THE EFFECTS OF LEUCINE OR DIFFERENT PROTEIN SUPPLEMENTS ON MUSCLE HYPERTROPHY AFTER 12 WEEKS OF RESISTANCE TRAINING IN UNTRAINED MEN

By

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A dissertation submitted to the Graduate Faculty of
Auburn University
In partial fulfillment of the
Requirements for the Degree of
Doctor of Philosophy of Exercise Science

Auburn, Alabama
December 16, 2017

Keywords: Resistance Exercise, Satellite Cells, Skeletal Muscle Hypertrophy, Leucine, Whey Protein, Soy Protein

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ABSTRACT

Purpose: We sought to determine the effects of L-leucine (LEU) or different protein supplements standardized to LEU (~3.0 g/serving) on changes in body composition, strength, and histological attributes in skeletal muscle and adipose tissue. **Methods:** Seventy-five untrained, college-aged males (mean±SE; age=21±1 yr, body mass=79.2±0.3 kg) were assigned to an isocaloric, lipid-, and organoleptically-matched maltodextrin placebo (PLA, n=15), LEU (n=14), whey protein concentrate (WPC, n=17), whey protein hydrolysate (WPH, n=14), or soy protein concentrate (SPC, n=15) group. Participants performed whole-body resistance training three days per week for 12 weeks while consuming supplements twice daily. Skeletal muscle and subcutaneous (SQ) fat biopsies were obtained at baseline (T1) and ~72 h following the last day of training (T39). Tissue samples were analyzed for changes in type I and II fiber cross sectional area (CSA), non-fiber specific satellite cell count, and SQ adipocyte CSA. **Results:** On average, all supplement groups exhibited similar training volumes and experienced statistically similar increases in total body skeletal muscle mass determined by dual x-ray absorptiometry (+2.2 kg; time $p=0.024$) and type I and II fiber CSA increases (+394 μm^2 and +927 μm^2; time $p<0.001$ and 0.024, respectively). Notably, all groups reported increasing Calorie intakes ~600-800 kcal/d from T1 to T39 (time $p<0.001$), and all groups consumed at least 1.1 g/kg/d of protein at T1 and 1.3 g/kg/d at T39. There was a

training, but no supplementation, effect regarding the reduction in SQ adipocyte CSA (-210 μm^2; time $p=0.001$). Interestingly, satellite cell counts within the WPC ($p<0.05$) and WPH ($p<0.05$) groups were greater at T39 relative to T1. **Conclusion:** In summary, LEU or protein supplementation (standardized to LEU content) does not provide added benefit in increasing whole-body skeletal muscle mass or strength following 3 months of training in previously untrained college-aged males that increase Calorie intakes with resistance training and consume above the recommended daily intake of protein throughout training. However, whey protein supplementation increases skeletal muscle satellite cell number in this population, and this phenomena may promote more favorable training adaptations over more prolonged periods.

ACKNOWLEDGMENTS

Fundraising for this study was done by the generous and painstaking efforts of Dr. Christopher M. Lockwood. Without his role, this study would not have been possible. Moreover, he has provided the large inspiration for the Molecular and Applied Sciences Laboratory with regards to their efforts in studying the physiological effects of different protein and amino acid supplements. Specifically, Dr. Lockwood procured funding from:

1) Dr. Chao Wu who is the Research and Design Director at Hilmar Ingredients. We are greatly appreciative of Dr. Wu and his team for allocating company funds and whey protein products to devote to this study.
2) Kevin Lawrence who is the C.E.O of Bionutritional Research Group (BNRG). We are greatly appreciative of Mr. Lawrence as well as Mr. Jeff Feliciano for allocating company funds and whey protein products to devote to this study.
3) Lockwood, LLC for formulating and supplying products to devote to this study.
4) JW Nutritional, LLC for formulating and manufacturing all five supplements used in the study.
5) Glambia Nutritionals for allocating leucine products to devote to this study.
6) Covance Laboratories for validating the nutritional facts and amino acid profiles of all five supplements.
7) Cellucor for allocating materials for this study.

I also want to take the time to thank the participants for devoting their time as well as their muscle and blood specimens and for completing the study. Without their participation, this would not have been possible. I would also like to thank all the lab personnel and volunteers who dedicating their time and contributing to the completion of this study because without their help this study would not have been able to function as it did without their help and contribution.

More importantly, I would like to thank God for His tremendous love, blessings and guidance because without Him, none of this would've been made possible. For Jeremiah 29:11 says, "'For I know the plans I have for you', declares the Lord, 'plans to prosper you and not to harm you, plans to give you hope and a future.'" I would like to thank my loving family for their unending love and support through the years. I love each of you very much. I would like to thank my mentor and best friend, Dr. Michael D. Roberts, for his friendship, mentorship, guidance and counsel. You are by far one of the best men that I know and will ever know. Your personality, generosity and intellectual character as a friend, a mentor and a scholar is nothing shy of remarkable. I would like to thank my committee members, Drs. David D. Pascoe, John J. McCarthy, John C. Quindry, Andreas N. Kavazis, and Jeffrey S. Martin, for their friendship, intellectual contribution, patience and counsel through this learning and growing process. Also, I would like to thank Dr. Kevin Huggins for accepting to be the outside reader for this project and for his time and intellectual contribution to the completion of this project.

Each of you have made a tremendous impact in my life. I look forward to future collaborations and good times. I would like to thank the Molecular and Applied Sciences Laboratory platoon, Dr. Kaelin Young, Dr. Darren Beck, Dr. Wesley C. Kephart, Petey W. Mumford, Cody T. Haun, Matthew A. Romero, Paul A. Roberson, Christopher G. Vann and Shelby C. Osburn, for their friendship, mentorship, counsel, and hard work in helping make this project a success and for helping me develop as a scientist and colleague. I would also like to thank Dr. Kenny V. Brock for his friendship and mentorship through the years. Thank you for helping point me in the direction of my academic future. Finally, I would like to thank the faculty, staff, and students of the School of Kinesiology and Auburn University for supporting me through the many years whilst here at Auburn.

Lastly, I would like to dedicate this body of work to my father, Albert Ridley Mobley. You are my worldly inspiration. I love you and miss you dearly each and every day. Your impact on me as a father, a friend, and a man of God has continued to inspire me to work hard at being a great husband, a good father, a better friend, and a humble man of God.

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LIST OF ABBREVIATIONS

g	grams
d	day
kg	kilograms
MPS	muscle protein synthesis
MPB	muscle protein breakdown
EAA	essential amino acid
BCAAs	branched chain amino acids
mTOR	mammalian target of rapamycin
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
mRNA	messenger RNA
kDa	kilodalton
p70s6k	70-kDa S6
RAPA	rapamycin
GβL	G-protein beta-subunit-like protein
Raptor	regulatory-associated protein of mTOR
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
Rictor	RAPA-insensitive companion of mTOR
Rheb	ras homologous protein enriched in brain
PI-3K	phosphatidylinositol-3 kinase
CSA	cross sectional area

TSC 1/2	tuberous sclerosis complex
Akt/PKB	protein kinase B
GTP	guanosine-5'-triphosphate
ATP	adenosine triphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate kinase
PLD	phospholipase D
PA	phosphatidic acid
FRB	FKBP12-rapamycin binding
TA	tibialis anterior
eIF4E/G	eukaryotic initiation factor 4E/G
4E-BP1	4E-binding protein-1
rps6	ribosomal protein small 6
RagA-D	Ras small GTPases
NEAA	nonessential amino acids
WPH	whey protein hydrolysate
Ser	serine
Thr	threonine
WPC	whey protein concentrate
WPI	whey protein isolate
LBM	lean body mass
IRB	institutional review board
T1	baseline/baseline testing

DXA	dual-energy X-ray absorptiometry
SQ	subcutaneous
T2	second visit/training familiarization
IMTP	isometric mid-thigh pull
3-RM	three repetition maximum
T38	last training bout
T39	week 12/post-testing
mL	milliliters
ppm	part per million
cm	centimeters
TBMM	total body skeletal muscle mass
mg	milligrams
OCT	optimal cutting temperature
H&E	hematoxylin and eosin
T3	first training bout
Hz	hertz
N	newtons
CSCS	Certified Strength and Conditioning Specialist
NSCA	National Strength and Conditioning Association
DUP	daily undulating periodization
RPE	rate of perceived exertion
kcal	total energy
DH	degree of hydrolysate

LEU	L-leucine
T20	week 6
µm -	micrometer
µL	microliter
DAPI	4,6-diamidino-2-phenylindole
C	Celsius
ms	milliseconds
µm2	microns-squared
SE	standard error of the mean
G*T	group*time
ANCOVA	analysis of covariance
ANOVA	analysis of variance
PLA	placebo
SPC	soy protein concentrate
yr	years
n	number
h	hour(s)
min	minutes
CONSORT	Consolidated Standards of Reporting Trials
MMP	matrix metalloprotease
Est. 1-RM	Estimated one repetition maximum
1-RM	one repetition maximum
IU	international units

CHAPTER I

INTRODUCTION

Investigating the effects of protein supplementation to facilitate skeletal muscle hypertrophy has been the focus of sports nutrition research for centuries. While more sophisticated techniques are currently used to assess the synthesis and breakdown rates of mixed-muscle specific protein sub-fractions, older studies relied upon nitrogen balance methods to assess the effects of dietary proteins on whole-body nitrogen retention. From a simplistic viewpoint, nitrogen balance is a measure of the amount of nitrogen retained by the body from dietary protein sources minus the amount of nitrogen released by the body via sweat, feces, and urine. Earlier studies used nitrogen balance methodologies for tracking whole-body protein metabolism muscle before, during, and after a meal and/or exercise. During the late 1800's, Atwater and Benedict [1] reported that nitrogen excretion during exercise did not increase. Their findings led to the notion that whole body nitrogen uptake increases following exercise in order to facilitate muscle growth; an idea that was later supported by other research groups [2-4].

These earlier investigations gave rise to studies in the 1950's like that of Calloway et al. [5] who investigated the effects of caloric and protein intake on nitrogen balance. Briefly, these authors reported that, in physically active military personnel, diets lacking in sufficient protein resulted in a negative nitrogen balance while diets with adequate protein intakes resulted in a positive whole-body nitrogen balance. Moreover, Consolazio et al. [6] reported that active young adults who increased protein intakes from

100 g/d to 190 g/d over a 40-d rigorous training period experienced up to a 2-fold greater increase in net nitrogen balance along with a 3.3 kg increase in estimated lean body mass (compared to 1.21 kg increase in those that maintained 100 g/d intakes). In essence, the authors determined that the increase in lean body mass was a result of an increase in net nitrogen balance.

Research that emerged in the late 1980's, specifically that of Rennie et al. [7], examined whole-body protein synthesis during the fed state versus the fasted state. These authors concluded that in the fed state, skeletal muscle protein synthesis (MPS) contributes to more than half of whole body protein synthesis. Later investigations [8-11] also examined whole body leucine metabolism during the fed or fasting state, and reported that resistance training may chronically increase the synthesis and breakdown of skeletal muscle. In the same era of the aforementioned studies, research in the area of protein consumption and exercise focused on how ingesting dietary protein and/or amino acids affected net nitrogen balance [12-15] or, more specifically, skeletal MPS versus skeletal muscle protein breakdown (MPB) rates. For example, in the 1990's Dr. Robert Wolfe and other laboratories began studying the effects of amino acid infusion [12, 16-18], amino acid oral ingestion [19-22], enriched protein meal ingestion [23-27] or protein supplement ingestion [28-33] on stimulating acute post-prandial anabolic responses in skeletal muscle. Through much of this work it became better understood that, during fasting states, MPS is low and MPB is increased which leads to a negative net protein balance (NPB). Over prolonged periods of fasting, a loss of skeletal muscle mass will

ensue [34]. In addition, the Wolfe laboratory and other researchers discovered that in order for an elevated MPS response to occur within skeletal muscle, two phenomena must occur: 1) nutrients, specifically essential amino acids from dietary protein sources, are needed [35-39], and/or 2) skeletal muscle must be mechanically-loaded [40-43]. It soon became appreciated that the dietary protein-driven MPS response depends upon the ingestion of a specific protein source given that dietary proteins contain distinct amino acid profiles [44]. Moreover, the type of exercise also is important for stimulating MPS and, in this regard, researchers have determined that higher-intensity contractions (such as resistance exercise) increases MPS for up to 48 hours following one exercise bout [45], whereas low-intensity and prolonged contractions (such as endurance exercise) depresses MPS. If these two respective training stimuli are applied over a chronic time course, then the molecular drivers of an increased MPS with resistance training lead to skeletal muscle hypertrophy, whereas the repetitive decrements in MPS with endurance training can lead to a somewhat modest but significant decrease in muscle mass [46-48].

There have now been numerous studies examining how different dietary protein supplements such as whey protein [29], soy protein [29], egg protein [33] and casein protein [32] affects whole-body nitrogen balance or skeletal muscle MPS versus MPB around an acute exercise bout. Many of these studies focused on the protein composition; specifically the essential amino acid (EAA) profile of these protein sources, given that the EAA composition of a protein source plays an integral role in stimulating MPS [35, 49-51]. Building upon previous investigations of EAA provision, more focus

was placed on the ‘anabolic’ amino acid profile; specifically branched chain amino acids (BCAAs; L-leucine, L-isoleucine, L-valine), and their anabolic effects on skeletal muscle [52, 53]. Later mechanistic studies [54, 55] determined that specifically L-leucine increases MPS by enhancing mammalian target of rapamycin (mTOR) signaling [56] and facilitating an increase in translation initiation [57-59]. Hence, these findings have led to the concept that, in order for diet-induced increase in MPS to occur in skeletal muscle, a ‘leucine threshold’ must be reached [44, 60-63].

The aforementioned amino acids or protein sources have been extensively researched with regards to their effects in stimulating post-feeding MPS and/or enhancing post-exercise MPS in an acute setting [19, 29, 32, 33, 64]. Generally, whey and egg protein elicit the greatest increases in MPS, and casein and soy protein elicit a lesser MPS response [29, 32, 33, 64-68]. The MPS response to BCAAs alone has been a bit more elusive given that: a) studies examining the acute anabolic response to BCAA ingestion around exercise have used a method that looks at leg amino acid uptake, but not MPS per se [69], and b) most of the BCAA literature in this area has examined how spiking whey protein with BCAAs or leucine affects skeletal muscle amino acid uptake or MPS [69-71]. In this regard, little research to our knowledge has compared the MPS-stimulating effects of intact protein supplements to BCAAs, although one recent animal study determined that whey protein was better able to stimulate post-exercise MPS in rats compared to an amino acid mixture that was contained within the whey protein supplement [72].

Although the aforementioned studies have unveiled valuable information as to how amino acid and/or protein ingestion acutely affects skeletal muscle MPS levels, there has been recent criticism that the acute MPS response to protein ingestion is not necessarily reflective of the long-term changes in muscle mass [47, 73]. However, numerous studies have examined how one or more amino acid and/or protein supplements affect muscle mass gains with chronic resistance exercise. For instance, Phillips authored a 2009 meta-review article which examined 9 placebo-controlled resistance exercise protein supplement studies (~250 subjects) and determined that those supplementing with milk or whey protein experienced, on average, an estimated 3 kg increase in muscle mass, while those supplementing with soy protein experienced a ~1.5 kg increase and those supplementing with a carbohydrate-based placebo experienced a ~1.0 kg increase [74]. Further support for the role of nutrient supplementation in muscle mass gains can be appreciated from a 10-week resistance training study comparing the effects of whey protein versus casein supplementation. In that study, the investigators reported that casein caused a 0.8 kg increase in lean body mass, whereas whey caused a 5.3 kg increase in lean body mass [65]. Studies incorporating chronic resistance training and EAA supplementation have yielded less than favorable results, with lean body mass changes being similar to those seen in placebo groups [75, 76] or unchanged after the training/supplementation period [77]. Similarly, while 8 weeks of egg protein supplementation and resistance training has been shown to increase in lean body mass,

muscle mass gains were not significantly different than those observed in the placebo group [78].

The aforementioned acute and long-term supplementation studies have led to a greater understanding regarding the anabolic properties of various dietary proteins and/or amino acids when consumed in the absence or presence of an exercise stimulus. However, there is a knowledge gap in regard to whether protein supplementation-induced increases in muscle mass with chronic training is chiefly due to the L-leucine content of said protein sources. Specifically, while meta-data exists which differentially compares the anabolic effects of various protein supplements [44, 74, 79, 80], no one scientific research study has conclusively assessed the chronic anabolic effects of various amino acid or protein sources such as leucine, whey, or soy in combination with resistance training. Therefore, the purpose of this study was to examine if leucine or other protein supplements (specifically whey or soy) enhances markers of skeletal muscle hypertrophy assessed from whole-body composition analyses and muscle biopsy analyses when ingested in conjunction with 12 weeks of chronic resistance training in previously untrained males. Specifically, all experimental groups received 2 doses per day of either a placebo supplement (no added leucine or protein), leucine, hydrolyzed whey protein concentrate, intact whey protein concentrate, or soy protein concentrate. As a secondary aim, we also examined how these different protein supplements affected subcutaneous fat cell size determined via gluteal fat biopsies.

Specific aims of this proposal

1. Examine pre- and post-intervention left-leg skeletal muscle biopsies to analyze potential anabolic effects of the following supplements during a 12-week whole body resistance training intervention: a) a maltodextrin placebo supplement, b) a leucine supplement, and c) whey protein concentrate, whey protein hydrolysate, and soy protein concentrate supplements.
2. Examine pre- and post-intervention body composition measures (i.e., total-body skeletal muscle mass, dual-leg and -arm lean muscle mass, and vastus lateralis muscle thickness) to analyze potential anabolic effects of the aforementioned supplements during a 12-week whole body resistance training intervention.
3. Examine pre- and post-intervention whole body (i.e., upper and lower) strength and peak force performance measures (i.e., isometric mid-thigh pull, maximum back and bench press) to analyze potential ergogenic effects of the aforementioned supplements during a 12-week whole body resistance training intervention.

Hypotheses

I hypothesize that:

1) Based upon the aforementioned literature showing that resistance training alone increases muscle mass, resistance training in the placebo group will elicit anabolic and ergogenic responses to resistance training.

2) Whey protein in combination with resistance training will provide the greatest anabolic and ergogenic response given that whey protein has been shown to outperform other protein supplements in the scientific literature [65, 74, 81].

CHAPTER II

LITERATURE REVIEW

Background

The anabolic response in skeletal muscle to an overload stimulus like that of heavy resistance exercise has been well documented from earlier investigations [40, 41, 82]. For instance, MacDougall et al. [4] reported a 98% increase in strength, a 10% increase in nuclei-to-fiber ratio, and 39% and 31% increase in fast twitch and slow twitch fiber areas, respectively, after 5-6 months of heavy resistance training. Likewise, McCall et al. [83] reported an increase in Type I and II muscle fibers, with the greatest increase occurring in type II fibers, after 12 weeks of intense resistance training. Further, investigations like that of Sale et al. [84] reported an 11% increase in left and right knee extensor cross-sectional area in males that trained 3 days/week for 19 weeks utilizing lower-body resistance exercise. Similarly, other studies like that of Higbie and colleagues [85] reported a 6.6% and 5.0% increase in quadriceps cross-sectional area after 10 weeks of concentric or eccentric unilateral knee extensor training. Hence, mechanical loading through resistance exercise promotes appreciable increases in skeletal muscle hypertrophy [86].

Literature using cell, animal and human models demonstrating the mechanisms involved with increased muscle protein synthesis

Understanding the mechanisms that drive increases in MPS have led to a greater appreciation of processes that influence muscle hypertrophy. In 1978, Laurent et al. [87] reported that 58 days of chronic stretch of the anterior and posterior latissimus dorsi in chickens caused a 140% increase in protein content with similar increases in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) concentrations in the stretched muscle. Interestingly, RNA and DNA concentrations peaked at 5-10 days following initiation of the stretch stimulus with an increase in protein content subsequently occurring at 28-58 days. Importantly, these authors demonstrated that the rate of protein synthesis following this stretch-loading model precedes changes in RNA accumulation. Similarly, Wong and Booth [88, 89] reported a 16-50% increase in protein synthesis 15 hours post-resistance exercise at a time point when RNA and DNA accumulation was unchanging in rats. They further concluded that the increase in total RNA, which represents ribosome content responsible for catalyzing MPS, was not predicative of protein synthesis but that translational and posttranslational mechanisms were responsible for increases in protein synthesis. Likewise, others [90, 91] have reported similar effects after acute resistance exercise with regard to increases in MPS synthesis not correlating with total RNA and messenger RNA (mRNA) concentrations. Using a rat model of acute and chronic resistance exercise, Baar and Esser [92] reported an increase in 70-kDa S6 (p70s6k) phosphorylation 6 hours after a single bout of resistance exercise. They also reported that the acute changes in skeletal muscle p70s6k phosphorylation following a single bout of resistance exercise correlated with percent changes in muscle mass after 6 weeks of

chronic resistance training. Moreover, these investigators employed polysome profiling to evaluate the effects of resistance training on ribosome activity and concluded that the rate of translation initiation increases following one bout of resistance exercise. Thus, their data suggested that p70s6k is a key regulator of translation initiation in skeletal muscle undergoing load-induced hypertrophy. Supporting Baar and Esser's model, Terzis et al. [93] reported that acute increases in p70s6k phosphorylation in response to the first bout of resistance exercise was closely correlated with increases in skeletal muscle mass after 14 weeks of resistance training in humans. Therefore, while the total RNA content (or number of ribosomes) may remain unaltered with acute resistance exercise, an increased translational efficiency accounts for the increase in MPS following nascent resistance training bouts [94]. Baar, Nader, and Bodine [47] have described these mechanisms by stating:

> *"Skeletal muscle hypertrophy occurs following repeated bouts of high resistance exercise. Whilst each individual bout of high resistance exercise is necessary, it is not sufficient to produce hypertrophy. This indicates that, following acute exercise, there is a transient alteration within the muscle that, when repeated, produces skeletal muscle hypertrophy. The most important acute response to resistance exercise is an increase in the rate of protein synthesis."*

It soon became appreciated that the upstream modulator of p70s6k, mammalian target of rapapmycin (mTOR), was a centralized hub for the initiation of MPS. Briefly, mTOR is a large (289-kDa) multi-domain enzyme that regulates a vast array of cellular processes. Since its discovery in the early 1990's [95, 96], researchers have discovered that mTOR is a highly conserved kinase that controls a vast array of homeostatic cell functions including cell growth, protein synthesis and translational control [97]. Earlier studies have reported [98, 99] that the antibiotic rapamycin (RAPA) selectively blocks mTOR and results in a diminished MPS/hypertrophic response in skeletal muscle cells. Later studies have shown that mTOR exists in two distinct multi-protein complexes with only one of the complexes being inhibited by RAPA. In this manner, Huang and Manning [100], along with Corradetti and Guan [101], reported that the RAPA-sensitive complex of mTOR consists of mTOR, G-protein beta-subunit-like protein (GβL) and regulatory-associated protein of mTOR (Raptor). Raptor is unique in that, when it is bound to mTOR, it promotes the assembly of the complex and helps in recruiting substrates to assist in the translocation of mTOR to the lysosome [102, 103]. Collectively, once the mTOR-GβL-Raptor complex translocates to the lysosome and becomes an active kinase, this complex is referred to as mTOR complex 1 (mTORC1). Of note, mTOR can exist in another cellular complex termed mTOR complex 2 (mTORC2) which consists of mTOR, GβL and RAPA-insensitive companion of mTOR (Rictor), though mTORC2 is not thought to play a direct role in protein synthesis regulation [101, 104].

Other small proteins or molecules have been shown to associate to the mTORC1 complex to increase its ability to up-regulate MPS. For instance, when overexpressed *in vitro*, Rheb (ras homologous protein enriched in brain) increases the activity of mTORC1 in a phosphatidylinositol-3 kinase (PI-3K)-independent manner. Furthermore, *in vivo* overexpression of Rheb increases cap-dependent translation resulting in a 64% increase in the cross sectional area (CSA) of muscle fibers [105]. One mechanism that may affect Rheb activation itself is the inactivation of tuberous sclerosis complex (TSC1/2). Interestingly, if TSC1/2 is inhibited by protein kinase B (Akt), this allows Rheb to become charged with guanosine-5'-triphosphate (GTP) which, in turn, increases mTORC1 activity [106]. If there is a loss in cellular energy secondary to an increased rate of adenosine triphosphate (ATP) consumption, and a subsequent increase in cellular adenosine monophosphate (AMP), AMP activated AMP kinase (AMPK) which, can activate TSC2 and inhibit Rheb-mediated activation of mTORC1 [107]. Hornberger and colleagues have recently reported that phospholipase D (PLD) could serve as a mechanically-induced activator of mTORC1. Specifically, activation of PLD increases intracellular phosphatidic acid (PA), and this occurs in response to mechanical stretch [108] or electrical stimulation [109]. Fang et al. [110] demonstrated that PA activates mTOR by binding to the FKBP12-rapamycin binding (FRB) domain on mTOR.

While numerous intracellular signals contribute to the phosphorylation/activation of mTORC1, downstream mTORC1 substrates must be activated for an MPS response to occur. For instance, Parkington et al. [111] reported that with acute resistance exercise

via electrical stimulation in rats, mTOR phosphorylation and downstream signaling (i.e., p70s6k), but not upstream signaling (i.e., Akt/PKB), were enhanced in type IIa muscle fibers (i.e., tibialis anterior, plantaris) for up to 6 hours following exercise. The research group concluded that mTOR activation is elevated after muscle contractions, and that this response was localized to type IIa muscle versus type I muscle fibers. Nader and Esser [112] have also reported that different exercise protocols (i.e., low frequency electrical stimulation vs. high frequency electrical stimulation vs. running) result in a selective activation of specific intracellular signaling pathways, which could very well determine the adaptive response to different forms of exercise. To this end, they reported a prolonged increase in p70s6k of 40% and 72% at 3 and 6 hours, respectively, following high-frequency electrical stimulation training in the tibialis anterior (TA; type IIa) muscle whereas the soleus (type I) muscle experienced no change. Others [113] have reported that several downstream mTORC1 substrates are transiently affected during the immediate recovery period (within 1 hour) following acute resistance exercise; specifically, increases in eukaryotic initiation factor 4E (eIF4E) association with eukaryotic initiation factor 4G (eIF4G) (292% above un-exercised), 4E-binding protein-1 phosphorylation (4E-BP1) (292% above un-exercised), p70s6k phosphorylation (336% above un-exercised) and ribosomal protein S6 (rps6) phosphorylation (647% above un-exercised). Collectively, these results indicate that an mTOR-dependent phosphorylation cascade facilitates translation initiation and MPS following acute resistance exercise.

Amino acid and/or protein ingestion for enhancing the acute anabolic response to resistance exercise

It is important to note that, while numerous exercise-dependent signals orchestrate the MPS response, the availability of amino acids is a rate-limiting factor in regulating protein synthesis [114]. Therefore, resistance exercise as well as amino acid provision following exercise is needed to optimally stimulate MPS. Tipton and Wolfe [50] have emphasized this point by stating:

> *"Exercise alone and the provision of amino acids alone both increase rates of muscle protein synthesis; exercise alone reduces the rates of muscle protein catabolism; and amino acids induce muscle anabolism. However, the potent initiator of muscle protein synthesis is the combination of resistance exercise and elevated amino acid availability."*

Beyond serving as building blocks for MPS, amino acids (specifically leucine) can stimulate mTORC1 activity to increase MPS independent of resistance exercise. While the in-depth mechanisms responsible for leucine-mediated increases in mTORC1 activity and eventual MPS is beyond the realm of this proposal, it is important to note what researchers have discovered thus far. Briefly, muscle cells contain four members of Rag subfamily of Ras small GTPases (e.g., RagA-D) [115], and these proteins are required for amino acids to acutely stimulate mTORC1 [116, 117]. However; unlike Rheb, Rag GTPases cannot stimulate mTORC1 directly [116]. Instead, amino acids

induce RagA or RagB-GTP binding, thereby causing them to associate with Raptor which, in turn, mediates mTORC1 translocation to the lysosome. As mentioned prior, once mTOR (and mTORC1-associated proteins) translocate to the lysosome, mTORC1 activity increases, downstream substrates are phosphorylated (p70s6k, 4EBP-1, and rps6), and MPS is up-regulated.

Empirical evidence for the aforementioned mechanisms exists. For instance, Borsheim et al. [22] reported a dose-dependent effect for EAA-mediated increases in MPS. Of note, leucine is an EAA and BCAA, so this effect may be due to the presence of leucine in EAAs. Rennie and colleagues [118] also reported that large doses of individual amino acids, particularly leucine but not nonessential amino acids (NEAA), were able to stimulate the uptake of an infused tracer into human muscle proteins suggestive of enhanced amino acid integration into the muscle.

There have been a plethora of studies also suggesting that amino acids, particularly leucine, enhances the anabolic response to resistance exercise. For example, Wolfe's lab [12] reported that amino acid infusion immediately after resistance exercise in humans contributed to a greater MPS response than an amino acid infusion administered up to 4 hours post-exercise. The research group concluded that, in the presence of an abundant extracellular amino acid supply, intramuscular processes that increase MPS is enhanced. Rasmussen et al. [20] reported a similar anabolic response in men and women at 1 and 3 hours post-resistance exercise after consuming 6 g of EAA in combination with 35 g of sucrose. Interestingly, Blomstrand et al. [57] also cite

numerous studies which have reported that BCAA administration (which contains leucine), activates key enzymes required for MPS (i.e., mTOR, p70s6k, 4E-BP1) within 1-2 hours following resistance exercise during the post-exercise recovery period.

As with acute amino acid administration and exercise studies, the acute MPS response to resistance exercise with various intact dietary protein sources has also been well documented. Tipton and colleagues [32] conducted a study whereby twenty-three young, healthy males and females were randomly assigned to consume a placebo treatment (water), 20 g of casein containing 1.7 g of leucine or 20 g of whey protein hydrolysate (WPH) containing 2.3 g leucine. Supplementation was provided 1 hour at the cessation of a heavy leg extension resistance exercise. Muscle biopsy samples were collected from the *vastus lateralis* muscle at baseline, 60, 120 and 300 minutes post exercise with blood samples collected at various time points throughout the length of the experiment. These authors concluded that acute whey protein ingestion resulted in a greater arterial and venous leucine concentration than casein. They further concluded that the acute ingestion of whey protein or casein protein resulted in similar increases in skeletal muscle uptake of amino acids, again suggestive of enhanced amino acid integration into the muscle. Tipton and colleagues [30] also investigated the benefits of whey protein plus additional leucine to enhance MPS following resistance exercise. These authors reported that when subjects consumed 16.6 g of WPH plus 3.4 g of additional leucine, both the placebo and WPH treatments resulted in an anabolic response. Hulmi et al. [119] compared the effects of whey protein versus a placebo 1

hour and 48 hours following lower-body resistance exercise. These authors reported that the phosphorylation of mTOR (Ser2448), p70s6k (Thr389), and rpS6 (Ser235/236) increased in the whey protein group versus placebo group, which is suggestive that MPS increased. Tang et al. [29] from Stuart Phillips's laboratory normalized whey, casein and soy protein doses to total EAA content (~10g) and had trained participants consume a whey (21.4 g), casein (21.9 g), or soy (22.2 g) protein drink immediately following a unilateral leg resistance exercise protocol. These authors reported that whey protein consumption resulted in a 93% and 18% greater MPS response over casein and soy, respectively. Moreover, it was speculated that whey was superior in increasing MPS due to its ability to facilitate post-prandial hyperaminoacidemia.

The aforementioned Tang et al. study was a landmark study in that it illustrated that whey protein optimally stimulates post-exercise MPS when compared to other 'high quality' protein sources. Whey protein makes up ~20% of the total protein content in commercial bovine milk, and casein makes up the remaining 80% of total milk protein [44, 120-122]. Whey protein exists in various forms such as whey protein concentrate (WPC; $\geq$ 30-80% intact protein fractions), whey protein isolate (WPI; $\geq$ 90% intact protein fractions), or WPH ($\geq$ 70% enzymatically pre-digested protein fractions) [123, 124]. Whey protein is unique compared to other intact protein sources given that it possesses a relatively high leucine content; approximately 12-14% of the whey protein amino acid profile is made up of leucine, whereas soy, egg and casein contain 8-9% leucine [125, 126]. Whey protein has a high biological value, or a measure of the

percentage of a given nutrient (e.g., protein) that is utilized by the body, [127] that exceeds egg protein by 15% as well as other protein sources such as meat, soy, casein, and fish [128]. Interestingly, when compared to the amino acid composition of skeletal muscle, whey protein possesses a similar amino acid composition [20, 129] allowing for ingested whey protein to provide similar proportions of amino acids for skeletal muscle protein synthesis and repair following exercise compared to other protein sources [121].

Chronic whey, egg, casein, soy, and BCAA supplementation studies with resistance exercise training in young men.

Several studies have examined the effects of different amino acid or protein sources on being able to enhance resistance exercise-induced increases in muscle mass (summarized in Table 1). However, chronic leucine, EAA or BCAA supplementation studies with resistance exercise are limited. Antonio et al. [130] reported that the ingestion of EAAs combined with 6 weeks of heavy-resistance exercise did not have a pronounced effect on enhancing muscle mass or muscular strength. 12-week resistance training and supplementation studies by Bird et al. [75] and Vieillevovye et al. [76] reported in that EAA supplementation promoted 3.0 and 1.0 kg increases in lean body mass (LBM), respectively, although these results were statistically equal to the placebo groups. Kerksick et al. [77] reported that 10 weeks of resistance training and BCAA supplementation yielded a non-significant 0.1 kg decrease in lean body mass. Only one chronic egg protein supplementation study with resistance training exists to our

knowledge and this was done in female athletes [78]. The authors reported that, with 8 weeks of resistance training and egg white protein supplementation, participants experienced a 1.5 kg increase in LBM which was statistically equal to a maltodextrin placebo group. Therefore, while different studies have examined how different amino acid or egg protein supplements affect resistance exercise-induced adaptations, the data suggests that these supplements are minimally effective in increasing muscle mass beyond the placebo groups.

Unlike the aforementioned EAA/BCAA/egg studies, more studies exist comparing the effects of whey protein supplementation to a single additional protein source and/or a placebo group. For instance, Cribb and colleagues [65] investigated 10 weeks of chronic supplementation with either WPI or casein in 19 male recreational bodybuilders. These authors concluded that participants who consumed 45 g/day of WPI had a 5 kg increase in LBM which was 4.2 kg greater than the casein group which experienced a 0.8 kg increase in LBM. Similarly, Burke and colleagues [131, 132] reported 2.3-2.5 kg increases in LBM with 6 weeks of resistance training combined with whey protein supplementation which was 1.4 kg greater than the maltodextrin placebo groups in these studies. Of note, a soy protein group was also examined in one of the two aforementioned studies [132], and this group experienced a 1.7 kg increase in LBM which was not statistically greater than the placebo group. Other studies like that by Joy et al. [133] reported a 3.2 kg increase in LBM with 8 weeks of resistance training and whey protein supplementation; however, no placebo group was reported. Hulmi et al.

[134] reported that 10.5 weeks of resistance training and whey protein supplementation elicited a 2.5 kg increase in LBM which was 0.5 kg greater than the non-energetic placebo. Recently, Volek et al. [81] reported that 9 months of resistance training combined with WPC or soy protein supplementation resulted in 3.6 kg and 2.6 kg increases in LBM, respectively. The resistance training mediated increase in LBM with whey protein was significantly greater than that with soy protein and maltodextrin placebo groups. However, the increases in LBM in the soy and placebo groups were similar.

Purpose statement

Collectively, the aforementioned acute and chronic studies suggest that various amino acids and protein sources can acutely initiate anabolic mechanisms which, over time, can enhance muscle mass. Moreover, protein source clearly plays a key role in facilitating muscle growth. However, there is a substantial knowledge gap within the scientific community with regards to whether or not the leucine content of supplemental protein is the driving factor in gains in muscle mass when supplementation is combined with resistance training. Specifically, while meta-data exists that differentially compares the anabolic effects of various protein supplements [44, 74, 79, 80], no one scientific research study has conclusively assessed the chronic anabolic effects of leucine, whey, and soy in combination with resistance training. Therefore, the purpose of this study was to examine which amino acid or protein supplement (specifically leucine, whey, and soy)

enhances markers of skeletal muscle hypertrophy (described in detail in Chapter III) when ingested in conjunction with 12 weeks of chronic resistance training in previously untrained males.

CHAPTER III

METHODS

Ethical approval and screening of participants

Prior to initiating this study, the protocol was reviewed and approved by the Auburn University Institutional Review Board (IRB), and will be conducted in accordance with the Declaration of Helsinki (approved protocol #: 15-320 MR 1508; IRB contact: irbadmin@auburn.edu). Healthy, untrained, college-aged male (i.e., 19-23) participants will be recruited for this study. All enrolled participants will be instructed to provide verbal and written study consent, complete a medical history form, and complete a 4-day food log to prior to starting the study. These screening forms will ensure that all eligible participants will apparently be healthy and recreationally active but: a) not engaging in any regular exercise program for at least 6 months prior to study initiation (<2 resistance training exercise or high-intensity aerobic exercise sessions/week), b) not currently consuming a high-protein diet (>2.0 g/kg/d), c) not using anabolic enhancing agents (e.g., anabolic steroids, supplemental protein, creatine monohydrate, or prohormones), or d) not presenting with any medical or orthopedic condition(s) that would hinder them from participating in the study. Once initial screening is complete, all eligible participants will be scheduled to return to the School of Kinesiology at Auburn University one week later for baseline testing (T1).

Study design

The study design implemented was double-blinded and placebo-controlled. Participants will be encouraged to refrain from rigorous physical activity for 4-5 days prior to baseline testing (T1). For T1, participants will be instructed to report to the laboratory in a well-hydrated, 4-hour fasted state whereby they will be subjected to the following assessments: a) urine specific gravity, b) height and body mass, c) body composition using dual-energy X-ray absorptiometry (DXA) (General Electric Lunar Prodigy enCORE, software version 10.50.086; Madison, WI, USA), d) vastus lateralis thickness using ultrasonography (General Electric LOGIQ S7 Expert; Chicago, IL, USA), e) venipuncture, f) percutaneous skeletal muscle biopsy collection from the vastus lateralis, and g) a percutaneous subcutaneous (SQ) fat biopsy from the gluteal region. Two to three days following T1, subjects will report back to the laboratory in a 4-hour fasted state for a second visit (T2) whereby maximal force production capacity will be assessed using an isometric mid-thigh pull (IMTP) test, lower body strength will be assessed using a three repetition maximum (3-RM) squat, and upper body strength will be assessed using a 3-RM bench press. Additionally, during T2, subjects will be familiarized with all lifts that are to be performed during the training intervention. Following T2, subjects will engage in 12 weeks of resistance training and supplementation. The last training bout (T38) will consist of IMTP as well as squat and bench press 3-RM re-assessments in a 4-hour fasted state. Seventy two hours following T38, subjects will report back to the laboratory in a 4-hour fasted state for post-testing (T39) which will consist of all body composition, and blood and biopsy collection

procedures noted for T1. All of the aforementioned testing procedures as well as training and supplementation procedures are described in greater detail below.

Body Composition Testing

During T1 and T39 participants will be instructed to submit a urine sample (~5 mL) to assess normal hydration specific gravity levels (1.005-1.020 ppm) using a handheld refractometer (ATAGO; Bellevue, WA, USA). Participants with a urine specific gravity >1.020 will be asked to consume tap water every 15 minutes for 30 minutes and then were re-tested. Following hydration testing, height and body mass will be assessed using a digital column scale (Seca 769; Hanover, MD, USA) with weights and heights collected to the nearest 0.1 kg and 0.5 cm, respectively. Next, participants will be subjected to a full body DXA scan while wearing general sports attire (i.e., athletic shorts or compression shorts and an athletic shirt) to assess various body composition characteristics. Dual arm and dual leg lean muscle mass, as assessed by the accompanying software, will be used to estimate total body skeletal muscle mass (TBMM) by employing the following equation: TBMM = (1.13 x ALST) – (0.02 x age) + 0.97.

Notably, body segmentation for each scan will be standardized prior to analyses by the same technician. Total body fat mass will also assessed by the accompanying software. According to previous data published by our laboratory, the same-day reliability of the DXA during a test-calibrate-retest on 10 participants produced intra-

class correlation coefficients of 0.998 for total body fat mass (mean difference between tests = 0.40±0.05 kg), 0.998 for total body lean mass (mean difference between tests = 0.29±0.13 kg), and 0.998 for dual-leg lean mass (mean difference between tests = 0.17±0.09 kg).

Following DXA scans, participants will be subjected to an ultrasound assessment to determine vastus lateralis muscle thickness. Measurements will be taken from the midway point between the iliac crest and patella of the right femur whereby subjects will be in a standing position and all weight will be placed on the left leg. All DXA scans and ultrasound assessments will be completed by the same investigator in order to minimize variability in testing procedures.

Venipuncture, and percutaneous skeletal muscle and fat biopsies

T1 and T39 venous blood samples will be aseptically collected from the antecubital vein and collected into a 5 mL serum separator tube (BD Vacutainer; Franklin Lakes, NJ, USA). Notably, this blood will be saved for further experimentation and variables assessed from these blood draws will not presented herein. Immediately following blood collection, participants will be instructed to lay in a supine position on a treatment table whereby a percutaneous skeletal muscle biopsy will be aseptically obtained from the left vastus lateralis muscle using a 5 gauge Bergstöm needle with suction. Approximately, 20-40 mg of skeletal muscle tissue for each time point will be placed in a cryomold with optimal cutting temperature media (OCT media) (Electron

Microscopy Sciences; Hatfield, PA, USA). Cryomolds will then be slow-frozen in liquid-nitrogen-cooled isopentane and stored at -80°C for immunohistochemistry analyses that are described below. Sections of SQ fat (1-2 cm) extracted from the gluteal aspect of the left hip will be placed in 10% formalin and preserved for hematoxylin and eosin (H&E) staining and histological analyses which are described in detail below. Following T1 testing procedures, subjects will be counterbalanced into one of five groups based upon DXA LBM values in order to ensure that baseline values do not differ between supplement groups. More details regarding supplementation are described below, and supplementation will begin immediately following the first training bout (T3).

Isometric mid-thigh pull, strength testing, and weightlifting familiarization

During T2 and T38, participants will be instructed to report back to the laboratory under well hydrated, 4-hour fasted conditions for strength testing and weight training familiarization (T2). First, each participant will complete an IMTP test which has been validated to approximate whole-body maximal voluntary strength. Briefly, knee and hip angles (125±5° and 175±5°, respectively) will be measured using a standard goniometer (Fabrication Enterprises; White Plains, NY, USA). A standard, 20 kg barbell (York Barbell; York, PA, USA) and STS Power Rack (York Barbell) will be used to conduct the IMTP. Dual OR6-7 force plates (AMTI; Watertown, MA, USA) with dual Gen 5 amplifiers (AMTI) sampling at 500 hertz (Hz) will be used to measure vertical force production in Newtons (N). Each participant will be allowed at least two attempts, and

up to four attempts if differences in vertical peak force between trials was >250 N. Manufacturer software will be used to calculate vertical peak force during the testing sessions, and a custom-written MATLAB script (Natick, MA, USA) will be employed to identify the greatest vertical force produced in N for each trial, post-hoc. Two trials within 250 N will be used to calculate an average vertical peak force across trials and function as a metric for maximal voluntary force production (i.e., strength) in this investigation.

Approximately 5 minutes following T2 and T38 IMTP testing, participants will perform 3-RM back squat and bench press assessments using a 20 kg barbell (York Barbell), STS Power Rack (York Barbell) and free weights. A demonstration of proper lifting technique as well as the implementation of progressive-loading 3-RM tests will be overseen by personnel who possess the Certified Strength and Conditioning Specialist (CSCS) credential from the National Strength and Conditioning Association (NSCA). Bench press and back squat repetitions will be considered successful if performed through the full range of motion (i.e., chest touch to full arm extension for bench press, and eccentric lowering past 90° knee flexion for back squat). A repetition will not be counted if subjects exhibit poor and/or unsafe technique or need assistance with a repetition during maximal testing.

Approximately 5 minutes following T2 3-RM testing, participants will be instructed to perform the other two major lifts implemented for training (i.e., deadlift and bent-over-row) in the presence of CSCS-certified personnel. The goal of this session will

be to familiarize each participant with appropriate lifting technique in order to minimize the risk of injury throughout the course of the study.

Training protocol

For visits 3-37 (T3-T37), a daily undulating periodization (DUP) training model will be employed over the 12-week training period. Specifically, participants will be instructed to perform free-weighted barbell squats, bench press, deadlifts, and bent-over-rows for 4 sets of 10 repetitions (Monday or Tuesday), 6 sets of 4 repetitions (Wednesday or Thursday), and 5 sets of 6 repetitions (Friday or Sunday). Immediately following each completed set, a rating of perceived exertion (RPE) score will be acquired from each participant (scale: 1-10) in order to monitor and progress each participant accordingly while minimizing the potential risk of injury. The RPE scale will be described to participants as the remaining number of repetitions that the participant would be able to complete while employing good technique (i.e., 1 = 9 remaining repetitions in reserve, 10 = 0 remaining repetitions in reserve). Participants will be instructed to attend all 36 resistance training sessions throughout the duration of the study, but those that miss more than 4 sessions will not be included in the analysis due to lack of training compliance. All participants will be supervised by laboratory personnel for each training session to ensure that proper lifting technique is executed, and training volumes for each session will be recorded.

Supplementation

As stated above, participants will be assigned to ingest either a PLA, LEU, WPC, WPH, or SPC supplement throughout the training intervention. On training days (T3-T37), participants will consume an individually-packaged serving in ~500 ml of tap water immediately following each training session under direct observation of the study personnel. Additionally, participants will be instructed to consume an individual serving within 30 minutes prior to bedtime on training days. On non-training days, participants will be instructed to consume an individual serving between a meal of their choosing and 30 minutes prior to bedtime. Supplements will be separated into individual ready-made supplement-coded packets for daily consumption, and participants will be given a 3-week supply. Study personnel will collect and count empty packets from each participant every 3 weeks before the next 3-week supply is distributed. Participants that do not consume >80% will not be included in the analysis due to lack of compliance.

Each supplement, except PLA, will be formulated to provide ~3 g of leucine, per serving. Furthermore, each supplement will be formulated to yield similar amounts of total energy (kcal) and fat (g), and will be double-blinded to laboratory personnel and participants for group, appearance, taste, texture, and packaging. The WPC supplement will be formulated using an agglomerated, 80% WPC (Hilmar™ 8010, Hilmar Ingredients; Hilmar, CA, USA). The WPH supplement will be formulated using an agglomerated, partially hydrolyzed [12.5% degree of hydrolysate (12.5% DH), yielding approximately 67% of peptides as <5 kDa in molecular weight] 80% whey protein

concentrate (Hilmar™ 8360, Hilmar Ingredients); SPC will use an agglomerated, 80% soy protein concentrate (ALPHA® 5812, Solae, LLC; St. Louis, MO, USA); LEU will use an agglomerated, L-Leucine (L-Leucine USP, Glambia Nutritionals; Carlsbad, CA, USA) and non-GMO, corn-derived maltodextrin (MALTRIN®-M100; Grain Processing Corporation; Muscantine, IA, USA); and, the PLA group will be formulated using maltodextrin (MALTRIN®-M100; Grain Processing Corporation). All five supplements will be manufactured at JW Nutritional, LLC (Allen, TX, USA), a United States Food and Drug Administration cGMP-compliant facility independently audited and pre-qualified by Obvium*Q, LLC (Phoenix, AZ, USA), a GMP regulatory compliance firm. Personnel at JW Nutritional, LLC and Lockwood, LLC (Draper, UT, USA) will formulate and maintain the blinding of groups, and each supplement will be assigned a randomly generated item number. Manufacturing batch records for production of each of the five supplements will be reviewed by a trained, independent expert in dietary supplement quality control and assurance before approval for use within the present study. All supplements will be independently validated for nutritional facts and total amino acids using validated, approved methods at Covance Laboratories, Inc. (Madison, WI, USA), a pre-qualified third-party analytical laboratory. Once analysis is complete, personnel not involved in the study will release the code for all treatments.

Nutritional intake monitoring

Participants will be instructed to maintain their normal dietary habits along with returning a 4-day food log (2 week days and both weekend days) at baseline (T1), week 6 (T20) and week 12 (T39). On each occasion, participants will be given detailed written and verbal instructions on completing the food logs. Dietary intake data will be analyzed using open-sourced software (http://www.myfitnesspal.com).

Immunofluorescent histochemistry for muscle fiber type-specific characteristics

Muscle sections will be analyzed for type I fiber cross sectional area (CSA), type II fiber CSA, type I fiber myonuclear number, type II fiber myonuclear number, and total (non fiber type-specific) satellite cell number. Briefly, sections from OCT-preserved samples will be cut at a thickness of 20 μm using a cryotome (Leica Biosystems; Buffalo Grove, IL, USA) and will be adhered to positively-charged histology slides. Once all samples are sectioned, batch processing will occur for immunofluorescent histochemistry. During batch processing, sections will be air-dried at room temperature for 30 minutes, fixed with 10% formalin for 10 minutes, permeabilized in a phosphate-buffered saline (PBS) solution containing 0.5% Triton X-100, and blocked with 100% Pierce Super Blocker (Thermo Fisher Scientific; Waltham, MA, USA) for 25 minutes.

For fiber type staining (following blocking), sections will be subsequently washed for 5 minutes in PBS and incubated for 1 hour with a primary antibody solution containing rabbit anti-dystrophin IgG (Thermo Fisher Scientific; 10 μL antibody per 1 mL of blocking solution) and mouse anti-myosin II IgG (catalog #: SC71; Hybridoma

Bank; 100 µL per 1 mL of blocking solution). Sections will then be washed for 5 minutes in PBS and incubated in the dark for 1 hour with a secondary antibody solution containing Texas Red-conjugated anti-rabbit IgG (Vector Laboratories; Burlingame, CA, USA), and Alexa Fluor 488-conjugated anti-mouse IgG (Thermo Fisher Scientific) (10 µL of all secondary antibodies per 1 mL of blocking solution). Sections will then be washed for 5 minutes in PBS, air-dried and mounted with fluorescent media containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Following mounting, slides will be stored in the dark at 4ºC until immunofluorescent images are obtained.

For satellite cell staining (following blocking), separate sections will be incubated for 1 hour with a pre-diluted commercially-available primary antibody solution containing rabbit anti-dystrophin IgG (Thermo Fisher Scientific), and 1:15 dilution of mouse anti-Pax7 IgG (Hybridoma Bank, Iowa City, IA, USA) for 1 hour. Sections will then be washed for 5 minutes in 1x PBS and incubated in the dark for 1 hour with a secondary antibody solution containing 1:100 dilution of Texas Red-conjugated anti-rabbit IgG (Vector Laboratories) and Alexa Fluor 488-conjugated anti-mouse IgG (Thermo Fisher Scientific). Sections will then be washed for 5 minutes in PBS, air-dried and mounted with fluorescent media containing DAPI (Vector Laboratories). Following mounting, slides will be stored in the dark at 4ºC until immunofluorescent images are obtained.

After staining is performed on all sections, digital images will be captured using a fluorescence microscope (Nikon Instruments; Melville, NY, USA) and 20x objective.

Approximate exposure times of 600 ms for red and green imaging and 30 ms for blue imaging will be employed. For fiber typing, our staining method will allow the identification of cell membranes (detected by the Texas Red filter), type II fiber green cell bodies (detected by the FITC filter), type I fiber black cell bodies (unlabeled), and myonuclei (detected by the DAPI filter). For satellite cell identification, our staining method will allow the identification of cell membranes (detected by the Texas Red filter), small green cell bodies as satellite cells (detected by the FITC filter), and myonuclei (detected by the DAPI filter). Measurements of type II fiber CSA will be performed using the open-sourced software CellProfiler™ per modified methods previously described whereby the number of pixels counted within the border of each muscle fiber are converted to a total area in microns-squared (μm2). Measurements of fiber type-specific myonuclear number will also be performed using open-sourced software CellProfiler™ to discriminate the fiber border that corresponds to each myonuclei. Satellite cells will be manually counted using a grid function in the NIS Elements software (Nikon Instruments) and handheld tally counter. At least 50 fibers per specimen will be quantified to obtain accurate CSA, myonuclear number and satellite cell values.

SQ fat CSA analysis

As mentioned above, gluteal fat will be obtained at T1 and T39 for SQ fat analysis. Following tissue processing and H&E staining, SQ fat CSA analysis will be performed. Briefly, SQ fat samples will be removed from formalin and then washed in

cold running tap water, embedded, and stored in 70% alcohol. Dehydration will be accomplished by gradually increasing percentages of ethyl alcohol to replace the water content in the tissue. Hemo-De will subsequently be used to clear the tissue from the ethyl alcohol to allow infiltration with paraffin. The paraffin tissue blocks will be sectioned into 6 µm slices and placed onto glass microscope slides. Paraffin will be removed with xylene, the mounted sections will be stained with hematoxylin and eosin, and sample sections will be enclosed with a coverslip and mounting media. Two 10x objective digital images per sample will be obtained using bright-field imaging (Nikon Instruments), and CSAs will be obtained from at least 50 adipocytes per image using ImageJ (National Institutes of Health; Bethesda, MD, USA).

Statistical analysis

All data will be presented in tables and figures as means ± standard error of the mean (SE) values. Statistics will be performed using SPSS v22.0 (IBM; Armonk, NY, USA) and Microsoft Excel when applicable. A Shapiro-Wilk's test will be employed for all dependent variables to test for distribution normality. If values are not normally distributed then values will be square root-transformed and re-tested using Shapiro-Wilk's tests to ensure that values are normally distributed. All raw and transformed dependent variables (except nutrition data) will then be compared between treatment groups using 5*2 group*time (G*T) two-way repeated measures analysis of covariance (ANCOVA) tests with T1 values for each respective dependent variable serving as the covariate. If a significant time effect is present then within-group dependent samples t-

test will be performed between T1 and T39 values. If a significant G*T interaction is present, within-group dependent samples t-tests will be performed between T1 and T39 values, and one-way analysis of variance (ANOVA) tests with Tukey post hoc tests will be performed at the T39 time point. All nutritional dependent variables will be compared between treatment groups using 5*3 (G*T) two-way repeated measures ANCOVAs with T1 values for each respective dependent variable serving as the covariate. If a significant time effect is present then within-group pairwise comparisons will be performed using Bonferroni post hoc tests. If a significant group*time interaction is present then within-group dependent-samples t-tests will be performed between T1 and T20 as well as T39 values, and one-way ANOVAs with Tukey post hoc tests will be performed at the T20 and T39 time points.

CHAPTER IV

RESULTS

MANUSCRIPT

Nutrients. 2017 Sep 4;9(9). pii: E972. doi: 10.3390/nu9090972. PMDI: 28869573

Received: 21 August 2017; Revised: 31 August 2017; Accepted: 1 September 2017; Published: 4 September 2017

Effects of whey, soy or leucine supplementation with 12 weeks of resistance training on strength, body composition, and skeletal muscle and adipose tissue histological attributes in college-aged males

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ABSTRACT

We sought to determine the effects of L-leucine (LEU) or different protein supplements standardized to LEU (~3.0 g/serving) on changes in body composition, strength, and histological attributes in skeletal muscle and adipose tissue. Seventy-five untrained, college-aged males (mean±standard error of the mean (SE); age=21±1 yr,

body mass=79.2±0.3 kg) were assigned to an isocaloric, lipid-, and organoleptically-matched maltodextrin placebo (PLA, n=15), LEU (n=14), whey protein concentrate (WPC, n=17), whey protein hydrolysate (WPH, n=14), or soy protein concentrate (SPC, n=15) group. Participants performed whole-body resistance training three days per week for 12 weeks while consuming supplements twice daily. Skeletal muscle and subcutaneous (SQ) fat biopsies were obtained at baseline (T1) and ~72 h following the last day of training (T39). Tissue samples were analyzed for changes in type I and II fiber cross sectional area (CSA), non-fiber specific satellite cell count, and SQ adipocyte CSA. On average, all supplement groups including PLA exhibited similar training volumes and experienced statistically similar increases in total body skeletal muscle mass determined by dual x-ray absorptiometry (+2.2 kg; time $p=0.024$) and type I and II fiber CSA increases (+394 μm2 and +927 μm2; time $p<0.001$ and 0.024, respectively). Notably, all groups reported increasing Calorie intakes ~600-800 kcal/d from T1 to T39 (time $p<0.001$), and all groups consumed at least 1.1 g/kg/d of protein at T1 and 1.3 g/kg/d at T39. There was a training, but no supplementation, effect regarding the reduction in SQ adipocyte CSA (-210 μm2; time $p=0.001$). Interestingly, satellite cell counts within the WPC ($p<0.05$) and WPH ($p<0.05$) groups were greater at T39 relative to T1. In summary, LEU or protein supplementation (standardized to LEU content) does not provide added benefit in increasing whole-body skeletal muscle mass or strength above PLA following 3 months of training in previously untrained college-aged males that increase Calorie intakes with resistance training and consume above the

recommended daily intake of protein throughout training. However, whey protein supplementation increases skeletal muscle satellite cell number in this population, and this phenomena may promote more favorable training adaptations over more prolonged periods.

Keywords: satellite cell; resistance training; leucine; whey; soy

1. Introduction

There is widespread evidence suggesting that protein supplementation enhances resistance training adaptations. For instance, Cribb et al. [1] reported that resistance trained participants who consumed 45 g/d of whey protein isolate following 10 weeks of resistance training achieved a 5 kg increase in lean body mass (LBM) which was 4.2 kg greater than a casein-supplemented group. Burke et al. [2,3] reported that whey protein supplementation promoted 2.3-2.5 kg increases in LBM with 6 weeks of resistance training which was ~1.4 kg greater than the effects observed in these studies' maltodextrin placebo (PLA) groups. Notably, a soy protein group was also examined in one of the two aforementioned studies [3], and this group experienced a 1.7 kg increase in LBM which was not statistically greater than the PLA group. Hulmi et al. [4] reported that 10.5 weeks of resistance training and whey protein supplementation elicited a 2.5 kg increase in LBM which was 0.5 kg greater than the non-energetic PLA group. Furthermore, Volek et al. [5] reported that 9 months of resistance training combined with

whey or soy protein supplementation resulted in 3.6 and 2.6 kg increases in LBM, respectively. While the aforementioned studies did not determine the mechanisms responsible for the reported phenotypic changes in skeletal muscle mass, others have postulated that protein supplementation reduces fast to slow isoform shifts [6] and promotes myogenic responses to resistance training [7]. A recent review by Morton et al. [8] provides additional studies reporting that protein supplementation in conjunction with resistance training enhances indices of skeletal muscle anabolism, although other studies have reported that protein supplementation has no added benefit when performed in conjunction with 8-12 weeks of resistance training [9,10].

Contrary to much of the positive data supporting whey protein supplementation, the data appears to be less favorable regarding the effects of amino acid-only supplementation in enhancing resistance training induced increases in LBM. For instance, Bird et al. [11] reported that essential amino acid (EAA) supplementation (6 g/d) led to ~3 kg increase in LBM following 12 weeks of resistance training twice a week in younger untrained males, albeit these increases were not significantly different from a PLA group. Vieillevoye et al. [12] reported similar findings in younger untrained males whereby 15 g/d of EAA supplementation during 12 weeks of resistance training did not increase LBM compared to a sucrose PLA group. Additionally, Aguilar et al. [13] recently reported that younger male subjects who supplemented with L-leucine (LEU; 3 g/d) during 8 weeks of resistance training experienced no additional increase in

quadriceps muscle size increases when compared to subjects consuming a cornstarch PLA.

In spite of the aforementioned literature suggesting that whey or soy protein supplementation may be more effective than EAA or LEU in promoting additional increases in LBM with resistance training, a prevailing hypothesis is that the LEU content of a given dietary protein determines the efficacy of how that protein potentiates muscle growth. However, this hypothesis is based on acute human, animal, or cell culture-based studies reporting that LEU or whey protein (which contains 8-11% LEU) optimally stimulates muscle protein synthesis [14-17]. To this end, there is no evidence which has directly compared the anabolic effects of LEU supplementation versus supplementation with other dietary protein sources that contain high levels of LEU (e.g., whey or soy). Therefore, the purpose of this study was to examine if supplementation with LEU, whey protein concentrate (WPC), whey protein hydrolysate (WPH) or soy protein concentrate (SPC) enhances markers of skeletal muscle hypertrophy with resistance training in previously untrained, college-aged males. A secondary aim was to also assess how these different supplements affected subcutaneous (SQ) fat cell size from biopsy specimens given that recent data from our group has demonstrated that WPH can elicit lipolytic effects [10,18,19]. Notably, the servings from all supplement groups (except the maltodextrin placebo described below) were standardized for LEU content (~3 grams per serving) and ingested twice daily. Based upon the supporting literature, we hypothesized that individuals consuming whey protein supplements would experience greater increases

in indices related to muscle anabolism compared to those consuming the LEU, SPC and PLA supplements.

2. Materials and Methods

2.1. Ethical approval and screening of participants

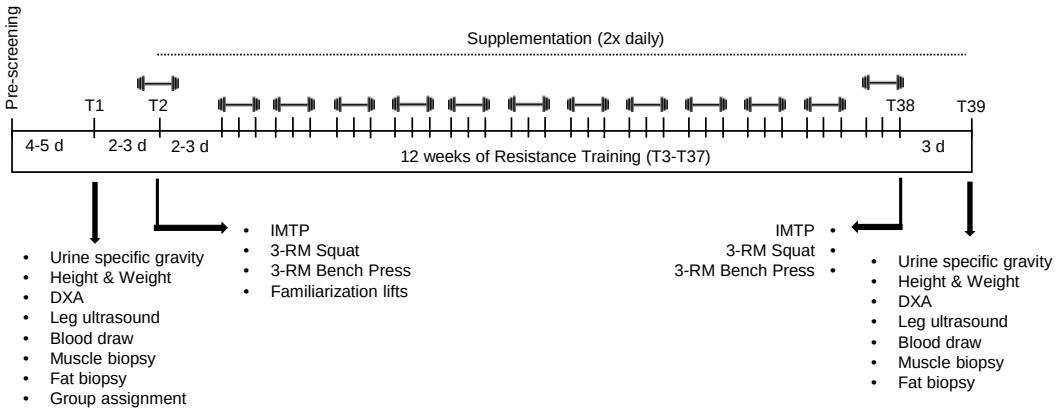
Prior to initiating this study, the protocol was reviewed and approved by the Auburn University Institutional Review Board (IRB), and was conducted in accordance with the Declaration of Helsinki (approved protocol #: 15-320 MR 1508; IRB contact: irbadmin@auburn.edu). Healthy, untrained, college-aged male (i.e., 19-23) participants were recruited for this study. All enrolled participants provided verbal and written study consent, completed a medical history form, and were given a 4-day food log to complete prior to initiating the study. These screening forms ensured that all eligible participants were healthy and recreationally active but: a) had not engaged in any regular exercise program for at least 6 months prior to study initiation (<2 resistance training exercise or high-intensity aerobic exercise sessions/week), b) were not currently consuming a high-protein diet (>2.0 g/kg/d), c) were not using anabolic enhancing agents (e.g., anabolic steroids, supplemental protein, creatine monohydrate, or prohormones), or d) did not have medical or orthopedic condition(s) that would hinder them from participating in the current study. Once initial screening was complete, all eligible participants were scheduled to return to the School of Kinesiology at Auburn University one week later for baseline testing (T1).

2.2 Study design

The study design implemented was double-blinded and placebo-controlled (Figure 1). Likewise, we followed guidelines established by the CONSORT Transparent Reporting of Trials established in 2010, and this trial was registered at ClinicalTrials.gov (unique protocol ID: 15-320 MR 1508). Participants were encouraged to refrain from rigorous physical activity for 4-5 days prior to baseline testing (T1). For T1, participants were instructed to report to the laboratory in a well-hydrated, 4-hour fasted state whereby they were subjected to the following assessments: a) urine specific gravity, b) height and body mass, c) body composition using dual-energy X-ray absorptiometry (DXA) (General Electric Lunar Prodigy enCORE, software version 10.50.086; Madison, WI, USA), d) vastus lateralis thickness using ultrasonography (General Electric LOGIQ S7 Expert; Chicago, IL, USA), e) venipuncture, f) percutaneous skeletal muscle biopsy collection from the vastus lateralis, and g) a percutaneous SQ fat biopsy from the gluteal region. Two to three days following T1, subjects reported back to the laboratory in a 4-hour fasted state for a second visit (T2) whereby maximal force production capacity was assessed using an isometric mid-thigh pull (IMTP) test, lower body strength was assessed using a three repetition maximum (3-RM) squat, and upper body strength was assessed using a 3-RM bench press. Additionally, during T2, subjects were familiarized with all lifts that were to be performed during the training intervention. Following T2, subjects engaged in 12 weeks of resistance training and supplementation. The last training bout

(T38) consisted of IMTP as well as squat and bench press 3-RM re-assessments in a 4-hour fasted state. Seventy two hours following T38, subjects reported back to the laboratory in a 4-hour fasted state for post-testing (T39) which consisted of all body composition, and blood and biopsy collection procedures noted for T1. All of the aforementioned testing procedures as well as training and supplementation procedures are described in greater detail below.

Figure 1. Study design



Abbreviations: DXA, dual x-ray absorptiometry; 3-RM, three-repetition maximum test; IMTP, isometric mid-thigh pull.

2.3. Body Composition Testing

During T1 and T39 participants were instructed to submit a urine sample (~5 mL) to assess normal hydration specific gravity levels (1.005-1.020 ppm) using a handheld refractometer (ATAGO; Bellevue, WA, USA). Participants with a urine specific gravity >1.020 were asked to consume tap water every 15 min for 30 min and then were re-

tested. Following hydration testing, height and body mass were assessed using a digital column scale (Seca 769; Hanover, MD, USA) with weights and heights collected to the nearest 0.1 kg and 0.5 cm, respectively. Next, participants were subjected to a full body DXA scan while wearing general sports attire (i.e., athletic shorts or compression shorts and an athletic shirt) to assess various body composition characteristics. Dual arm and dual leg lean muscle mass, as assessed by the accompanying software, were used to estimate total body skeletal muscle mass (TBMM) by employing the equation from Kim et al. [20], as reported by our group previously [10]. Notably, body segmentation for each scan was standardized prior to analyses by the same technician. Total body fat mass was also assessed by the accompanying software. According to previous data published by our laboratory, the same-day reliability of the DXA during a test-calibrate-retest on 10 participants produced intra-class correlation coefficients of 0.998 for total body fat mass (mean difference between tests = 0.40±0.05 kg), 0.998 for total body lean mass (mean difference between tests = 0.29±0.13 kg), and 0.998 for dual-leg lean mass (mean difference between tests = 0.17±0.09 kg) [21].

Following DXA scans, participants were subjected to an ultrasound assessment to determine vastus lateralis muscle thickness. Measurements were taken from the midway point between the iliac crest and patella of the right femur whereby subjects were in a standing position and all weight was placed on the left leg. All DXA scans and ultrasound assessments were completed by the same investigator as suggested by

previous research interventions [10,22] in order to minimize variability in testing procedures.

2.4 Venipuncture, and percutaneous skeletal muscle and fat biopsies

T1 and T39 venous blood samples were aseptically collected from the antecubital vein and collected into a 5 mL serum separator tube (BD Vacutainer; Franklin Lakes, NJ, USA). Notably, this blood was saved for further experimentation and variables assessed from these blood draws are not presented herein. Immediately following blood collection, participants were instructed to lay in a supine position on a treatment table whereby a percutaneous skeletal muscle biopsy was aseptically obtained from the left vastus lateralis muscle using a 5 gauge Bergstöm needle with suction as previously described by our laboratory [23-26]. Approximately, 20-40 mg of skeletal muscle tissue for each time point was placed in a cryomold with OCT media (Electron Microscopy Sciences; Hatfield, PA, USA). Cryomolds were then slow-frozen in liquid-nitrogen-cooled isopentane and stored at -80°C for immunohistochemistry analyses that are described below. Sections of SQ fat (1-2 cm) extracted from the gluteal aspect of the left hip were placed in 10% formalin and preserved for hematoxylin and eosin (H&E) staining and histological analyses which are described in detail below. Following T1 testing procedures, subjects were counterbalanced into one of five groups based upon DXA LBM values in order to ensure that baseline values did not differ between

supplement groups. More details regarding supplementation are described below, and supplementation began immediately following the first training bout (T3).

2.5 Isometric mid-thigh pull, strength testing, and weightlifting familiarization

During T2 and T38, participants were instructed to report back to the laboratory under well hydrated, 4-hour fasted conditions for strength testing and weight training familiarization (T2). First, each participant completed an IMTP test which has been validated to approximate whole-body maximal voluntary strength [27-29]. Briefly, knee and hip angles (125±5° and 175±5°, respectively) were measured using a standard goniometer (Fabrication Enterprises; White Plains, NY, USA). A standard, 20 kg barbell (York Barbell; York, PA, USA) and STS Power Rack (York Barbell) were used to conduct the IMTP. Dual OR6-7 force plates (AMTI; Watertown, MA, USA) with dual Gen 5 amplifiers (AMTI) sampling at 500 Hz were used to measure vertical force production in Newtons (N). Similar to other investigations [29-32], each participant was allowed at least two attempts, and up to four attempts if differences in vertical peak force between trials was >250 N. Manufacturer software was used to calculate vertical peak force during the testing sessions, and a custom-written MATLAB script (Natick, MA, USA) was employed to identify the greatest vertical force produced in N for each trial, post-hoc. Two trials within 250 N were used to calculate an average vertical peak force across trials and functioned as a metric for maximal voluntary force production (i.e., strength) in this investigation.

Approximately 5 min following T2 and T38 IMTP testing, participants performed 3-RM back squat and bench press assessments using a 20 kg barbell (York Barbell), STS Power Rack (York Barbell) and free weights. The demonstration of proper technique as well as the implementation of progressively-loaded 3-RM tests were overseen by C.B.M. and C.T.H. who possess the Certified Strength and Conditioning Specialist (CSCS) credential from the National Strength and Conditioning Association (NSCA). Bench press and back squat repetitions were considered to be successful when performed through the full range of motion (i.e., chest touch to full arm extension for bench press, and eccentric lowering past 90° knee flexion for back squat). A repetition was not counted if subjects exhibited poor and/or unsafe technique or needed assistance with a repetition during maximal testing.

Approximately 5 min following T2 3-RM testing, participants were instructed to perform the other two major lifts that were implemented for training (i.e., deadlift and bent-over-row) in the presence of CSCS-certified personnel. The goal of this session was to familiarize each participant with appropriate lifting technique in order to minimize the risk of injury throughout the course of the study.

2.6 Training protocol

For visits 3-37 (T3-T37), a daily undulating periodization (DUP) training model was employed over the 12-week training period given that this model has been shown to be more beneficial in eliciting greater increases in strength [33,34] and hypertrophy

[35,36] than traditional linear periodization training models. Specifically, participants were instructed to perform free-weighted barbell squats, bench press, deadlifts, and bent-over-rows for 4 sets of 10 repetitions (Monday or Tuesday), 6 sets of 4 repetitions (Wednesday or Thursday), and 5 sets of 6 repetitions (Friday or Sunday). Immediately following each completed set, a rating of perceived exertion (RPE) score was acquired from each participant (scale: 1-10) in order to monitor and progress each participant accordingly while minimizing the potential risk of injury [37-40]. The RPE scale was described to participants as the remaining number of repetitions that the participant would be able to complete while employing good technique (i.e., 1 = 9 remaining repetitions in reserve, 10 = 0 remaining repetitions in reserve). More information on relative training intensities and progression can be found in Table 1. Participants were instructed to attend all 36 resistance training sessions throughout the duration of the study, but those that missed more than 4 sessions were not included in the analysis due to lack of training compliance. All participants were supervised by laboratory personnel for each training session to ensure that proper lifting technique was executed, and training volumes for each session were recorded.

Table 1. Training load progression

Week	**Training Paradigm**
0	Days 1-3 Familiarization session, IMTP & 3-RM Testing

1	Day 1: 4x10	51 % of Est. 1-RM
	Day 2: 6x4	60 % of Est. 1-RM
	Day 3: 5x6	56 % of Est. 1-RM
2	Day 1: 4x10	60 % of Est. 1-RM
	Day 2: 6x4	70 % of Est. 1-RM
	Day 3: 5x6	65 % of Est. 1-RM
3	Day 1: 4x10	70 % of Est. 1-RM
	Day 2: 6x4	79 % of Est. 1-RM
	Day 3: 5x6	74 % of Est. 1-RM
4	Day 1: 4x10	73 % of Est. 1-RM
	Day 2: 6x4	89 % of Est. 1-RM
	Day 3: 5x6	84 % of Est. 1-RM
5	Day 1: 4x10	78 % of Est. 1-RM
	Day 2: 6x4	95 % of Est. 1-RM
	Day 3: 5x6	90 % of Est. 1-RM
6	Day 1: 4x10	82 % of Est. 1-RM
	Day 2: 6x4	100 % of Est. 1-RM
	Day 3: 5x6	94 % of Est. 1-RM
7	Day 1-3: 4x5 (de-load)	60% of Est. 1-RM
8	Day 1: 4x10	74 % of Est. 1-RM
	Day 2: 6x4	90 % of Est. 1-RM
	Day 3: 5x6	85 % of Est. 1-RM
9	Day 1: 4x10	83 % of Est. 1-RM
	Day 2: 6x4	101 % of Est. 1-RM
	Day 3: 5x6	96 % of Est. 1-RM
10	Day 1: 4x10	87 % of Est. 1-RM
	Day 2: 6x4	107 % of Est. 1-RM
	Day 3: 5x6	98 % of Est. 1-RM
11	Day 1: 4x10	90 % of Est. 1-RM
	Day 2: 6x4	109 % of Est. 1-RM
	Day 3: 5x6	102 % of Est. 1-RM
12	Day 1-2: 4x5 (de-load)	60% of Est. 1-RM
	Day 3: IMTP & 3-RM Testing	108% of Est. 1-RM

Legend: Estimated one repetition maximum (Est. 1-RM) was calculated per the NSCA's recommended guidelines (i.e., 3-RM/0.93). Abbreviations: IMTP, isometric mid-thigh

pull; 3-RM three repetition maximum; 1-RM, one repetition maximum; NSCA, National Strength and Conditioning Association.

2.7 Supplementation

As stated above, participants were assigned to ingest either a PLA, LEU, WPC, WPH, or SPC supplement throughout the training intervention. More information regarding the macronutrient profile for a serving size of each supplement can be found in Table 2. On training days (T3-T37), participants consumed an individually-packaged serving in ~500 ml of tap water immediately following each training session under direct observation of the study personnel. Additionally, participants were instructed to consume an individual serving within 30 min prior to bedtime on training days given that this strategy has been shown to be effective for stimulating overnight muscle protein synthesis [41]. On non-training days, participants were instructed to consume an individual serving between a meal of their choosing and 30 min prior to bedtime. Supplements were separated into individual ready-made supplement-coded packets for daily consumption, and participants were given a 3-week supply. Study personnel collected and counted empty packets from each participant every 3 weeks before the next 3-week supply was distributed. Participants that did not consume >80% were not included in the analysis due to lack of compliance.

Each supplement, except PLA, was formulated to provide ~3 g of leucine, per serving. Furthermore, each supplement was formulated to yield similar amounts of total

energy (kcal) and fat (g), and was double-blinded to laboratory personnel and participants for group, appearance, taste, texture, and packaging. The WPC supplement was formulated using an agglomerated, 80% WPC (Hilmar™ 8010, Hilmar Ingredients; Hilmar, CA, USA). The WPH supplement was formulated using an agglomerated, partially hydrolyzed [12.5% degree of hydrolysate (12.5% DH), yielding approximately 67% of peptides as <5 kilodaltons (kDa) in molecular weight] 80% whey protein concentrate (Hilmar™ 8360, Hilmar Ingredients); SPC used an agglomerated, 80% soy protein concentrate (ALPHA® 5812, Solae, LLC; St. Louis, MO, USA); LEU used an agglomerated, L-Leucine (L-Leucine USP, Glambia Nutritionals; Carlsbad, CA, USA) and non-GMO, corn-derived maltodextrin (MALTRIN®-M100; Grain Processing Corporation; Muscantine, IA, USA); and, the PLA group was formulated using maltodextrin (MALTRIN®-M100; Grain Processing Corporation). All five supplements were manufactured at JW Nutritional, LLC (Allen, TX, USA), a United States Food and Drug Administration cGMP-compliant facility independently audited and pre-qualified by Obvium*Q, LLC (Phoenix, AZ, USA), a GMP regulatory compliance firm. Personnel at JW Nutritional, LLC and C.M.L. (Lockwood, LLC; Draper, UT, USA) formulated and maintained blinding of groups, and each supplement was assigned a randomly generated item number. Manufacturing batch records for production of each of the five supplements were reviewed by a trained, independent expert in dietary supplement quality control and assurance (C.M.L.) before approval for use within the present study. All supplements were independently validated for nutritional facts and total amino acids

using validated, approved methods at Covance Laboratories, Inc. (Madison, WI, USA), a pre-qualified third-party analytical laboratory, and results reviewed by C.M.L. prior to the supplements being approved for use within the present study. Once analysis was complete, a Lockwood, LLC representative not involved in the study released the code for all treatments.

Table 2. Nutritional components per serving for the different supplements

Variable	**PLA**	**LEU**	**WPC**	**WPH**	**SPC**
Calories	204	200	184	192	266
Total Fat (g)	2.8	2.0	3.5	4.6	4.5
Saturated Fat (g)	2.3	1.6	2.3	3.3	2.6
Trans Fat (g)	0.0	0.0	0.1	0.2	0.2
Cholesterol (mg)	3.8	2.9	74.0	74.3	5.3
Total Carbohydrate (g)	44.4	43.1	12.0	12.2	17.2
Dietary Fiber (g)	1.6	1.8	1.8	2.2	1.5
Sugars (g)	6.0	5.1	5.9	3.2	6.2
Protein (g)	0.4	2.3	26.3	25.4	39.2
Alanine (mg)	7	7	1,397	1,430	1,646
Arginine (mg)	8	8	766	773	2,969
Aspartic Acid (mg)	15	16	2,881	3,010	4,537
Cystine (mg)	0	0	651	728	536
Glutamic Acid (mg)	36	35	4,530	4,730	7,154
Glycine (mg)	6	6	489	543	1,597
Histidine (mg)	0	0	470	477	910
Isoleucine (mg)	8	28	1,736	1,820	1,842
Leucine (mg)	15	2,871	2,794	2,910	2,960
Lysine (mg)	11	79	2,386	2,640	2,362
Methionine (mg)	0	8	598	611	540
Phenylalanine (mg)	8	9	861	908	1,980
Proline (mg)	14	13	1,630	1,670	2,029
Serine (mg)	10	9	1,348	1,400	1,950
Threonine (mg)	7	7	1,853	1,900	1,499
Tryptophan	0	0	482	525	501
Tyrosine (mg)	7	7	808	839	1,480
Valine (mg)	11	14	1,465	1,530	1,754

Total EAAs (mg)	60	3,016	12,645	13,321	14,348
Total BCAAs (mg)	34	2,913	5,995	6,260	6,556
Calcium (mg)	15	15	155	152	165
Iron (mg)	0.38	0.35	0.63	1.04	5.21
Potassium (mg)	32	37	230	464	961
Sodium (mg)	91	105	133	310	217
Vitamin D3 (IU)	0.0	0.0	0.0	0.0	0.0
Degree of hydrolysis (%)	N/A	N/A	N/A	12.5	N/A
M.W. range (%)					
>10.0 kD	-	-	74.3	29.0	86.0
5.0-10.0 kD	-	-	5.1	5.3	3.6
2.0-5.0 kD	-	-	15.4	10.2	2.6
1.0-2.0 kD	-	-	1.6	10.8	1.2
0.5-1.0 kD	-	-	0.9	15.5	0.9
<0.5 kD	-	-	2.7	29.3	5.6

Abbreviations: PLA, maltodextrin placebo; LEU, L-leucine; WPC, whey protein concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate; g, grams; mg, milligrams; IU, international units; kD, kilodaltons; N/A, not applicable.

2.8 Nutritional intake monitoring

Participants were instructed to maintain their normal dietary habits along with returning a 4-day food log (2 week days and both weekend days) at baseline (T1), week 6 (T20) and week 12 (T39). On each occasion, participants were given detailed written and verbal instructions on completing the food logs. Dietary intake data were analyzed using the open-sourced software myfitnesspal, which has been employed to analyze food intake data in other studies [42-48].

2.9 Immunofluorescent histochemistry for muscle fiber type-specific characteristics

Muscle sections were analyzed for type I fiber cross sectional area (CSA), type II fiber CSA, type I fiber myonuclear number, type II fiber myonuclear number, and total (non fiber type-specific) satellite cell number. Methods for immunofluorescent histochemistry have been employed previously in our laboratory and described elsewhere [25, 49]. Briefly, sections from OCT-preserved samples were cut at a thickness of 20 μm using a cryotome (Leica Biosystems; Buffalo Grove, IL, USA) and were adhered to positively-charged histology slides. Once all samples were sectioned, batch processing occurred for immunofluorescent histochemistry. During batch processing, sections were air-dried at room temperature for 30 min, fixed with 10% formalin for 10 min, permeabilized in a phosphate-buffered saline (PBS) solution containing 0.5% Triton X-100, and blocked with 100% Pierce Super Blocker (Thermo Fisher Scientific; Waltham, MA, USA) for 25 min.

For fiber type staining (following blocking), sections were subsequently washed for 5 min in PBS and incubated for 1 hour with a primary antibody solution containing rabbit anti-dystrophin IgG (Thermo Fisher Scientific; 10 μL antibody per 1 mL of blocking solution) and mouse anti-myosin II IgG (catalog #: SC71; Hybridoma Bank; 100 μL per 1 mL of blocking solution). Sections were then washed for 5 min in PBS and incubated in the dark for 1 hour with a secondary antibody solution containing Texas Red-conjugated anti-rabbit IgG (Vector Laboratories; Burlingame, CA, USA), and Alexa Fluor 488-conjugated anti-mouse IgG (Thermo Fisher Scientific) (10 μL of all secondary antibodies per 1 mL of blocking solution). Sections were then washed for 5 min in PBS,

air-dried and were mounted with fluorescent media containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Following mounting, slides were stored in the dark at 4ºC until immunofluorescent images were obtained.

For satellite cell staining (following blocking), separate sections were incubated for 1 hour with a pre-diluted commercially-available primary antibody solution containing rabbit anti-dystrophin IgG (Thermo Fisher Scientific), and 1:15 dilution of mouse anti-Pax7 IgG (Hybridoma Bank, Iowa City, IA, USA) for 1 hour. Sections were then washed for 5 min in 1x PBS and incubated in the dark for 1 hour with a secondary antibody solution containing 1:100 dilution of Texas Red-conjugated anti-rabbit IgG (Vector Laboratories) and Alexa Fluor 488-conjugated anti-mouse IgG (Thermo Fisher Scientific). Sections were then washed for 5 min in PBS, air-dried and were mounted with fluorescent media containing DAPI (Vector Laboratories). Following mounting, slides were stored in the dark at 4ºC until immunofluorescent images were obtained. After staining was performed on all sections, digital images were captured using a fluorescence microscope (Nikon Instruments; Melville, NY, USA) and 20x objective. Approximate exposure times of 600 ms for red and green imaging and 30 ms for blue imaging. For fiber typing our staining method allowed the identification of cell membranes (detected by the Texas Red filter), type II fiber green cell bodies (detected by the FITC filter), type I fiber black cell bodies (unlabeled), and myonuclei (detected by the DAPI filter). For satellite cell identification our staining method allowed the identification of cell membranes (detected by the Texas Red filter), small green cell

bodies as satellite cells (detected by the FITC filter), and myonuclei (detected by the DAPI filter). Measurements of type II fiber cross sectional area (CSA) were performed using the open-sourced software CellProfiler™ [50] per modified methods previously described whereby the number of pixels counted within the border of each muscle fiber were converted to a total area in microns-squared (μm2). Measurements of fiber type-specific myonuclear number were also performed using open-sourced software CellProfiler™ to discriminate the fiber border that corresponded to each myonuclei. Satellite cells were manually counted using a grid function in the NIS Elements software (Nikon Instruments) and handheld tally counter. Per the recommendations of Mackey et al. [51], at least 50 fibers per specimen were quantified to obtain accurate CSA, myonuclear number and satellite cell values.

2.10 SQ fat CSA analysis

As mentioned above, gluteal fat was obtained at T1 and T39 for SQ fat analysis. Following tissue processing and H&E staining, SQ fat CSA analysis was performed as previously published by our laboratory [52,53]. Briefly, SQ fat samples were removed from formalin and then washed in cold running tap water, embedded, and stored in 70% alcohol. Dehydration was accomplished by gradually increasing percentages of ethyl alcohol to replace the water content in the tissue. Hemo-De was subsequently used to clear the tissue from the ethyl alcohol to allow infiltration with paraffin. The paraffin tissue blocks were sectioned into 6 μm slices and placed onto glass microscope slides.

Paraffin was removed with xylene, the mounted sections were stained with hematoxylin and eosin, and sample sections were enclosed with a coverslip and mounting media. Two 10x objective digital images per sample were obtained using bright-field imaging (Nikon Instruments), and CSAs were obtained from at least 50 adipocytes per image using ImageJ (National Institutes of Health; Bethesda, MD, USA).

2.11 A priori sample size calculations and statistical analyses

Based upon meta-data compiled by Phillips [14], whey protein-supplemented subjects participating in resistance training for at least 8 weeks experienced, on average, gained an estimated 3.0 ± 0.6 kg increase in muscle mass, while those supplementing with soy protein experiences a ~1.4 ± 0.3 kg increase and those supplementing with a carbohydrate-based placebo presented a ~1.0 ± 0.2 kg increase. To obtain an adequately-powered sample-size for each treatment, a priori calculations (non-centrality parameter = 2.8, power = 0.80, pooled standard deviation values of 0.5) suggested that a sample-size of 3 participants per group would be needed to detect a significant difference between whey protein versus soy and/or other potential treatments. However, in order to sufficiently power the trial, we attempted to enroll 15-20 subjects per treatment.

All data are presented in tables and figures as means ± standard error of the mean (SE) values. Statistics were performed using SPSS v22.0 (IBM; Armonk, NY, USA) and Microsoft Excel when applicable. A Shapiro-Wilk's test was employed for all dependent variables to test for distribution normality. If values were not normally distributed then

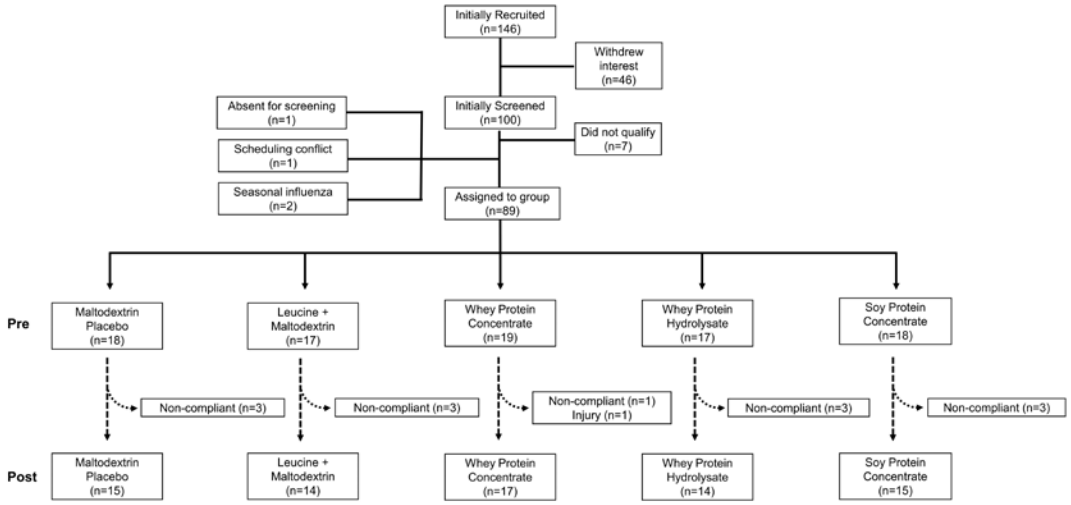
values were square root-transformed and re-tested using Shapiro-Wilk's tests to ensure that values were normally distributed. All raw and transformed dependent variables (except nutrition data) were then compared between treatment groups using 5*2 group*time (G*T) two-way repeated measures analysis of covariance (ANCOVA) tests with T1 values for each respective dependent variable serving as the covariate. If a significant time effect was present then within-group dependent samples t-test were performed between T1 and T39 values. If a significant G*T interaction was present, within-group dependent samples t-tests were performed between T1 and T39 values, and one-way analysis of variance (ANOVA) tests with Tukey post hoc tests were performed at the T39 time point. All nutritional dependent variables were compared between treatment groups using 5*3 (group*time) two-way repeated measures ANCOVAs with T1 values for each respective dependent variable serving as the covariate. If a significant time effect was present then within-group pairwise comparisons were performed using Bonferroni post hoc tests. If a significant group*time interaction was present then within-group dependent-samples t-tests were performed between T1 and T20 as well as T39 values, and one-way ANOVAs with Tukey post hoc tests were performed at the T20 and T39 time points.

3. Results

3.1. Subject compliance and baseline characteristics

The Consolidated Standards of Reporting Trials (CONSORT) diagram for this study is presented in Figure 2.

Figure 2. CONSORT diagram of the study



Legend: Details regarding this Consolidated Standards of Reporting Trails (CONSORT) diagram can be found in the results.

Briefly, a total of 146 potential participants were recruited for the study. Of these individuals, 46 withdrew interest and 100 were pre-screened. Of these 100 individuals, 4 did not consent due to scheduling conflicts or illness, and 7 did not qualify for the study. Of the 89 participants that provided consent and began the study, a total of 75 successfully completed the intervention (PLA n=15, LEU n=14, WPC n=17, WPH n=14, and SPC n=15). Notably, 13 participants were removed from the study due to lack of compliance with supplementation or resistance training. One subject in the WPC group

had to withdraw from the study due to a musculoskeletal injury sustained during training which was reported to the Auburn University IRB.

There were no baseline differences between supplement groups for select dependent variables related to age, body composition, or strength (see Table 3 for p-values). Overall, supplement compliance was 95% and did not differ between groups (ANOVA, p=0.203) Furthermore, overall training compliance was 94% and did not differ between groups (ANOVA, p=0.296).

Table 3. Baseline characteristics between groups

Variable	**PLA (n=15)**	**LEU (n=14)**	**WPC (n=17)**	**WPH (n=14)**	**SPC (n=15)**	**ANOVA p-value**
Age (yr)	21±1	20±1	21±1	21±1	21±1	0.811
Height (cm)	183±2	179±1	179±2	182±2	182±2	0.454
Body Mass (kg)	79±3	75±2	81±3	79±3	81±3	0.600
Lean body mass (kg)	58±4	57±3	59±4	59±5	59±4	0.899
Total Fat Mass (kg)	18±3	15±3	19±5	16±3	20±5	0.378
Strength 3-RM (kg)						
Squat	70±8	83±12	82±11	79±14	82±10	0.369
Bench press	66±8	67±11	68±8	73±9	65±7	0.650
IMTP (N)	3,247±215	3,205±170	3,476±141	3,461±130	3,192±117	0.488

Legend: Values are presented as means±SE. Abbreviations: PLA, maltodextrin placebo; LEU, L-leucine; WPC, whey protein concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate; 3-RM, 3-repetition maximum; IMTP, isometric mid-thigh pull; yr, years; cm, centimeters kg, kilograms; N, newtons.

3.2. Self-reported nutritional intakes

All self-reported food intakes during the intervention are reported in Table 4. Caloric and macronutrient intakes (i.e., total and relative calories, protein, carbohydrates and fats) did not differ between groups at T1 (all ANOVA p-values >0.50). Furthermore, a significant main effect of time for Caloric intake existed whereby T20 and T39 were greater than T1 ($p<0.001$; Table 4). However, there was no G*T interaction ($p=0.847$).

A significant main effect of time existed for total daily protein intake whereby T20 and T39 was greater than T1 ($p<0.001$; Table 4). Additionally, there was a G*T interaction ($p<0.001$) whereby: a) WPC/WPH/SPC ingested more protein at T20 and T39 relative to T1 ($p<0.001$), b) LEU ingested more protein at T39 relative to T1 ($p<0.01$), c) at T20 WPC/WPH/SPC > LEU/PLA ($p<0.01$) and SPC > WPC ($p<0.01$), and d) at T39 WPC/WPH/SPC > LEU/PLA ($p<0.05$). A significant main effect of time also existed for relative protein (body mass-adjusted) intake whereby T20 and T39 was greater than T1 ($p<0.001$; Table 4). Additionally, there was a G*T interaction ($p<0.001$) whereby: a) WPC/WPH/SPC ingested more protein at T20 and T39 relative to T1 ($p<0.001$), b) LEU ingested more protein at T39 relative to T1 ($p<0.05$), c) at T20 WPC/WPH/SPC > LEU/PLA ($p<0.05$), and d) at T39 WPC/SPC > LEU/PLA ($p<0.05$).

A significant main effect of time existed for total daily carbohydrate intake whereby T20 and T39 was greater than T1 ($p<0.001$; Table 4). There was also a G*T interaction ($p<0.001$) whereby: a) PLA/LEU/WPH/SPC ingested more carbohydrates at T20 and T39 relative to T1 ($p<0.05$), b) WPC ingested more carbohydrates at T39 relative to T1 ($p<0.05$), c) at T20 PLA > WPC/WPH/SPC ($p<0.05$), and d) at T39 PLA >

WPC/WPH/SPC ($p<0.05$). A significant main effect of time also existed for relative (body mass-adjusted) carbohydrate intake whereby T20 and T39 was greater than T1 ($p<0.001$; Table 4). Additionally, there was a G*T interaction ($p<0.01$) whereby: a) PLA/LEU ingested more carbohydrates at T20 and relative to T1 ($p<0.01$), b) WPH/SPC ingested more carbohydrates at T39 relative to T1 ($p<0.05$), c) at T20 LEU > WPC/SPC ($p<0.05$), and d) at T39 PLA > WPC ($p<0.05$).

A significant main effect of time existed for total daily fat intake whereby T20 and T39 was greater than T1 ($p<0.001$). However, there was no G*T interaction for total daily fat intake or relative (body mass-adjusted) fat intake ($p=0.549$ and $p=0.809$, respectively).

Table 4. Self-reported nutrient intakes between groups

Variable Group	Baseline	Week 6	Week 12	ANCOVA p-values
Energy intake (kcal/d)				
PLA	2109±166	2756±236*	2812±232*	
LEU	1835±116	2303±165*	2488±132*	
WPC	1866±115	2305±116*	2389±177*	Time p<0.001 G*T p=0.865
WPH	2039±149	2611±156*	2617±144*	
SPC	1853±136	2461±129*	2611±158*	
Protein intake (g/d; g/kg/d)				
PLA	94±7; 1.2±0.1	109±8[c]; 1.3±0.1[b]	111±11[b]; 1.3±0.1[b]	Time p<0.001 G*T p<0.001

LEU	87±6; 1.2±0.1	96±8[c]; 1.3±0.1[b]	108±10[*,b]; 1.4±0.1[b]	
WPC	88±6; 1.1±0.1	142±5[*,a]; 1.8±0.1[*,a]	145±6[*a]; 1.8±0.1[*,a,b]	
WPH	94±8; 1.2±0.1	160±7[*,a,b]; 2.0±0.1[*,a]	153±7[*,a]; 1.9±0.1[*,a]	
SPC	88±6; 1.1±0.1	176±7[*,b]; 2.1±0.1[*,a]	179 ±10[*,a]; 2.1±0.1[*,a]	
Carbohydrate intake (g/d; g/kg/d)				
PLA	244±23; 3.1±0.3	337±21[*,a]; 4.2±0.3[*,a,b]	348±29[*,a]; 4.2±0.3[*,a]	
LEU	206±17; 2.8±0.3	303±24[*,a,b]; 4.0±0.4[*,a]	310±21[*,a,b]; 4.1±0.4[*,a,b]	
WPC	215±14; 2.8±0.3	231±18[b]; 2.9±0.3[b]	244±16[*,b]; 3.0±0.2[b]	Time p<0.001 G*T p=0.002
WPH	208±20; 2.7±0.3	247±15[*,b]; 3.1±0.3[*,a,b]	255±16[*,b]; 3.3±0.3[*,a,b]	
SPC	203±18; 2.6±0.3	238±16[*,b]; 2.9±0.2[b]	256±20[*,b]; 3.1±0.3[*,a,b]	
Fat intake (g/d; g/kg/d)				
PLA	83±9; 1.1±0.1	106±12[*]; 1.3±0.1	110±12[*]; 1.3±0.1[*]	
LEU	73±5; 1.0±0.1	79±5; 1.0±0.1	92±6; 1.2±0.1	Time p<0.001 G*T p=0.549
WPC	71±5; 0.9±0.1	87±5[*]; 1.1±0.1[*]	93±12; 1.2±0.1	
WPH	81±6; 1.1±0.1	102±9[*]; 1.3±0.2	101±7; 1.3±0.1	
SPC	73±6; 0.9±0.1	90±7[*]; 1.1±0.1[*]	101±10[*]; 1.2±0.1[*]	

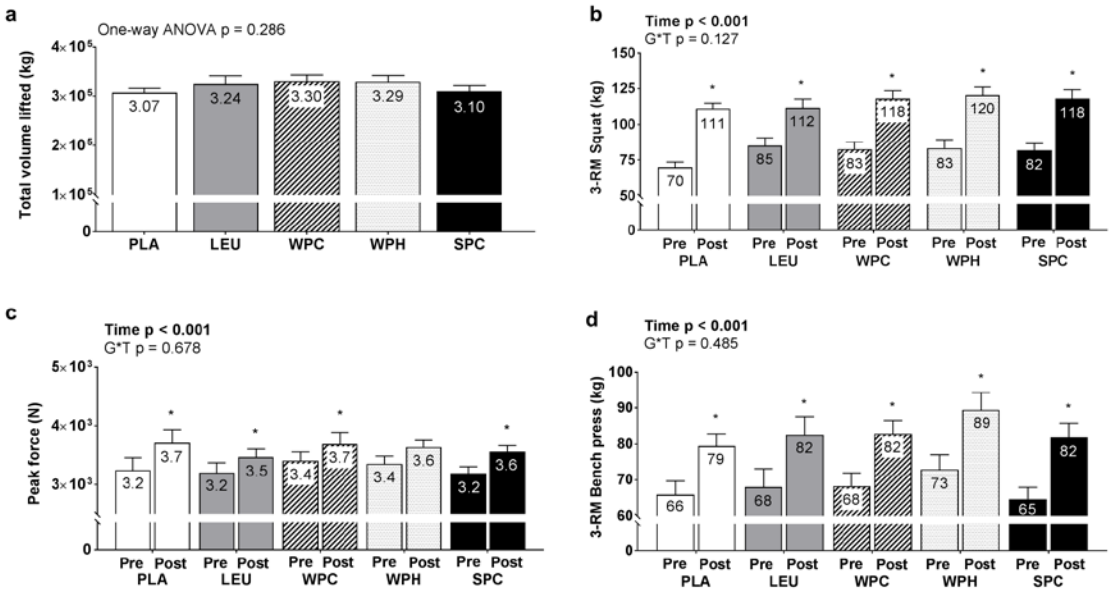
Legend: Values are means±SE. Symbols: *, indicate within-group increases from baseline ($p<0.05$); values that do not share superscript ([a,b,c]) letters represent between-group significance at a given time point ($p<0.05$). Abbreviations: PLA, maltodextrin

placebo; LEU, L-leucine; WPC, whey protein concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate; G*T, group*time interaction.

3.3 Training volume, 3-RM strength, IMTP

Training volume during the intervention did not differ between groups (ANOVA, p=0.286; Figure 3a). Significant main effects of time existed for 3-RM squat (p<0.001; Figure 3b), 3-RM bench press (p<0.001; Figure 3c) and IMTP (p<0.001; Figure 3d) whereby T39 values were greater than T1 values. However, no significant G*T interactions existed for these variables (p=0.127, 0.485, and 0.684 for 3-RM squat, 3-RM bench press, and IMTP, respectively).

Figure 3. Total volume lifted and changes in strength measures between groups

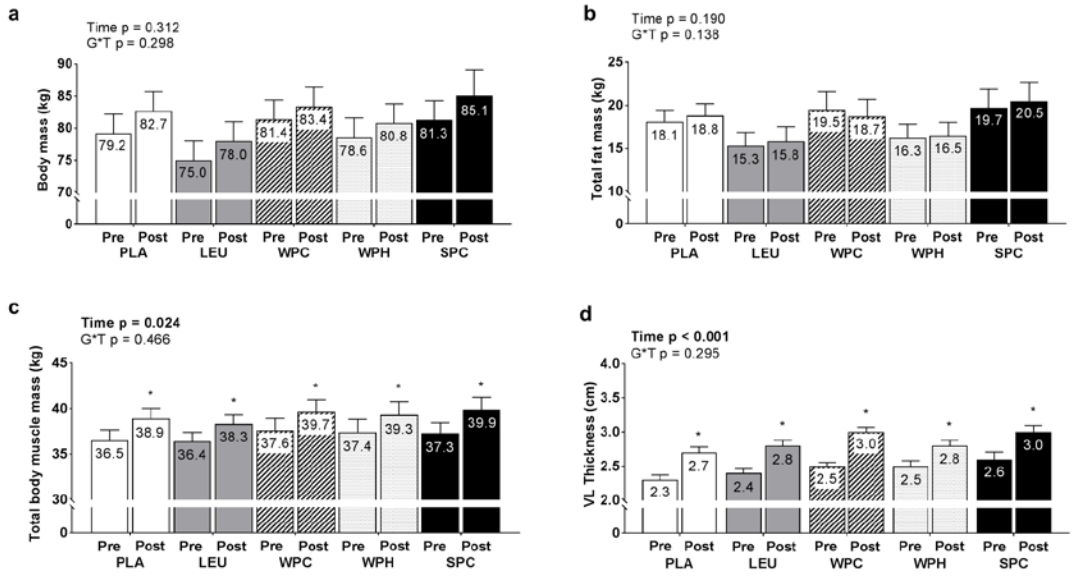


Legend: Data include total volume lifted during the 12-week training intervention (panel a) as well as pre- and post-intervention 3-repetition maximum (RM) squat values (panel b), 3-RM bench press values (panel c), and isometric mid-thigh pull (IMTP) peak force values (panel d). Each bar graph depicts group averaged data presented as mean+standard error values, and mean values are presented within each bar. Additional abbreviations: PLA, maltodextrin placebo; LEU, L-leucine; WPC, whey protein concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate. Symbol: *, within-group increase from pre- to post training ($p<0.05$).

3.4 Changes in body mass, TBMM, fat mass, and vastus lateralis muscle thickness between groups

No significant main effects of time or G*T interactions existed for changes in total body mass (Figure 4a) or fat mass (Figure 4b). Significant main effects of time did exist for changes in TBMM ($p<0.001$; Figure 4c) and vastus lateralis muscle thickness ($p<0.001$; Figure 4d) whereby T39 values were greater than T1 values. However, there were no G*T interactions for these variables ($p=0.847$ and 0.295 for TBMM and vastus lateralis muscle thickness, respectively).

Figure 4. Changes in body composition variables and vastus lateralis muscle thickness between groups

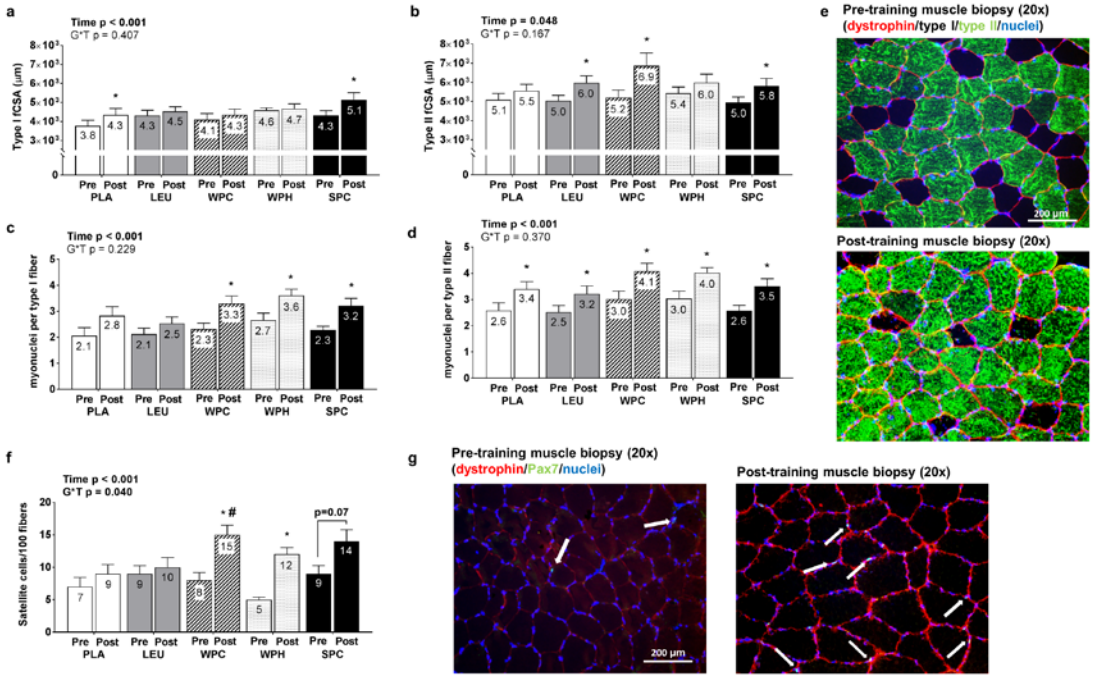


Legend: Data include pre- and post-intervention body mass values (panel a), total fat mass values determined by dual x-ray absorptiometry (DXA;panel b), total body muscle mass (TBMM) values determined by DXA (panel c), and vastus lateralis (VL) thickness determined by ultrasonography (panel d). Each bar graph depicts group averaged data presented as mean+standard error values, and mean values are presented within each bar. Additional abbreviations: PLA, maltodextrin placebo; LEU, L-leucine; WPC, whey protein concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate. Symbol: *, within-group increase from pre- to post training ($p<0.05$).

3.5 *Changes in fiber type-specific CSA and myonuclear number as well as total satellite cell number between groups*

Significant main effects of time existed for changes in type I fiber CSA ($p<0.001$; Figure 5a) and type II fiber CSA ($p=0.048$; Figure 5b) whereby T39 values were greater than T1 values, although no G*T interactions for these variables existed ($p=0.407$ and $p=0.167$, respectively). Significant main effects of time also existed for changes in type I fiber myonuclear number ($p<0.001$; Figure 5c) and type II fiber myonuclear number ($p<0.001$; Figure 5d) whereby T39 values were greater than T1 values, although no G*T interactions for these variables existed ($p=0.370$ and 0.229 for type I and II fiber myonuclear number, respectively). A significant main effect of time existed for changes in total satellite cell counts whereby T39 was greater than T1 ($p<0.001$; Figure 5f). Additionally, there was a G*T interaction ($p<0.05$) whereby: a) WPC and WPH prompted more satellite cells at T39 relative to T1 ($p<0.05$) and b) WPC expressed a significantly greater number of satellite cells than PLA at T39 ($p=0.033$).

Figure 5. Changes in muscle fiber cross sectional area, myonuclear number and satellite cell counts between groups



Legend: Data include pre- and post-intervention type I and type II fiber cross sectional area (CSA) values (panels a&b), type I and type II fiber myonuclear number values (panels c&d), and total satellite cell counts (panel f). Due to poor tissue quality on select subjects, n-sizes were as follows: PLA n=13, LEU n=13, WPC n=15, WPH n=12, and SPC n=14. Representative 20x objective histology images from one subject demonstrating myofiber hypertrophy and increases in satellite cell counts are presented in panels e and g, respectively. Each bar graph depicts group averaged data presented as mean+standard error values, and mean values are presented within each bar.

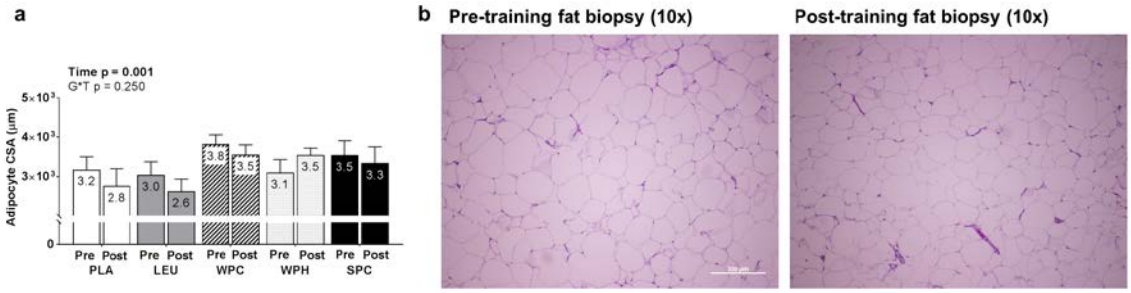
Abbreviations: PLA, maltodextrin placebo; LEU, L-leucine; WPC, whey protein

concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate. Symbols: *, ithin-group increase from pre- to post training ($p<0.05$); #, WPC > PLA at T39 ($p<0.05$).

3.6 Changes in SQ adipocyte CSA between groups

A significant main effect of time for adipocyte CSA existed whereby T39 was less than T1 ($p=0.001$; Figure 6). However, within-group dependent samples t-tests did not reveal any significant effect for time between T1 and T39 within groups (all p-values >0.200). Likewise, no G*T interaction existed ($p=0.250$).

Figure 6. Changes in gluteal subcutaneous adipocyte cross sectional area between groups



Legend: Pre- and post-training subcutaneous adipocyte cross sectional area (CSA) values are presented in panel a. Representative 10x objective histology images from one subject demonstrating a reduction in fat cell size is presented in panel b. Due to poor tissue quality on select subjects, n-sizes were as follows: PLA n=14, LEU n=12, WPC n=14, WPH n=10, and SPC n=13. The bar graph depicts group averaged data presented as mean+standard error values, and mean values are presented within each bar.

Abbreviations: PLA, maltodextrin placebo; LEU, L-leucine; WPC, whey protein concentrate; WPH, whey protein hydrolysate; SPC, soy protein concentrate.

4. Discussion

We sought to determine the effects of LEU or different protein supplements standardized to ~3.0 g LEU, consumed twice daily, on changes in body composition, strength, and histological changes in skeletal muscle and adipose tissue attributes in previously untrained, college-aged males when combined with 12 weeks of resistance training. The main findings for our study included the following: a) there was a training effect, but no effect of supplementation, for increases in TBMM, strength (i.e., IMTP, 3-RM squat, 3-RM bench press), vastus lateralis muscle thickness, and type I and II fiber CSA, type I and II fiber myonuclear number b) WPC and WPH, but not LEU or PLA, significantly increased satellite cell counts, and increases in the SPC group approached significance, and c) there was a time/training effect for decrements in SQ fat cell size ($p=0.001$).

Contrary to our hypotheses our data indicated that there was a training effect, but no supplementation effect, on increases in TBMM, vastus lateralis thickness, and type I/II fiber CSA values. Several studies have demonstrated that whey protein supplementation during resistance training increases indices of muscle anabolism compared to placebo [1-4, 54-56] or soy [5] supplementation. However, our findings are in agreement with other literature reporting that protein supplementation (i.e., WPC/WPH/SPC) provides no

added benefit to increasing muscle mass when consumed over an 8 to 16 week resistance training period [6,9,13,57-60]. Our null findings may have been due to a variety of factors. For instance, younger males have been reported to experience robust hypertrophic responses to resistance exercise when compared to middle-aged and older males and females (younger and older) over the first 4 months of training [61]. Hence, many of our null findings could be attributed to our examining the effects of these supplements in younger, untrained males who seemingly respond the most favorably to resistance training. We also posit that our training model was very advanced for novice lifters (i.e., 30 sets/wk for upper and lower body muscles). Consequently, the employed training model could have obscured any additive anabolic effects that additional LEU or protein supplementation may have otherwise provided. It is also notable that all groups reported increasing Calorie intakes ~600-800 kcal/d from T1 to T39 (time $p<0.001$), and all groups consumed at least 1.1 g/kg/d of protein at T1 and 1.3 g/kg/d at T39. Thus, in lieu of hypotheses put forth by Hoffman et al. [62] suggesting that 1.2 g/kg/d of protein is adequate to support muscle anabolism with resistance training, we posit that all of the participants herein were in adequately-fed states throughout the study and may minimally benefit from additional LEU or protein supplementation. Finally, given that others have reported that whey protein enhances muscle anabolism in resistance trained individuals [1,2], along with evidence indicating that trained individuals require additional protein intake to maintain a net neutral protein balance [63-65], it is plausible that whey protein

supplementation may only benefit those that undergo more prolonged, strenuous resistance training.

Satellite cells have the capacity to divide and fuse to pre-existing muscle fibers in order to promote further increases in muscle fiber growth [66-70]. Furthermore, it has been suggested that resistance training-induced increases in satellite cell number are obligatory for skeletal muscle hypertrophy to occur in humans [71]. Interestingly, relative to T1 the WPC and WPH protein supplemented groups experienced significant increases in satellite cell counts at T39, whereas the SPC group trended towards significance ($p=0.07$) and the LEU and PLA groups did not exhibit this effect. Collectively, our data suggesting that whey protein, rather than LEU, stimulates myogenesis are in agreement with Olsen et al. [72] who reported a 50% increase in total satellite cell number following 16 weeks of strength training and whey protein supplementation. Likewise, Farup et al. [73] reported a 132% and 78% increase in type II and type I fiber satellite cell number, respectively with 12 weeks of concentric exercise and whey protein supplementation. While the mechanisms of action were not directly examined herein or in any of the abovementioned studies, it is notable that Hulmi et al. [4] reported a 120% increase in cdk2 mRNA expression (a regulator of satellite cell proliferation) following 21 weeks of resistance training and whey protein supplementation. Similarly, Roberts et al. [68] reported that whey protein supplementation prior to one bout of lower body resistance exercise in younger males robustly increased MyoD mRNA expression levels 6 hours following exercise which

potentially indicated an increase in satellite cell activation. Thus, it is possible that protein supplementation, or increasing protein intake levels in general, may up-regulate genes within satellite cells responsible for enhanced proliferation in an acute and chronic manner which act to increase satellite cell number. Other evidence has also suggested that matrix metalloprotease (MMP) enzymes stimulate satellite cell activation and migration [74]. In this regard, our group has previously reported that the WPH utilized herein possesses MMP2/9 activity [75]. Moreover, others have reported that a variety of proteins and enzymes are contained within dairy-derived exosomal cargos [76], and dairy-derived exosomes can traverse the digestive system and target numerous tissues in vivo [77]. Hence, it also remains possible that whey protein-derived MMPs can traverse the digestive system via exosomal cargos to stimulate satellite cell activity. It should be noted, however, that a more recent investigation by Reidy et al. [7] indicated that whey or dairy-soy protein blend supplementation did not enhance satellite cell number following 12 weeks of resistance training. Thus, more data are needed to examine how increasing dietary protein intake mechanistically affects satellite cell turnover and if protein-induced increases in satellite cell number provide any added benefit to resistance-trained individuals (i.e., reducing recovery time between training bouts due to satellite cell-mediated recovery mechanisms).

This study is unique in that it is the first study to examine how LEU or protein supplementation affects SQ adipocyte CSA values. Although a secondary aim, our rationale for performing these analyses were due to our prior work which has

demonstrated that WPH supplementation acutely increases lipolysis markers in rodents [18,71] and reduces fat mass in younger males following 10 weeks of resistance training [78]. While mechanisms for these prior findings have not been characterized, we have previously posited that the lipolytic effects observed with WPH supplementation may be due to unidentified peptides (produced during the hydrolysis manufacturing process) being absorbed from the digestive system and acting as ligands for fat cell membrane receptors [71]. Notwithstanding, we report that, while there was a training effect for the reduction in SQ adipocyte CSA levels (-210 μm2; time p=0.001), WPH supplementation did not affect total body fat mass or SQ fat CSA values. It is difficult to reconcile the discrepancies between studies and, our hypotheses regarding WPH supplementation and SQ fat histological attributes require more research.

5. Experimental Considerations

One notable limitation to the current study is the relatively small sample size per group (n=14–17), and this limitation was primarily due to resource constraints rather than faulty experimental design. In this regard, others have suggested that >20 subjects per group are needed in order to determine a significant between-treatment effect regarding protein supplementation and changes in muscle mass [5, 79, 80]. A second limitation was the relatively shorter intervention time (i.e., 12 weeks) employed for the current study. Limited evidence exists regarding the anabolic effects of resistance training with protein supplementation over a >6 month period [5]. Thus, implementing the current

study design with more sampling time points and larger group sizes is warranted. Finally, we posit that the age and gender of our studied population limits the scope of our conclusions per our discussion above regarding the robust responses that younger males in well-fed states typically exhibit in response to resistance training. Thus, more research is needed with replicating the current study design in older males and younger and older female participants.

6. Conclusions

In conclusion, our study demonstrates that neither LEU nor protein supplementation (standardized to LEU) in previously untrained, college-aged males provide added benefit in increasing whole-body skeletal muscle mass or whole-body strength. We do report, however, that whey protein supplementation significantly increases skeletal muscle satellite cell number with resistance training; this being a finding that requires further elucidation.

Acknowledgements

We graciously thank Kevin Lawrence at Bionutritional Research Group and Dr. Chao Wu at Hilmar Ingredients for their involvement in garnering generous donations of research funds and protein. We also graciously thank JW Nutritional for donating all manufacturing, packaging, non-active and soy protein ingredients, and for all costs involving analytical testing of raw materials and finished products for identity, purity,

potency, and composition. We graciously thank Dr. John Quindry, Dr. Andreas Kavazis, and Dr. John J. McCarthy for their intellectual insight into the development and execution of this project. We also thank Dr. Melissa Pangelinan for her critical assistance with IMTP data acquisition, and Dr. Keith Lohse for assistance with statistical analyses. Finally, we graciously thank our participants for devoting time to the study, and our training staff including Jessica Nendze, Casey Sexton, Chester Davis, Romil Patel, David Baumohl and Corey Chandler for their contributions.

Author contributions

1) This person has made substantial contributions to the conception and design, or acquisition of data, or analysis and interpretation of data.

2) This person primarily was involved in drafting the manuscript or revising it critically for important intellectual content.

3) This person provided substantial edits to the manuscript and approved submission for publication.

4) This person agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

C.B.M.: 1, 2, 3, 4

C.T.H: 1, 3, 4

P.A.R.: 1, 3, 4

P.W.M.: 1, 3, 4

M.A.R.: 1, 3, 4

W.C.K.: 1, 3, 4

R.G.A.: 1, 3, 4

C.G.V.: 1, 3, 4

S.C.O.: 1, 3, 4

C.D.P. 1, 3, 4

J.S.M.: 1, 3, 4

M.D.G.: 1, 3, 4

D.D.P.: 1, 3, 4

C.M.L.: 1, 3, 4

M.D.R.: 1, 2, 3, 4

Conflicts of interest

The authors have no conflicts of interest to disclose regarding the publication of these data. Funding for participant compensation and reagents were provided by gift monies donated to M.D.R. through Hilmar Ingredients (Hilmar, CA, USA) and Bionutritional Research Group (Irvine, CA, USA), and Lockwood, LLC (C.M.L., Draper, UT, USA) solicited these donations.

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