb protein is reduced
with age and thus “faster” proteins (i.e., whey) are recommended over “slower” proteins (i.e.,
casein). Fast versus slow proteins refers the rate at which amino acids (i.e., from the
breakdown of the proteins) appear in the blood stream and the speed at which the protein is
absorbed in the small intestine.\textsuperscript{115} Whey protein, over casein protein, has been reported to increase MPS in healthy older adults.\textsuperscript{116}

Furthermore, with age there is a reduction in the ability to metabolize amino acids and, thus, a larger dietary intake of amino acids is suggested. Essential amino acids (EAAs), over non-essential amino acids, have been found to be the primary stimulus for MPS in healthy older adults.\textsuperscript{117} EAAs, in particular the branched chain amino acid (BCAA) leucine, can directly stimulate MPS by activating mTOR.\textsuperscript{118} As mentioned above, mTOR turns on the translational machinery responsible for protein synthesis and, thus, regulates muscle mass.\textsuperscript{34} Further, several studies\textsuperscript{119,120} have reported that the combination of RT and protein or EAA supplementation has a greater effect on increases in MPS and strength in older adults than the gains made by the two independently.

Inadequate nutrition in itself may contribute to the development of sarcopenia and dynapenia.\textsuperscript{121,122} Also, this relationship can work in reverse, as the age-associated loss of muscle mass and strength can lead to inadequate nutrition and, thus, cause a vicious cycle that amplifies one another.\textsuperscript{109} Overall, the increase in energy intake and the increase in quality protein intake, specifically the EAA leucine, can help to counteract or even prevent the losses of muscle mass and strength. Further research is needed on additional nutritional interventions to counteract sarcopenia and dynapenia.

\textit{Hormone Supplementation (Androgens, Growth Hormone, and Insulin-Like Growth Factor 1).} Hormone supplementation as an intervention to counteract or prevent the age-related declines in muscle mass and strength has grown in popularity in recent years. Estrogen supplementation in women has been reported to have a small effect on increasing muscle mass,\textsuperscript{123} but can also cause an increase in oxidative cancers (i.e., breast cancer).\textsuperscript{124}
The two most prevalently used and studied hormones against the loss of muscle mass and strength are testosterone and human growth hormone (HGH).

Testosterone is a crucial hormone involved in regulation of skeletal muscle mass and strength with age. Blood testosterone levels decline with age in males. This decline in testosterone is linked to a loss in muscle mass, strength, and physical function and a decreased rate of MPS. Testosterone supplementation can increase muscle mass and strength in hypogonadal older adults. However, the amount of testosterone hormone needed to produce increases in muscle mass and strength in older adults is above physiological levels, and can lead to serious side effects such as cardiovascular disease, prostate enlargement, and certain cancers.

HGH regulates the growth of tissues throughout the body, in particular skeletal muscle. HGH regulates skeletal muscle growth in part by activating IGF-1. IGF-1 works through the PI3k-Akt pathway to directly influence MPS. HGH can also independently promote skeletal muscle cell growth through a separate signaling cascade that is beyond the scope of this review. HGH supplementation in older adults to counteract sarcopenia and dynapenia has shown both positive and negative results. HGH supplementation, like testosterone, can lead to adverse side effects.

IGF-1 supplementation has also had positive and negative results on MPS in aged human and rodent models. The significant decline in plasma IGF-1 observed in aged adults is negatively correlated with the rate of MPS. Even with the ability to promote MPS, IGF-1 also activates a plethora of other signaling pathways, including pathways that lead to tumor growth and certain cancers. Thus, IGF-1 supplementation needs further examination of whether it can be an appropriate countermeasure against sarcopenia and
dynapenia. The negative side effects associated with these treatments emphasize the need of finding non-toxic and non-hormonal treatments to help increase muscle mass and strength. The current body of literature pertaining to hormone supplementation as a countermeasure to reverse or reduce the age-associated loss of muscle mass and strength has produced conflicting results indicating that further research is needed to determine if these treatments are viable countermeasures against sarcopenia and dynapenia.

**Ajuga turkestanaica and Human Health.** *Ajuga turkestanaica*, a wild plant native to Uzbekistan and other regions in Central Asia, is a traditional folk remedy used to enhance muscular strength and physical endurance. Leaves of *A. turkestanaica* contain an array of bioactive phytochemicals, in particular phytoecdysteroids and iridoids. Phytoecdysteroids isolated from *A. turkestanaica* include ajugalactone, ajugasterone B, cyasterone, cyasterone-22-acetate, ecdysterone and most predominantly, 20-hydroxyecdysone (20E) and turkesterone. The phytoecdysteroids from *A. turkestanaica* are reported to contain an abundance of biological, pharmacological, and medicinal properties such as anabolic, analgesic, anti-inflammatory, antihypertensive, antioxidant, antibacterial, increased skin hydration (aquaporin production), renoprotective, hepatoprotective, increased red blood cell production and regeneration, reversal of mitochondrial dysfunction, normalization of cell energetics and lipid metabolism, and increased insulin sensitivity in diabetic animals.

Furthermore, these compounds are considered to be adaptogens. Adaptogens enhance mental and physical capabilities by promoting vitality and increasing the organism’s resistance to stress and aging. Iridoids isolated from *A. turkestanaica* include harpagide and 8-O-acetylharpigide. These particular iridoids contain a plethora of biological, pharmacological, and medicinal properties such as antitumor, fungicidal, antimicrobial,
antioxidant, anti-inflammatory, cholegic (promotes bile secretion), and hepatoprotective.\textsuperscript{10,137} There is evidence that the acute toxicity of the compounds in \textit{A. turkestanica} to mammals is extremely low with relatively no side effects.\textsuperscript{137,139}

Recent research has demonstrated than an extract produced from \textit{A. turkestanica} was able to stimulate protein synthesis by 120\% in cultured C2C12 skeletal muscle myotubes.\textsuperscript{140} Additionally, when the PI3k inhibitor LY294002 was administered to the cell cultures protein synthesis was completely abolished,\textsuperscript{140} suggesting that the extract from \textit{A. turkestanica} works through the PI3k-Akt pathway to stimulate MPS. Finally, the authors demonstrated that the same compounds elicited their affects in vivo, by reporting that young rats fed 20E for 28 days increased muscular grip strength by 18\%.\textsuperscript{140} The same group administered 20E to C2C12 skeletal muscle cells and reported an increase in Akt phosphorylation in a dose-dependent manner.\textsuperscript{35} The authors also reported that 20E Akt activation may be working through a G-protein coupled-receptor (GPCR) – phospholipase C (PLC) – inositol 3-phosphate receptor (IP\textsubscript{3}R) – PI3k pathway via increases of Ca\textsuperscript{2+} influx into the skeletal muscle cells (for a review of PLC-IP\textsubscript{3}R in skeletal muscle see Rozengurt et al.\textsuperscript{141}). This study demonstrates that activation of the PI3k-Akt pathway by 20E may not be working through an IGF-1 dependent mechanism.\textsuperscript{35}

Toth et al.\textsuperscript{142} demonstrated that 20E administration to rats not only increased muscle mass (by increasing both fast and slow twitch muscle fiber size), but also increased the total number of fiber myonuclei, suggesting activation of regenerative mechanisms (i.e., satellite cells) involved in skeletal muscle. Other investigators\textsuperscript{10,137,143} have demonstrated that the compounds in \textit{A. turkestanica} promote similar anabolic effects as androgens (i.e., testosterone), but have not shown any of the adverse side effects such as atrophy of the testes.
or enlargement of tissues other than just skeletal muscle (i.e., prostate gland and cardiac muscle) that are experienced with exogenous hormone supplementation. Furthermore, the compounds in *A. turkestanica* are non-androgenic and work through an androgen receptor independent pathway. *A. turkestanica* could be a potential non-toxic, non-hormonal intervention to counteract the age-associated loss of skeletal muscle mass and strength. Additionally, no studies to date have investigated the effects of phytoecdysteroid supplementation on improving skeletal muscle mass and strength in 20-month old animals.

**SUMMARY**

Sarcopenia and dynapenia affect a large proportion of our global population and cause severe financial burdens on our health care system. Countermeasures to alleviate the physical losses associated with sarcopenia and dynapenia require further research. A countermeasure is needed than can stimulate MPS (PI3k-Akt pathway) or mitochondrial biogenesis (AMPK and p38-MAPK pathways) in aged skeletal muscle without adverse side effects. *A. turkestanica* presents itself as a potential intervention to both decrease and prevent the age-associated loss of skeletal muscle mass and strength in a non-toxic, non-hormonal way.

**STATEMENT OF PROBLEM**

Neither the anti-sarcopenic nor anti-dynapenic effects of *A. turkestanica* or 20E on aged skeletal muscle are known. Therefore, the purpose of this study is to determine the effects of either ATE, 20E, or control (CT; vehicle only of 100% non-denatured ethanol, EtOH) fed to 20-month old male mice for 28 days on the ability to (1) increase strength and muscle mass, (2) activate the key control point of protein synthesis, the PI3k-Akt pathway,
and (3) activate key upstream control points for mitochondrial biogenesis, the AMPK and p38-MAPK pathways.

HYPOTHESIS

I hypothesize that ATE will (1) increase strength and muscle mass, (2) activate the key control point of protein synthesis, the PI3k-Akt pathway, and (3) activate key upstream control points for mitochondrial biogenesis, the AMPK and p38-MAPK pathways in 20-month old male mice. The efficacy of ATE will be tested against 20E and CT.
CHAPTER 2: EXPERIMENT

The loss of muscle mass (sarcopenia) and strength (dynapenia) with advanced age are associated with decreased physical function. The loss of physical function can accelerate the loss of muscle mass and strength and create a vicious cycle that continuously builds on one another and can eventually lead to loss of independence and premature death. Therefore, it is critical to study countermeasures that can attenuate the outcomes associated with sarcopenia and dynapenia in the elderly.

The ability to stimulate MPS and mitochondrial biogenesis are impaired with aging, both of which contribute to the loss of muscle mass and strength with age. Blunted MPS in older adults is due, in large part, to reductions in signaling pathways directly involved in protein synthesis (specifically the PI3k/Akt/mTOR/p70S6k; PI3k-Akt pathway), amino acid and protein intake, hormone levels, and physical activity. Whereas, blunted mitochondrial biogenesis in older adults is due, in large part, to reductions in signaling pathways directly involved in mitochondrial biogenesis, upregulation of RONS, decreased heat shock protein expression, as well as reductions in antioxidant enzyme content and activity. Taken collectively, a countermeasure that can both activate MPS and mitochondrial biogenesis pathways would be very important for reducing or even reversing the effects of sarcopenia and dynapenia. Current countermeasures for sarcopenia and dynapenia include RT, increasing food or energy intake, increasing protein or essential amino acid intake, and pharmaceuticals (i.e., hormone-replacement therapy); however, for multiple
reasons these interventions are not entirely effective at attenuating sarcopenia and dynapenia in the elderly.\textsuperscript{109,122}

\textit{Ajuga turkestanica}, a wild plant native to Uzbekistan and other regions in Central Asia, is a traditional folk remedy used to enhance muscular strength and physical endurance. Leaves and shoots of \textit{A. turkestanica} contain an array of bioactive phytochemicals, in particular phytoecdysteroids. Phytocdysteroids are plant-produced analogues of ecdysteroids, insect molting hormones that control cell proliferation, growth, and development.\textsuperscript{139} The structures of phytoecdysteroids vary according to the number, location, and position of hydroxyl substituents on a steroid backbone.\textsuperscript{10,145} The phytoecdysteroids from \textit{A. turkestanica} are reported to have anabolic, analgesic, anti-inflammatory, antihypertensive, antioxidant, antibacterial, and hepatoprotective properties.\textsuperscript{10,137} Recent research has demonstrated that an extract, enriched in phytoecdysteroids, produced from \textit{A. turkestanica} stimulates protein synthesis by 120\% via the PI3k-Akt pathway in cultured C2C12 skeletal muscle myotubes.\textsuperscript{140} In addition, young rats fed 20E at 50 milligrams per kilogram per day (mg/kg/day) for 28 days increased muscular grip strength by 18\%.\textsuperscript{140} These data suggest that \textit{A. turkestanica} may possess the properties to increase MPS and strength. Furthermore, the acute toxicity of phytoecdysteroids is very low to mice: the LD\textsubscript{50} of 20E is >9 grams per kilogram (g/kg) body weight when given orally and 6.4 g/kg body weight if injected intraperitoneally.\textsuperscript{146,147} It is unknown if \textit{A. turkestanica} works to combat sarcopenia and dynapenia in aged skeletal muscle. Therefore, the purpose of the current study was to determine the effects of ATE, 20E, or vehicle only (CT) fed to 20-month old male mice for 28 days on their ability to (1) increase strength and muscle mass, (2) activate
the key control point of protein synthesis, the PI3k-Akt pathway, and (3) activate key upstream control points for mitochondrial biogenesis, the AMPK and p38-MAPK pathways.
MATERIALS AND METHODS

**Plant Materials and Crude Extraction.** Whole *A. turkestanica* plant material was harvested in Las Palmas, Spain (PoliNat; Las Palmas, Spain) in April-June 2008. The plant material was air dried and milled to 1-2 mm particle size. A crude extraction consisting of a 30:70 water-ethanol solvent solution at 60°C to extract phytoecdysteroids and sterilize the plant material was performed by P.L. Thomas, Inc. (Morristown, NJ, USA). High-pressure liquid chromatography (HPLC) analysis of the sterilized and extracted *A. turkestanica* plant material, normalized to a 20E standard, revealed a 6% milligrams per milliliter (mg/mL) total phytoecdysteroid content in the initial extract. The initial extract was air dried and sieved through a 60 millimeter (mm) mesh screen. P.L. Thomas, Inc. kindly donated 500 grams (g) of the initial extract for this project and shipped it to the Appalachian State University’s Human Performance Laboratory in Kannapolis, NC. The initial extract was received and placed in a -20°C freezer until further extraction and analysis.

**Phytoecdysteroid Enrichment and Standardization.** Creation of an extract enriched in phytoecdysteroids from the initial ATE was performed using methods established by Cheng et al.\(^ {145} \), with modifications. Briefly, (1st extraction) 100 g of the initial extract was suspended in 500 mL of sterile double distilled water (ddH\(_2\)O) and sonicated for 10 minutes. The suspension was defatted by partition (liquid-liquid extraction) with n-hexane (500 mL x 4). The defatted aqueous layer was then partitioned with ethyl acetate (500 mL x 8). The combined ethyl acetate extract was dried over anhydrous sod-sulfate and solvent was removed via rotary evaporation to afford the ethyl acetate extract. The dried ethyl acetate extract was analyzed by thin-layer chromatography (TLC) silica gel 60 F\(_{254}\) 250 µm pre-coated plates (EMD Chemicals, Inc.; Gibbstown, NJ, USA) with a solvent system of ethyl
acetate-methanol-water at 77:13:19 ratio. The TLC plates were monitored by short wave ultraviolet lamps (254 nm) and visualized with vanillin reagent (4 g vanillin, 100 mL methanol and 2.5 mL H₂SO₄). Following identification of phytoecdysteroids by TLC visualization, HPLC analysis was performed for quantification of phytoecdysteroids. A commercial HPLC grade standard of 20E (Sigma Aldrich; St. Louis, MO, USA) at concentrations of 250, 500, and 1000 µg/ml with 10 µL injection volumes was used to determine the concentrations of phytoecdysteroids in the extract. Analysis was performed using an Agilent 1200 HPLC system (Agilent Technologies, Inc.; Wilmington, DE, USA) with autosampler, DAD (247 nm) and Synergi 4 um Hydro-RP 80A reversed phase column (250 mM x 4.6 mm x 5 um, Phenomenex; Torrance, CA, USA). The mobile phase solvents consisted of 2% acetic acid in H₂O (solvent A) and 0.5% acetic acid in 50% aqueous acetonitrile (solvent B). Gradients of 10%, 15%, 25%, 35%, 55%, 100%, and 10% of solvent B were used at 0, 10, 13, 20, 50, 54, and 60 minutes, respectively, with 1.0 mL/min flow rate. Samples were filtered through 0.2 mm PTFE filters (GE Healthcare; Pittsburg, PA, USA) before injecting 10 µl on the HPLC column (25°C). Identification of phytoecdysteroids was performed in reference to compounds isolated previously from A. turkestanica. Quantification of compounds was performed from the peak areas recorded at 247 nm for phytoecdysteroids in reference to the calibration curve obtained with a purified commercial 20E standard (i.e., 100% total phytoecdysteroids). Concentrations were calculated by taking the average of two HPLC runs.

The aqueous layer remaining after the ethyl acetate partition was then partitioned with butanol (750 mL x 3). Butanol was removed via rotary evaporation and the residue obtained was freeze-dried to afford the butanol extract. Following identification of
phytoecdysteroids by TLC visualization, HPLC analysis was performed for quantification of phytoecdysteroids. The aqueous layer after butanol extraction was discarded. The dried butanol extract was pulverized with a porcelain mortar and pestle, heated in 60°C water bath and sonicated for 10 minutes to help dissolution and then vacuum filtered through Whatman #4 (GE Healthcare; Pittsburg, PA, USA). One-half of the dried butanol extract was then extracted with 0.5% methanol in ethyl acetate (100 mL x 4). The solvent was removed via rotary evaporation and the combined ethyl acetate extracts were dehydrated over anhydrous sod-sulfate and lyophilized. Based on HPLC results, the 0.5% methanol in ethyl acetate dried extract was then combined with the remaining one-half dried butanol extract in a ratio of 2:3 (i.e., 2:3 mixture). The 2:3 mixture was first extracted with methanol (100 mL x 4) and then butanol (100 mL x 4). The solvents from the 2:3 extract were rotatory evaporated and lyophilized. From the concentrations determined by HPLC, the dried methanol 2:3 mixture extract and butanol 2:3 mixture extract were then combined to create a 1:4 ratio of 20E to turkesterone, ATE. The 1:4 ATE was analyzed by TLC as described above and then analyzed for phytoecdysteroid content by HPLC. The final amount remaining from extraction was not sufficient for 28 days of supplementation, so a second extraction had to be performed (Figure 1).

The 2nd extraction was simplified based on the results obtained from the 1st batch extraction. Briefly, 100 g of the initial ATE was suspended in 500 mL of ddH₂O and sonicated for 10 minutes. The suspension was then partitioned with ethyl acetate (500 mL x 10). The ethyl acetate was dried over anhydrous sod-sulfate, the solvent was removed via rotary evaporation, and the residue was lyophilized. The remaining aqueous layer was then extracted with butanol (500 mL x 5). The butanol was removed via rotary evaporation, and
the remaining residue was lyophilized. The dried butanol extract was then pulverized, heated, sonicated, and filtered using the same methods described above. The dried butanol extract was then extracted with 0.2% methanol in ethyl acetate (100 mL x 4). The 0.2% methanol in ethyl acetate extract was dried over anhydrous sod-sulfate, the solvents were removed via rotary evaporation, and the residue was lyophilized. From the concentrations determined by HPLC, the dried 0.2% methanol in ethyl acetate was then combined with the dried ethyl acetate and the 1:4 ATE from extraction 1 to create the final 1:4 ATE that was used for the supplementation protocol (Figure 2). The final 1:4 ATE was analyzed by TLC as described above and then analyzed for phytoecdysteroid content by HPLC.

**Experimental Design.** Aged male C57BL/6 (20 months-old) mice were supplemented with 50mg/kg/day of ATE, 20E, or vehicle only (CT) for 28 consecutive days (Figure 3). Following 28 days of supplementation, a force-frequency curve and low-frequency fatigue protocol were utilized to determine in vivo contractile function of the gastrocnemius-plantaris-soleus (GPS) complex. Following contractile function measurements, animals were euthanized and skeletal muscle and organ tissues were harvested, weighed, and stored at -80°C until further analysis. MPS and mitochondrial biogenesis pathway signaling analyses were performed on muscle lysates and muscle fiber cross-sectional area (CSA) analysis was performed on transverse sectioned muscle.

**Animals.** Male C57BL/6 mice (n = 36), 20-months old, were randomly assigned to one of three treatment groups: CT (50 mg/kg/day; n = 12), ATE (50 mg/kg/day; n = 12), or 20E (50 mg/kg/day; n = 12). The ATE used for the ATE treatment was from the enriched phytoecdysteroid extract described above, and 20-hydroxyecdysone (20E) was purchased from Bosche Scientific (E6425-HE; New Brunswick, NJ, USA). ATE and 20E were
suspended in EtOH. The ATE, 20E or CT was aliquoted onto one-190 mg rodent enrichment treat (Fruit Crunchies; BioServ, Frenchtown, NJ, USA) the day before feeding it to the animal in order to allow the EtOH to evaporate. The previous day’s body mass was used to calculate the amount of ATE, 20E, or vehicle needed. For example, the measured body mass of a given mouse on Sunday was used to calculate the volume of ATE or 20E (50 mg/kg) that was applied to the Fruit Crunchie, air dried, and fed to that mouse on Monday. Mice were housed and tested within the David H. Murdock Research Institute Center for Laboratory Animal Sciences at the North Carolina Research Campus, Kannapolis, NC, an Association for Assessment and Accreditation of Laboratory Animal Care facility. The room was maintained at 21°C, 50% humidity, with a 12:12-hour reverse light cycle. Food and water were provided ad libitum. At the end of each light cycle (0800 hours), body mass was measured. Mice were then individually placed in an empty cage without bedding and fed one treated Fruit Crunchie. Complete consumption of the dosed Fruit Crunchie was visually confirmed for each mouse. All experiments were approved by the North Carolina Research Campus Institutional Animal Care and Use Committee and the experiments were conducted within the guidelines set forth by the American Physiological Society (Appendix A).

**In vivo Contractile Function.** In vivo determination of contractile function was obtained from the GPS complex. Force output of the plantar flexors (i.e., GPS complex) was measured using a muscle lever system (Model 300C, Aurora Scientific; Aurora, ON, Canada) as previously described. Briefly, each mouse was initially anesthetized with 4% inhaled isofluorane with 800 mL oxygen (O₂) output and maintained at 2% inhaled isofluorane with 500 mL O₂ output for the entire contractile function data collection period. Hair was removed from both hindlimbs with a standard depilatory product (Nair™) and placed on a
heated platform (37°C). The knee joint was kept stationary and the foot was firmly fixed to a footplate with adhesive wrapping, which was connected to the shaft of the force transducer (Model 402A, Aurora Scientific; Aurora, ON, Canada). Contraction of the GPS complex was elicited by electrical stimulation using a model 701C stimulator (Aurora Scientific; Aurora, ON, Canada) via needle electrodes (Model F-E7-30, Grass Technologies; West Warwick, RI) inserted parallel to the tibial nerve. Electrode placement was tested via short stimulation of the nerve to cause plantar flexion. When stimulated, the foot was checked for plantar flexion without any visible eversion or inversion of the foot.

**Force-Frequency Curve and Fatigue Protocol.** Optimal voltage ($V_o$) and optimal length ($L_o$) were determined as the maximal voltage and length that produced the greatest amount of force during a single submaximal twitch contraction. A force-frequency curve was then determined by stimulating the GPS complex with single 500 ms trains, 200 µsec pulses every 3 minutes at increasing stimulation frequencies (1, 15, 30, 60, 100, 160 Hertz; Hz). At five minutes post force-frequency, a low-frequency fatigue protocol was implemented (250 ms trains, 200 µsec pulses every 3.5 seconds at 30 Hz for 30 minutes). At five minutes post low-frequency fatigue protocol, a single 100 Hz contraction was performed to determine fatigue level following the protocol.

**Data Acquisition and Analysis.** The muscle lever system, stimulator and force transducer were connected to a signal interface (Model 604A, Aurora Scientific; Aurora, ON, Canada) that sent the analog signal to an analog to digital convertor card (Model PCI-6221, National Instruments; Austin, TX, USA) on a computer with Dynamic Muscle Control software (Aurora Scientific; Aurora, ON, Canada). Force output data was analyzed using Dynamic Muscle Analysis software (Aurora Scientific; Aurora, ON, Canada).
Dissection of Skeletal Muscles and Organs. Following the contractile function procedure mice were euthanized by exsanguination via cardiac puncture. Skeletal muscles were dissected from both contracted and non-contracted hindlimbs (including extensor digitorum longus, tibialis anterior, soleus, plantaris, gastrocnemius, and quadriceps) and wet weight (mass) was measured. Triceps brachii muscles were dissected from front limbs. In addition, organs (including heart, liver, spleen, kidneys, and testes) were removed and wet weight was measured. Skeletal muscles and organs were immediately snap frozen in liquid nitrogen (N2) or liquid N2 cooled isopentane (plantaris and triceps brachii) and stored at -80°C freezer until subsequent analysis.

Western Blotting. Both hindlimb contracted and non-contracted gastrocnemius (100 mg) were pulverized on dry ice using a porcelain mortar and pestle and homogenized on ice in 0.3 mL of homogenization buffer containing 1.0 mM NaCl, 1.0% Triton-X, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.6 with protease and phosphatase inhibitor cocktails (Sigma Aldrich; St. Louis, MO, USA). After homogenization, the samples were cleared by centrifugation for 15 min at 5,000 x gravity. The supernatant was removed and protein concentration was determined using the BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.; Rockford, IL, USA). Total protein (30 µg) was prepared in Laemmli buffer and subjected to electrophoretic separation by SDS-PAGE on 7.5% acrylamide gels (#116-1118; Bio-Rad; Hercules, CA, USA) as previously described. Following electrophoretic separation, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore; Bradford, MA, USA) and stained with 0.1% Ponceau S in 0.5% acetic acid (Sigma Aldrich; St. Louis, MO, USA) to ensure equal loading and transferring of proteins. The SNAP i.d. protein detection system (Millipore; Bradford, MA, USA) was used for
blocking and antibody incubation as previously described. Briefly, non-specific binding was blocked with 1% bovine serum albumin (BSA) in TBS-T (Tris buffered saline, 0.1% Tween 20) and immediately vacuumed through. Following blocking, the membrane was washed three times and immediately vacuumed through with TBS-T and exposed to the following primary antibodies in 1% BSA/TBS-T for 10 minutes at room temperature: phosphorylated p70S6k<sup>Thr389</sup> (Cell Signaling; Danver, MA, USA - 9234; 1:500); total p70S6k (Cell Signaling; Danver, MA, USA - 2708; 1:1000); phosphorylated Akt<sup>Ser473</sup> (Cell Signaling; Danver, MA, USA - 9271; 1:500); total Akt (Cell Signaling; Danver, MA, USA - 9272; 1:1000); phosphorylated AMPK<sup>Thr172</sup> (Cell Signaling; Danver, MA, USA - 2531; 1:500); total AMPK (Cell Signaling; Danver, MA, USA - 2532; 1:1000); phosphorylated p38-MAPK<sup>Thr180/Tyr182</sup> (Cell Signaling; Danver, MA, USA - 9210, 1:2500); and total p38-MAPK (Cell Signaling; Danver, MA, USA - 9212, 1:5000). The primary antibody was vacuumed through, the membrane was washed three times, and was immediately vacuumed through with TBS-T. The membrane was then incubated for 10 minutes with horseradish peroxidase (HRP)-linked IgG anti-rabbit (Cell Signaling; Danver, MA, USA - 7074; 1:2000) secondary antibody and vacuumed through. The membrane was then washed three times and immediately vacuumed through with TBS-T. Membranes were exposed to SuperSignal West Dura ECL chemiluminescent detection HRP reagents (Pierce; Thermo Fisher Scientific, Inc.; Rockford, IL, USA) mixed 1:1 for 5 minutes. Membranes were covered in plastic wrap and exposed to radiographic film. Films were developed in a dark room using Konica Minolta SRX-101A film processor (Konica Minolta; Ramsey, NJ, USA). Densitometry of specific protein target bands was determined using Image J software (National Institutes of Health; Bethesda, MD, USA). All values obtained from one blot were normalized to the blot average.
density. Membranes were stripped with Restore western blot stripping buffer (Thermo Fisher Scientific, Inc.; Rockford, IL, USA) for 10 minutes, washed with TBS-T and re-probed for additional antibodies as outlined earlier. Complete stripping of antibodies was verified by application of the secondary antibody to the blot and developed as described above.

**Histological Analysis.** Non-contracted plantaris and triceps brachii muscles were mounted on cork in a tragacanth gum/optimal cutting temperature (OCT, Thermo Fisher Scientific, Inc.; Rockford, IL, USA) mixture and frozen in liquid N₂-cooled isopentane. Muscles were then cut into 10 µm cross-sections using a cryoSTAT (Model Microm HM 505, Thermo Fisher Scientific, Inc.; Rockford, IL, USA). The cross-sections were stained with hemotoxylin and eosin (H&E) and light micrographs were captured (10X) with an Olympus IX81 microscope (Olympus America, Inc.; Center Valley, PA, USA). Muscle fiber CSA of ~200 muscle fibers for each section were measured with Olympus MicroSuite software (Olympus America, Inc.; Center Valley, PA, USA) by three individual investigators blinded to the treatment.

**Statistical Analysis.** A 1 x 3 (maximal tetanic force x treatment) one-way ANOVA was performed to determine differences between maximal force output (grams) amongst treatments for the force-frequency curve. A 1 x 3 (fatigue index x treatment) one-way ANOVA was performed to determine differences between fatigue index (%), the percent difference from the maximal tetanic tension force output during the force-frequency curve and the tetanic tension force output from a single 100 Hz contraction after 5 minutes of rest following the fatigue protocol between treatments. A 1 x 3 one-way ANOVA was performed to determine if differences exist in phosphorylation status of AMPK, p38-MAPK, Akt, and
p70S6K in the non-contracted gastrocnemius muscles with treatment. Additionally, a 1 x 3 (contraction x treatment) repeated measures two-way ANOVA was performed to determine if differences exist in contraction-induced phosphorylation status of AMPK, p38-MAPK, Akt, and p70S6K in the gastrocnemius muscle with treatment. A 1 x 3 one-way ANOVA was performed to determine if differences exist in CSA (µm) of the triceps brachii and plantaris muscles with treatment. A 1 x 3 one-way ANOVA was performed to determine differences between muscle or organ wet weight (normalized to grams of body mass) between treatments. Following a significant F-ratio, Tukey’s post-hoc analyses were performed to determine where specific differences in conditions or treatments occurred. Significance was determined, a priori, at a p-value of ≤ 0.05, and GraphPad Prism 5 (GraphPad; La Jolla, CA, USA) software was used for the analyses.
CHAPTER 3: RESULTS

Isolation of Phytoecdysteroids from A. turkestanica. The 200 g (100 g x 2) ethyl acetate, butanol, and methanolic extraction of initial dried A. turkestanica plant material yielded a 3.89 g enriched phytoecdysteroid extract. TLC analysis of the final 1:4 ATE revealed that the major phytoecdysteroid compounds were present in the extract. Peak run time ($R_t$) identities were recorded at a wavelength of 247nm for the identified phytoecdysteroids: turkesterone ($R_t$ 22.7 min), 20E ($R_t$ 29.6 min), cyasterone ($R_t$ 36.7 min), ajugasterone ($R_t$ 40.3 min), ajugalactone ($R_t$ 50.0 min), and cyasterone 22-acetate ($R_t$ 51.9 min), determined by HPLC (Figure 4). These results are consistent with the commercial standards. The identified phytoecdysteroids contained within the 1:4 ATE dry weight included: turkesterone (5.85%, 0.29 mg/mL), 20E (23.61%, 1.18 mg/mL), cyasterone (3.07%, 0.15 mg/mL), ajugasterone (2.02%, 0.10 mg/mL), ajugalactone (3.98%, 0.20 mg/mL), and cyasterone 22-acetate (2.23%, 0.11 mg/mL; Figure 5). Peak identity of the commercial 20E used for supplementation occurred at $R_t$ 29.4 min and contained 80.11% total phytoecdysteroid and 0.4 mg/mL dry weight, determined by HPLC (Figure 5).

Body, Muscle, and Organ Mass. Body mass was determined for each treatment prior to day 1 and day 28 of supplementation. No significant differences (NSD; $p > 0.05$) were observed in body mass between CT, ATE, and 20E on day 1 and day 28; 32.7 ± 1.0 g, 33.0 ± 0.9 g, 33.4 ± 0.4 g and 32.4 ± 1.1 g, 32.8 ± 0.7 g, 32.5 ± 0.6 g, respectively (Table 1). Skeletal muscle and organ wet mass were determined as mass of muscle tissue (mg) normalized to total body mass (mg/g body mass). NSD ($p > 0.05$) were observed in muscle
tissue wet mass/body mass between CT, ATE, and 20E in the soleus, plantaris, gastrocnemius, tibialis anterior, or extensor digitorum longus muscles (Table 2). In addition, NSD ($p > 0.05$) were observed in organ tissue wet mass/body mass between CT, ATE, and 20E for the heart, liver, spleen, kidneys, and testes (Table 3).

**In vivo Contractile Function.** Twitch tension was determined as the force during a single stimulated submaximal twitch (1 Hz) contraction and force output data are presented as Newtons per gram of GPS mass (N/g GPS). There were NSD ($p > 0.05$) in twitch tension between CT, ATE, and 20E; $11.6 \pm 0.7$ N/g GPS, $10.2 \pm 1.0$ N/g GPS, $10.8 \pm 0.8$ N/g GPS, respectively. Maximal tetanic tension was determined as the highest force output achieved during the force-frequency curve. The average maximal tetanic tension for all animals occurred between 60 to 100 Hz. Maximal tetanic tension force output data are presented as N/g GPS. There were NSD ($p > 0.05$) in maximal tetanic tension between CT, ATE, and 20E; $42.8 \pm 2.7$ N/g GPS, $41.4 \pm 3.5$ N/g GPS, $34.8 \pm 3.0$ N/g GPS, respectively. Fatigue index (%) was determined as the percent difference from the maximal tetanic tension force output during the force-frequency curve and the tetanic tension force output from a single 100Hz contraction after 5 minutes of rest following the fatigue protocol. There were NSD ($p > 0.05$) in fatigue index (%) between CT, ATE, and 20E; $23.1 \pm 2.4\%$, $21.2 \pm 2.0\%$, $23.6 \pm 1.7\%$, respectively (Table 4).

**Muscle Fiber Cross-Sectional Area.** Plantaris and triceps brachii muscle cross-sections were visualized using H&E staining techniques. Each muscle fiber cross-sectional area was measured on ~200 complete muscle fibers from digital images of H&E stained muscle cross-sections. Results revealed that 20E-treated mice had 30% larger plantaris muscle fiber CSA, compared to CT ($p = 0.018$; $2180.0 \pm 152.4 \mu m^2$ versus $1676.9 \pm 138.4$
µm²); however plantaris muscle fiber CSA in ATE-treated mice was not significantly different than CT ($p = 0.497; 1810.5 \pm 114.4$ µm² versus $1676.9 \pm 138.4$ µm²; Figure 6A and 6B). Furthermore, 20E-treated mice had 41% larger triceps brachii muscle fiber CSA, compared to CT ($p = 0.014; 2557.2 \pm 209.8$ µm² versus $1811.0 \pm 90.0$ µm²). Triceps brachii muscle fiber CSA in ATE-treated mice tended to be larger, but was not significantly different than CT ($p = 0.088; 2285.4 \pm 215.6$ µm² versus $1811.0 \pm 90.0$ µm²; Figure 6C and 6D).

**Protein Synthesis Pathway Signaling.** Markers for protein synthesis pathway signaling activity (Akt and p70S6k) were measured by Western blot analysis and determined as the density (arbitrary units; AU) of the target protein band, normalized to the blot average. Total measured samples for non-contracted limb treatment for the phosphorylation status of Akt$^{\text{Ser473}}$ and p70S6k$^{\text{Thr389}}$ was $n = 6$ for each group. NSD ($p > 0.05$) were observed in phosphorylation status of Akt$^{\text{Ser473}}$ and p70S6k$^{\text{Thr389}}$ in basal (non-contracted) skeletal muscles between CT, ATE and 20E (Figures 7A and 7B, respectively). Further, NSD ($p > 0.05$) were observed in contraction-induced (contracted versus non-contracted; C:NC) phosphorylation status of Akt$^{\text{Ser473}}$ and p70S6k$^{\text{Thr389}}$ between CT, ATE, and 20E (Figures 7C and 7D, respectively). NSD ($p > 0.05$) were seen between CT, ATE, and 20E for total protein content of non-contracted Akt and p70S6k (Figures 8A and 8B, respectively) and contraction-induced Akt and p70S6k (Figures 8C and 8D, respectively).

**Mitochondrial Biogenesis Pathway Signaling.** Upstream markers for mitochondrial biogenesis pathway signaling (AMPK and p38-MAPK) were measured by Western blot analysis and determined as the density (AU) of the target protein band, normalized to the blot average. Total measured samples for non-contracted limb treatment for the phosphorylation status of AMPK$^{\text{Thr172}}$ and p38-MAPK$^{\text{Thr180/Tyr182}}$ was $n = 6$ for each group. NSD ($p > 0.05$)
were observed in phosphorylation status of AMPK$^{\text{Thr172}}$ and p38-MAPK$^{\text{Thr180/Tyr182}}$ in basal (non-contracted) skeletal muscles between CT, ATE, and 20E (Figures 9A and 9B, respectively). Further, NSD ($p > 0.05$) were observed in contraction-induced (C:NC) phosphorylation status of p38-MAPK$^{\text{Thr180/Tyr182}}$ (Figure 9C); however, there was a significant increase ($p < 0.001$) in contraction-induced phosphorylation of AMPK$^{\text{Thr172}}$ in 20E-treated mice, compared to CT and ATE (Figure 9D). NSD ($p > 0.05$) were seen between CT, ATE, and 20E for total protein content of non-contracted AMPK and p38-MAPK (Figures 10A and 10B, respectively) and contraction-induced AMPK and p38-MAPK (Figures 10C and 10D, respectively).
CHAPTER 4: DISCUSSION

The purpose of the current study was to determine the effects of ATE, 20E, or vehicle only (CT) fed to 20-month old male mice for 28 days on their ability to (1) increase strength and muscle mass, (2) activate the key control point of protein synthesis, the PI3k-Akt pathway, and (3) activate key upstream control points for mitochondrial biogenesis, the AMPK and p38-MAPK pathways. The novel findings of this study were the increase in muscle fiber size and contraction-induced AMPK activation in aged mouse skeletal muscle with 20E supplementation.

**Anabolic Effects of Phytoecdysteroids in Aged Muscle.** The anabolic effects of 20E supplementation are well documented.\textsuperscript{10,35,137-139,140,142,152} There are conflicting results between 20E supplementation and increases in muscle and body mass.\textsuperscript{142,152-153} The variances in anabolic effects lie within the differences in route, dose, length of treatment, and strain or species of 20E administration. Further, all previous studies that have reported anabolic effects of phytoecdysteroids in rodents have all been in young animals.\textsuperscript{140,142,152} This is the first study to demonstrate the effects of 28 days of phytoecdysteroid supplementation on aged mouse skeletal muscle.

20E-treated sedentary aged mice had a significant 30% and 41% larger fiber CSA of plantaris and triceps brachii muscles, respectively, compared to CT. The increases in muscle fiber CSA are similar to the findings of Toth et al.\textsuperscript{142}, who reported significant increases in extensor digitorum longus and soleus muscle fiber CSA when young rats were administered subcutaneous injections of 20E (5 mg/kg/day) for 8 days. Additionally, the authors
demonstrated that 20E increases fiber size in a muscle-specific fashion (i.e., dependent on the fiber type composition of the muscle), with type I and IIa (i.e., slower, smaller force producing fiber subtype and larger, faster force producing subtype, respectively) and IIx (i.e., fastest, largest force producing fiber subtype) demonstrating the greatest gains in CSA, in soleus and extensor digitorum longus muscles, respectively.\textsuperscript{142}

ATE supplemented mice had a non-significant 8\% and 26\% increase in fiber CSA of plantaris ($p = 0.497$) and triceps brachii ($p = 0.088$) muscles, respectively, compared to CT. The lack of significance in plantaris and triceps brachii in fiber CSA of ATE is an interesting finding. The 50 mg/kg/day dose of phytoecdysteroids of the ATE treated mice contained 28.96 mg/kg/day 20E. Whereas the 20E treated mice received 50 mg/kg/day 20E, which is a difference of 21.04 mg/kg/day (Figure 5). This may partially explain the differences in both signaling and fiber CSA between ATE and 20E. Toth et al.\textsuperscript{142} demonstrated that the increases in body and muscle mass were greater with 5 mg/kg dosages than 0.5 mg/kg dosages of subcutaneous injections of 20E. In addition, Gorelick-Feldman et al.\textsuperscript{35} demonstrated that 20E increased Akt phosphorylation in a dose-dependent (i.e., from 0-10 \mu M) manner in C2C12 cells. Taken together, a higher dosage of 20E in the ATE treatment may have been required in order to see increases in muscle fiber size and signaling.

The mechanisms for increases in muscle fiber size with age are believed to be due, in part, to either increases in anabolic or protein synthesis signaling (i.e., PI3k-Akt signaling) or transitions from slower, smaller fiber subtypes (i.e., type I) to faster, larger fiber subtypes (i.e., type IIa and IIb or IIx). The effect of phytoecdysteroid treatment on fiber type is currently being analyzed in our lab. To test PI3k-Akt signaling, upstream (i.e., Akt\textsuperscript{Ser473}) and downstream (i.e., p70S6k\textsuperscript{Thr389}) markers for this pathway were analyzed in both non-
contracted and contracted GPS. We were unable to detect differences in non-contracted or contracted limb phosphorylation of either Akt\textsuperscript{Ser473} or p70S6k\textsuperscript{Thr389} between treatments. While 20E and ATE have been found to work through the PI3k-Akt pathway,\textsuperscript{35,140} no study has investigated activation of this pathway by phytoecdysteroid treatment from in vivo supplementation. In the current study, the increases in fiber CSA seen with 20E would suggest activation of anabolic (i.e., PI3k-Akt) signaling. The reason for no differences in non-contracted PI3k-Akt signaling could have been due to missed peak signaling for both Akt and p70S6k. The animals were not supplemented the day of contractile function and sacrifice (i.e., day 29). Therefore, we hypothesize that the acute signaling response of phytoecdysteroid supplementation was missed. In support of this muscle protein synthesis, signaling through the PI3k-Akt pathway has been shown to peak within 45-90 minutes following oral feeding of the amino acid leucine, an activator of the PI3k-Akt pathway, in young animals.\textsuperscript{154} In addition, 20E has been found to be eliminated rapidly in young mice. Dzhukharova et al.\textsuperscript{155} reported that a 50 mg/kg caudal vein infusion of 20E with radioactively labeled \textsuperscript{3}H-thymidine had ~90\% eliminated in 30 minutes. The authors also reported that 20E has a half-life of 8.15 min.\textsuperscript{155} This may explain the reason behind the missed signaling differences of the PI3k-Akt pathway between treatments. However, the goal of the current study was to examine the chronic (i.e., multiple days; 28 days) and not acute (i.e., single day or hours) effects of phytoecdysteroid supplementation on PI3k-Akt signaling. Future investigations should examine the acute effects of 20E supplementation of PI3k-Akt signaling in aged skeletal muscle.

There were no differences between treatments in contracted gastrocnemius phosphorylation of Akt and p70S6k. These findings are similar to other studies.\textsuperscript{89,156}
Skeletal muscle has been shown to have two distinct pathways involved in hypertrophy or anabolic signaling and energy or mitochondrial signaling.\textsuperscript{88,156-157} With the 30 minute low-frequency fatigue protocol, it can be speculated that there were high AMP:ATP ratios creating a low cellular energy state. During low cellular energy states, AMPK will directly inhibit large energy consuming pathways (i.e., PI3k-Akt) and up-regulate pathways involved in increasing cellular energy production (i.e., mitochondrial biogenesis).\textsuperscript{157} Therefore, that no differences were seen in PI3k-Akt signaling following contraction was to be expected.

**Phytoecdysteroids Effects on Mitochondrial Biogenesis Pathway Activation.** To test the effects of phytoecdysteroid supplementation on increasing mitochondrial biogenesis, two upstream markers (i.e., p38-MAPK\textsuperscript{Thr180/Tyr182} and AMPK\textsuperscript{Thr172}) that are directly linked to activating the “master regulator” of mitochondrial biogenesis, peroxisome proliferators-activated receptor co-activator 1 (PGC-1\textsubscript{α}), were analyzed. No differences were seen between treatments in non-contracted limb phosphorylation of p38-MAPK\textsuperscript{Thr180/Tyr182} and AMPK\textsuperscript{Thr172}. The acute signaling response for these two markers has been found to peak within 30-60 minutes following pharmaceutical activation via 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) in skeletal muscle.\textsuperscript{158,159} Therefore, with the rapid elimination of 20E demonstrated by Dzhukharova et al.\textsuperscript{155} may explain the reason for the no differences in resting mitochondrial biogenesis signaling. Again, the goal of the current investigation was to examine the effects of chronic (i.e., 28 days) mitochondrial biogenesis signaling and not acute (i.e., single day or hours) mitochondrial biogenesis signaling. Future investigations should examine the acute effects of phytoecdysteroid supplementation on mitochondrial biogenesis signaling in aged skeletal muscle.
AMPK’s low energy sensing ability is impaired with aging.\textsuperscript{6-7,76} In the current study, 20E treated mice were able to activate AMPK following a 30 minute low-frequency fatigue protocol. Therefore, one can speculate that 20E may be able to correct AMPK’s sensing ability in aged muscle. However, further research is needed to either prove or disprove this finding. Conversely, no differences were observed in p38-MAPK\textsuperscript{Thr180/Tyr182} phosphorylation following the low-frequency fatigue protocol. p38-MAPK has been found to be activated during low cellular energy states to subsequently activate molecules (i.e., PGC-1\textalpha) involved in increasing cellular energy production (i.e., mitochondrial biogenesis).\textsuperscript{92} It is interesting that 20E was able to activate AMPK following contraction, but not p38-MAPK. However, these findings are similar to Ljubicic and Hood.\textsuperscript{6} The authors demonstrated a difference in high oxidative red muscle (i.e., high mitochondrial content) and low oxidative white muscle (i.e., low mitochondrial content) activation of AMPK and p38-MAPK following a low-frequency fatigue protocol of 1 Hz for 5 minutes of muscle isolated from the tibialis anterior (TA) of aged rats. Only the high oxidative red TA muscle was shown to activate both p38-MAPK\textsuperscript{Thr180/Tyr182} and AMPK\textsuperscript{Thr172} following the fatigue protocol. In addition, the isolated low oxidative white TA muscle did not activate either p38 MAPK\textsuperscript{Thr180/Tyr182} or AMPK\textsuperscript{Thr172} following the fatigue protocol. In the current study, the entire gastrocnemius muscle, a predominantly low oxidative muscle, was utilized for mitochondrial biogenesis pathway signaling. The use of the entire gastrocnemius muscle may partially explain the reason for no differences in p38 MAPK\textsuperscript{Thr180/Tyr182} activation following the fatigue protocol. Conversely, the observed AMPK\textsuperscript{Thr172} activation by 20E following the fatigue protocol is opposite of the findings by Ljubicic and Hood.\textsuperscript{6} The differences between AMPK activation in low oxidative muscle by Ljubicic and Hood\textsuperscript{6} and the current study could be due to the frequency (1 Hz
versus 30 Hz) and duration (5 minutes versus 30 minutes) of the fatigue protocols. More research is needed to analyze the effects of phytoecdysteroid treatment on contraction-induced mitochondrial biogenesis signaling, specifically in determining the differences in both low and high oxidative muscles/fibers.

**Novel Method for Dietary Phytoecdysteroid Supplementation.** To minimize distress and chance of injury to the animals, a new method for phytoecdysteroid supplementation was employed. The method of applying the 50 mg/kg/day dosage of either vehicle only (CT), ATE, or 20E to one-190 mg rodent enrichment treat (Fruit Crunchies, BioServ; Frenchtown, NJ, USA) one-day prior to supplementation proved to be very effective. This newly established method could be of great use for investigators in order to dose mice with phytoecdysteroids solubilized in non-denatured ethanol for long supplementation protocols and to minimize stress and chance of injury, especially in aged rodents where there is an even greater concern due to the high mortality rates of aged animals.

**CONCLUSIONS**

This is the first study to demonstrate that 20E increases muscle fiber CSA in 21-month old male mice. In addition, we report that 20E increases contraction-induced AMPK activity in aged mouse skeletal muscle. However, further research is needed to determine the consequence of this finding on mitochondrial biogenesis. Further, 20E may be a potent, non-hormonal countermeasure to reverse the loss of muscle mass that occurs with aging. Findings from this study warrant further research on phytoecdysteroids effects on the age-associated loss of muscle mass.
REFERENCES


54. Funai K, Parkington JD, Carambula S, Fielding RA. Age-associated decrease in contraction-induced activation of downstream targets of Akt/mTor signaling in skeletal muscle. Am J Physiol Regul Integr Comp Physiol 2006;290:R1080-1086.


Table 1. Animal mass data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Mass Day 1 (g)</th>
<th>Body Mass Day 28 (g)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>32.7 ± 1.0</td>
<td>32.4 ± 1.1</td>
<td>0.44</td>
</tr>
<tr>
<td>ATE</td>
<td>33.0 ± 0.9</td>
<td>32.8 ± 0.7</td>
<td>0.40</td>
</tr>
<tr>
<td>20E</td>
<td>33.4 ± 0.4</td>
<td>32.5 ± 0.6</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Data are presented as mean (grams; g) ± standard error of the mean by treatment: control (CT), *Ajuga turkestanica* extract (ATE) and 20-hydroxyecdysone (20E). No significant differences between groups, $p > 0.05$. 
### Table 2. Muscle wet weight of non-contracted hindlimb.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>CT</th>
<th>ATE</th>
<th>20E</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>0.27 ± 0.00</td>
<td>0.27 ± 0.01</td>
<td>0.28 ± 0.00</td>
<td>0.36</td>
</tr>
<tr>
<td>Plantaris</td>
<td>0.62 ± 0.01</td>
<td>0.60 ± 0.01</td>
<td>0.65 ± 0.02</td>
<td>0.27</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>4.13 ± 0.13</td>
<td>3.96 ± 0.11</td>
<td>4.07 ± 0.11</td>
<td>0.60</td>
</tr>
<tr>
<td>Tibialis Anterior</td>
<td>1.74 ± 0.08</td>
<td>1.69 ± 0.03</td>
<td>1.74 ± 0.03</td>
<td>0.72</td>
</tr>
<tr>
<td>Extensor Digitorum Longus</td>
<td>0.39 ± 0.02</td>
<td>0.36 ± 0.01</td>
<td>0.36 ± 0.10</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Data are presented as mean (mg tissue/g body mass) ± standard error of the mean by treatment: control (CT), *Ajuga turkestanica* extract (ATE) and 20-hydroxyecdysone (20E). No significant differences between groups, *p* > 0.05.
<table>
<thead>
<tr>
<th>Organ</th>
<th>CT</th>
<th>ATE</th>
<th>20E</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>5.0 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>4.9 ± 0.1</td>
<td>0.66</td>
</tr>
<tr>
<td>Liver</td>
<td>46.4 ± 3.6</td>
<td>40.4 ± 0.9</td>
<td>41.6 ± 1.2</td>
<td>0.16</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.8 ± 1.4</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>0.47</td>
</tr>
<tr>
<td>Kidneys</td>
<td>13.6 ± 0.4</td>
<td>14.1 ± 0.5</td>
<td>14.5 ± 0.5</td>
<td>0.44</td>
</tr>
<tr>
<td>Testes</td>
<td>5.7 ± 0.2</td>
<td>5.5 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Data are presented as mean (mg tissue/g body mass) ± standard error of the mean by treatment: control (CT), *Ajuga turkestanica* extract (ATE) and 20-hydroxyecdysone (20E). No significant differences between groups, *p* > 0.05.
Table 4. In vivo contractile function data of the gastrocnemius-plantaris-soleus (GPS) complex.

<table>
<thead>
<tr>
<th>Contractile Measurement</th>
<th>CT</th>
<th>ATE</th>
<th>20E</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twitch Tension</td>
<td>11.6 ± 0.7</td>
<td>10.2 ± 0.2</td>
<td>10.8 ± 0.8</td>
<td>0.51</td>
</tr>
<tr>
<td>Maximal Tetanic Tension</td>
<td>42.8 ± 2.7</td>
<td>41.4 ± 3.6</td>
<td>34.8 ± 3.0</td>
<td>0.16</td>
</tr>
<tr>
<td>Fatigue Index (%)</td>
<td>23.1 ± 2.4</td>
<td>21.2 ± 2.0</td>
<td>23.6 ± 1.7</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Data are presented as mean (Newtons per gram of GPS mass; N/g GPS) ± standard error of the mean by treatment: control (CT), *Ajuga turkestanica* extract (ATE) and 20-hydroxyecdysone (20E). No significant differences between groups, \( p > 0.05 \).
FIGURE 1. *Ajuga turkestanica* 1st batch flow chart. The first extraction of the 6% “crude” *A. turkestanica* extract was suspended in double distilled water (ddH$_2$O) and partitioned with n-hexane. The n-hexane layer was then discarded. The remaining aqueous (AQ) layer was partitioned with ethyl acetate (EthAc). The ethyl acetate extract (6.27 g) was dried and set aside. The remaining AQ layer was then extracted with butanol (BuOH). The BuOH extract (35.13 g) was split and ½ of the extract (17.57 g) was extracted with EthAc in 0.5% methanol (MeOH). The 0.5% MeOH in EthAc extract (2.47 g) was then combined with 1.64 g of the remaining ½ BuOH extract at 2:3 EthAc extract to BuOH extract ratio (i.e., 2:3 mixture) to obtain a 1:4 20E to turkesterone ratio. The 2:3 mixture was then extracted first with EthAc and then BuOH. The EthAc extract (1.65 g) was dried and combined with the dried BuOH extract (0.99 g) to make a 1:4 20E to turkesterone ratio.
FIGURE 2. *Ajuga turkestanica* 2nd batch flow chart. The second extraction of 6% “crude” *A. turkestanica* extract (ATE) was suspended in double distilled water (ddH₂O) and partitioned with ethyl acetate (EthAc). The EthAc extract (6.79 g) was dried and set aside. The remaining aqueous (AQ) layer was then partitioned with butanol (BuOH). The AQ layer from the BuOH partitioning was discarded. The dried BuOH extract was then extracted with 0.2% methanol (MeOH) in EthAc. The 0.2% MeOH in EthAc extract (1.02 g) was then combined with the EthAc extract (1.56 g) and the 1st extraction 1:4 mixture (1.31 g) to create a final 1:4 ATE (3.89 g). TLC analysis of the final 1:4 ATE revealed that the major phytoecdysteroid compounds were present in the extract.
**FIGURE 3.** Experimental design schematic. The effects of phytoecdysteroid supplementation on 20-month old male mouse skeletal muscle was tested. Three treatment groups were employed for the 28-day supplementation protocol: control (CT; vehicle only of 100% non-denatured ethanol, EtOH), *Ajuga turkestanica* extract (ATE), and 20-hydroxecdysone (20E). Following 28 days of consecutive supplementation with ATE, 20E, or vehicle only (CT) each animal was tested for in vivo contractile function of the gastrocnemius-plantaris-soleus (GPS) complex. Skeletal muscles and organs were then dissected and utilized for Western blot analysis, muscle and organ wet weights, or histological and cross-sectional area (CSA) analysis.
**FIGURE 4.** HPLC chromatogram of the final *Ajuga turkestania* extract (ATE). Peak run time ($R_t$) identities were recorded at a wavelength of 247nm for the identified phytoecdysteroids: turkesterone ($R_t$ 22.7 min), 20E ($R_t$ 29.6 min), cyasterone ($R_t$ 36.7 min), ajugasterone ($R_t$ 40.3 min), ajugalactone ($R_t$ 50.0 min), and cyasterone 22-acetate ($R_t$ 51.9 min).
FIGURE 5. ATE and 20E phytoecdysteroid contents. The commercial 20-hydroxecdysone (20E; i.e., 80.11% total phytoecdysteroid) utilized for the supplementation protocol was different from the purified commercial 20E (i.e., 100% total phytoecdysteroid) utilized for the phytoecdysteroid quantification calibration curve. The identified phytoecdysteroids contained within the 1:4 Ajuga turkestana extract (ATE) dry weight include: turkesterone (5.85%), 20E (23.61%), cyasterone (3.07%), ajugasterone (2.02%), ajugalactone (3.98%), and cyasterone 22-acetate (2.23%) for a total of 40.77% total phytoecdysteroid. The commercial 20E used for supplementation contained 80.11% total phytoecdysteroid based on dry weight, as determined by HPLC.
FIGURE 6. Plantaris and Triceps Brachii fiber cross-sectional area (CSA). Skeletal muscle fiber CSA in 20-month old male mice after the 28-day supplementation period by treatment: control (CT), Ajuga turkestanica extract (ATE), and 20-hydroxyecdysone (20E). Quantification of CSA from ~200 fibers in Plantaris (A) and Triceps Brachii (B) muscles, respectively. Representative Hematoxylin and Eosin stained sections from Plantaris (C) and Triceps Brachii (D) muscles, respectively. *Denotes 20E significantly different from CT (p < 0.05).
FIGURE 7. Akt and p70S6k resting and contracted phosphorylation. Protein analysis of markers of protein synthesis signaling in aged (20-month old) mouse gastrocnemius muscle via Western blot after the 28-day supplementation period by treatment: control (CT), *Ajuga turkestanica* extract (ATE), and 20-hydroxyecdysone (20E). Quantification of non-contracted phosphorylation of Akt$^{\text{Ser473}}$ (A) and p70S6k$^{\text{Thr389}}$ (B) by treatment, respectively, No significant differences (NSD) between groups ($p < 0.05$). Quantification of contracted versus non-contracted (C: NC) fold change from control (CT) phosphorylation of Akt$^{\text{Ser473}}$ (C) and p70S6k$^{\text{Thr389}}$ (D) by treatment, respectively. NSD between groups ($p < 0.05$). Above A-D are representative Western blot protein bands by treatment.
FIGURE 8. Akt and p70S6k resting and contracted total protein content. Protein analysis of markers of protein synthesis signaling in aged (20-month old) mouse gastrocnemius muscle via Western blot after the 28-day supplementation period by treatment: control (CT), Ajuga turkestanica extract (ATE), and 20-hydroxyecdysone (20E). Quantification of non-contracted total protein content of Akt (A) and p70S6k (B) by treatment, respectively. No significant differences (NSD) between groups (p < 0.05). Quantification of contracted versus non-contracted (C: NC) fold change from CT total protein content of Akt (C) and p70S6k (D) by treatment, respectively. NSD between groups (p < 0.05). Above A-D are representative Western blot protein bands by treatment.
FIGURE 9. p38-MAPK and AMPK resting and contracted phosphorylation. Protein analysis of markers of mitochondrial biogenesis signaling in aged (20-month old) mouse gastrocnemius muscle via Western blot after the 28-day supplementation period by treatment: control (CT), Ajuga turkestania extract (ATE), and 20-hydroxyecdysone (20E). Quantification of non-contracted phosphorylation of p38-MAPK\(^{\text{Thr180/Tyr182}}\) (A) and AMPK\(^{\text{Thr172}}\) (B) by treatment, respectively. No significant differences (NSD) between groups \((p < 0.05)\). Quantification of contracted versus non-contracted (C: NC) fold change from CT phosphorylation of p38-MAPK\(^{\text{Thr180/Tyr182}}\) (C) and AMPK\(^{\text{Thr172}}\) (D) by treatment, respectively. *Denotes significantly different from CT and # denotes significantly different from ATE, respectively. Above A-D are representative Western blot protein bands by treatment.
FIGURE 10. p38-MAPK and AMPK resting and contracted total protein content. Protein analysis of markers of mitochondrial biogenesis signaling in aged (20-month old) mouse gastrocnemius muscle via Western blot after the 28-day supplementation period by treatment: control (CT), *Ajuga turkestanaica* extract (ATE), and 20-hydroxyecdysone (20E). Quantification of non-contracted total protein content of p38-MAPK\(^{\text{Thr180/Tyr182}}\) (A) and AMPK\(^{\text{Thr172}}\) (B) by treatment, respectively. No significant differences (NSD) between groups \((p < 0.05)\). Quantification of contracted versus non-contracted (C: NC) fold change from CT total protein content of p38-MAPK\(^{\text{Thr180/Tyr182}}\) (C) and AMPK\(^{\text{Thr172}}\) (D) by treatment, respectively. NSD between groups \((p < 0.05)\). A-D are representative Western blot protein bands by treatment.
**ANIMAL RESEARCH PROTOCOL**

**DATE SUBMITTED:** MARCH 17, 2011  
**DATE REVIEWED:** APRIL 6, 2011

For IACUC Use Only:  

<table>
<thead>
<tr>
<th>IACUC No.</th>
<th>Date of Approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-008</td>
<td>4/8/2011</td>
</tr>
</tbody>
</table>

NOTE: Use a separate form for each species. DO NOT include individual appendices if they are not relevant to the protocol being described.  

ACRONYMS: Define all abbreviations the first time they are used. TABLES/ROWS: To add a row to a table, click inside one of the existing table cells, then select Table, Insert, Rows from the main menu of the program.

### SECTION A: APPLICATION INFORMATION

Complete items A.1- A.8 below; then proceed to item B.

**CHECK ALL THAT APPLY:**

- Animals Housed Outside the CLAS  
- Breeding  
- Major Survival Surgery  
- Category E studies  
- Free-ranging Wildlife  
- Hazardous Agents  
- Chemical Hazards  
- Physical Restraint  
- Exceptions to The Guide  
- Comments:  

1. **PRINCIPAL INVESTIGATOR:** R. Andrew Shanely, Ph.D.

2. **DHMRI ADDRESS & CONTACT INFORMATION:**  
   - PHONE: 704-250-5357  
   - EMAIL: shanelyra@appstate.edu  

3. **PROPOSAL TITLE:** Naturally-produced phytoecdysteroids from Ajuga turkestanica as a countermeasure against sarcopenia

4. **ANIMAL SPECIES COVERED BY THIS PROTOCOL:** Mouse

5. **FUNDING SOURCE:**
   - Department of Veterans Affairs  
   - U. S. Public Health Service (e.g. NIH)  
   - Private or Charitable Foundation. Identify:  
   - University Departmental Funds. Identify University and Department:  
   - Private Company Identify:  
   - Other: Identify: Internal (ASU) funding via research indirects

6. **Is this a new protocol for a new project?**
   - Yes. If YES, proceed to item 7.  
   - No. If NO, answer A.6.a.-d. below.
     - a. Indicate the status of this protocol below:  
       - This is an unchanged, approved protocol intended for a new funding source.
Notice of Initial Protocol Approval

TO: Dr. R. Andrew Shanely
600 Laureate Way, NCRC

FROM: Dr. Lawrence Mays
NCRC IACUC Chair

SUBJECT: Approval of Protocol #11-008
TITLE: “Naturally-produced phytoecdysteroids from Ajuga turkestanica as a countermeasure against sarcopenia”

DATE: April 8, 2011

The North Carolina Research Campus Institutional Animal Care and Use Committee (IACUC) approved the protocol “Naturally-produced phytoecdysteroids from Ajuga turkestanica as a countermeasure against sarcopenia” on April 8, 2011.

Approvals are valid for one (1) year and may be renewed before the anniversary of the original approval date for a total of three (3) years of study. A renewal/status report is due annually for IACUC protocols and must be submitted along with the renewal request form. After three years, a new protocol application must be submitted to continue the study. Please note the following information:

Protocol #: 11-008
Title: “Naturally-produced phytoecdysteroids from Ajuga turkestanica as a countermeasure against sarcopenia”

Renewal #1 due and approved before: April 8, 2012
Renewal #2 due and approved before: April 8, 2013
Expiration date: April 8, 2014

Please note that it is the investigator’s responsibility to promptly inform the committee of any proposed changes in this study, as well as any unanticipated problems that may occur involving care and use of animals.

All changes (i.e., adding or removing personnel from this study, changing strains, changes in procedure or in prescribed animal care, etc.) must be submitted to the IACUC by way of an Amendment. All amendment and renewal forms are available through the IACUC office.

It is also the investigator’s responsibility to maintain detailed surgical records if the IACUC-approved study involves surgical procedures. These records must be available to the Center for Laboratory Animal Sciences (CLAS) Director and the Attending Veterinarian and a copy must be kept with the animals at all times.

If you need additional information or assistance, please contact Dixie Airey at 704-687-3311.

Approval Authorized by:

__________________________________  __________________
Dr. Lawrence Mays, NCRC IACUC Chair       Date
VITA

Marcus Michael Lawrence was born in Modesto, California to Michael J. Lawrence and Anne E. Lawrence. Following graduation at Orestimba High School in Newman, California he attended California State University Monterey Bay in Seaside, California to obtain a Bachelor of Science in Kinesiology.

In August 2010, Marcus accepted a research assistantship in the Biochemistry, Neuromuscular, and Biomechanics Laboratories at Appalachian State University in Boone, North Carolina and began study toward a Master of Science in Exercise Science. In May 2011, Marcus accepted a Kannapolis Scholars fellowship through the USDA National Institute of Food and Agriculture to conduct research in the Human Performance and Plants for Human Health Institute Laboratories at the North Carolina Research Campus in Kannapolis, North Carolina. His Master of Science was awarded in August 2012. Following graduation Mr. Lawrence commenced work toward his Ph.D. in Biology at the University of North Carolina at Charlotte in Charlotte, North Carolina.