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LEUCINE IS A CRITICAL FACTOR DETERMINING PROTEIN QUANTITY AND  
QUALITY OF A COMPLETE MEAL TO INITIATE MUSCLE PROTEIN SYNTHESIS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in Nutritional Sciences  
in the Graduate College of the  
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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## ABSTRACT

Emerging research suggests that high protein diets may have a role in preventing obesity, sarcopenia, diabetes, and optimizing muscle mass and bone health. Evidence is mounting that these benefits may be in part due to the amino acid leucine (leu) and its unique ability to stimulate muscle protein synthesis (MPS). Current dietary protein recommendations for protein quantity and quality are defined as the minimum daily amount to prevent deficiencies of limiting amino acids and increases in plasma amino acids are regarded as ‘excess’ or inefficient use of amino acids due to increased amino acid oxidation. This view does not account for the meal responses of MPS associated with intracellular signaling in response to increasing plasma leu.

The goal of this research was to elucidate the physiological role that leu plays in complete meals and to determine if the meal distribution of protein or leu is important for optimization of muscle mass. My hypothesis was that optimum MPS would equate to maximum signaling response of the kinase known as mammalian target of rapamycin (mTOR) and directly related to post-prandial plasma leu concentration. Ultimately, the leu content of individual meals and the number of meal per day with sufficient leu to increase MPS will influence muscle mass, lean body mass and long term body composition

In vivo work with purified solutions established that leu has a regulatory role for initiating MPS by activating mTOR and there is a clear threshold of leu required to increase plasma leu and stimulate MPS. In order to test the role of leu in complete meals we examined the duration and peak activation of MPS and mTOR signaling in response to complete test meals containing 10, 20, and 30% of total energy from wheat and whey protein (6.8 and 10.9% leu) respectively. We determined that whey increased plasma leu, mTOR signaling, and MPS more than wheat at all protein intakes. Stimulation of MPS after meals last for 3 hours with a peak at 90 minutes, however plasma leu and mTOR signaling remained at a plateau for 3 hours before decreasing. Meal leu content and the increases in postprandial plasma leu were closely associated with peak activation of

mTOR signaling and MPS but did not determine the duration of protein synthesis which demonstrated a refractory response to the constant elevations in plasma leu.

Experiment 2 examined the potential to use leu to predict the quality of individual proteins to stimulate mTOR signaling and MPS. Adult rats were adapted for 2 weeks to a meal-feeding protocol with complete diets containing 16% protein but with different leu contents using wheat, soy, egg, or whey proteins (6.8, 8.0, 8.8, and 10.9% leu). Animals fed egg and whey significantly increased plasma leu and MPS whereas animals fed wheat and soy did not. The leu content of the meals predicted mTOR signaling and peak MPS responses and were closely related to changes in plasma leu. These findings support the hypothesis that there is a meal leu threshold for increasing plasma leu.

While experiment 2 provided proof of concept that there is a meal threshold for leu stimulation of MPS, the critical outcomes are changes in muscle mass or body composition. This study tested the hypothesis that long-term meal-feeding of isonitrogenous/isocaloric diets with 16/54/30% of energy from protein/carbohydrates/lipids, respectively, using protein sources (wheat, soy, egg, whey) with different leu contents could produce body composition and muscle weight outcomes in relation to the changes in postprandial MPS and plasma leu observed in the previous experiments. Based on the leu threshold observed in the previous experiment, the diets were designed such that animals fed egg and whey proteins would receive sufficient leu during each of the three meals to achieve three stimulations of MPS per day as opposed to animals fed wheat and soy proteins with lower leu contents that would achieve the leu threshold only at the larger dinner meal. After 11 weeks of meal-feeding, body composition, lean body mass, and gastrocnemius muscle weights were positively related to the leu content of the diet and reflected the ability of the respective diets to increase postprandial plasma leu and MPS.

Finally, we tested the hypothesis that long-term distribution of protein/leu could make a difference in body composition by feeding isocaloric/isonitrogenous meals containing whey protein evenly distributed to achieve the leu thresholds at each of 3 meals (ED-Whey) daily or unevenly distributed over 3 meals (UD-Whey) with only the

dinner meal exceeding the leu threshold. The ED-Whey treatment with evenly distributed leu produced multiple stimulations of MPS throughout the day and larger gastrocnemius muscle weights compared to the UD-whey that only achieved stimulation of MPS at a single meal. While muscle mass was larger in the ED-Whey treatment, total lean body mass was not different between groups. This may have been due to the large protein (i.e. nitrogen) content of the dinner meal in the UD-Whey group producing a shift in lean body mass deposition to the liver and visceral tissues, which were larger in the UD-Whey group.

In summary, the leu content of complete meals is an important regulator of MPS and produces different body composition outcomes with long term feeding of diets differing in leu contents and leu/protein distribution. These results cannot be explained by limiting amino acids and are not accounted for by current protein quantity and quality recommendations.

## **DEDICATION**

*I would like to dedicate this dissertation to my beautiful wife, Isabel. Without her I never would have been able to see this through on my own. I would also like to dedicate it to my family for their support and for ingraining a strong work ethic in me. Finally, I want to dedicate this dissertation to anyone who believes that strong work ethic, not talent, is the key to success.*

## ACKNOWLEDGEMENTS

This dissertation is the result of an effort by a team of individuals and could certainly not have been achieved by myself without the support of various key individuals who sacrificed their time and energy to help me. I must first and foremost mention my lab colleagues; Chris Moulton, Suzanne Devkota, Dee Walker, and Gabriel Wilson. I must also thank our undergraduate assistants Ann Kruk and Adam Schiltz.

I must extend thanks to several individuals in other labs including Piyawan Bunpo, Indu Rupassara, Matt Thorpe, and Rudy Valentine whose technical expertise was crucial for my research. I would also like to thank my committee members Dr. Manabu T Nakamura, Dr. Yuan-Xiang Pan, and especially Dr. Peter Garlick who has been like a second advisor to me. I would also like to extend a special thank you to Dr. Tracy Anthony for her help, advice, and talking me off of the ledge at various points throughout my graduate studies.

An inordinate amount of thanks is due to my advisor, Dr. Donald Layman. He showed patience with me; when others might have kicked me out of the door. He always knew how to push me hard enough to motivate me, but not so hard that I felt undo pressure. He knew how to 'get on my case' when I needed it, but never in such a way that made me lose confidence in myself. I can probably never thank him enough for allowing me to form my own hypotheses and take my research in an independent direction.

Finally I have to thank my family and friends. I was the first member of my family to go to college, graduate from college, and then pursue a graduate degree. My family never pressured me to go in a certain direction, only to follow my heart and my dreams and make well-thought out decisions. I'm sure this degree means just as much to them as it does to me. I cannot put into words how fortunate I am to have such a great family, and especially, a great wife.

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## LIST OF ABBREVIATIONS

4E-BP1, eukaryotic initiation factor 4E binding protein 1  
AMPK, AMP Protein Kinase  
BCAA, branched chain amino acid  
BCAT, branched-chain aminotransferase  
BV, biological value  
CHO, carbohydrate  
DAAO, direct amino acid oxidation  
DXA, dual energy X-ray absorptiometry  
EAA, essential amino acids  
ED-Whey, evenly distributed whey protein group fed 16% energy from protein at each meal  
eEF1, eukaryotic elongation factor 1  
eEF2, eukaryotic elongation factor 2  
eIF4A, eukaryotic initiation factor 4A  
eIF4E, eukaryotic initiation factor 4E  
eIF4F, eukaryotic initiation factor 4F  
eIF4G, eukaryotic initiation factor 4G  
eIFs, eukaryotic initiation factors  
FAO, Food and Agriculture Organization  
FSR, fractional rates of protein synthesis  
GC-MS, gas chromatography mass spectroscopy  
HPLC, high performance liquid chromatography  
IAAO, indispensable amino acid oxidation  
Leu, leucine  
MPS, muscle protein synthesis  
mTOR, mammalian target of rapamycin  
NPU, net protein utilization

NRC, National Research Council

PDCAAS, Protein Digestibility-Corrected Amino Acid Score

PER, protein efficiency ratio

PI3-kinase, phosphoinositide 3-kinase

RDA, recommended daily allowance

RIA, radioimmunoassay

rpS6, ribosomal protein S6

S6K, ribosomal protein 70 S6 kinase

SPE, solid phase extraction

UD-Whey, unevenly distributed whey protein group fed 8, 8, and 27.5% energy from protein at meals 1, 2, and 3 respectively

WHO, World Health Organization

## CHAPTER 1: Introduction

### Background

#### *Concerns with current dietary protein guidelines*

Dietary protein needs are defined as both the amount and quality of protein to meet metabolic requirements. However, current nutrition guidelines for dietary protein use the Recommended Dietary Allowance (RDA) which provides only the minimum amount of protein to maintain short-term nitrogen balance based largely on data from young adults in ideal health and energy balance. Information is increasing that these guidelines are not adequate to meet metabolic needs or maintain optimum long-term health for adults in environments with sedentary lifestyles and excess dietary energy.

Current standards for protein quality use measurements of animal growth (protein efficiency ratio or PER), or in humans, nitrogen balance, where both digestibility and the suitability of the amino acid pattern of absorbed amino acids, (biological value or BV), determines net protein utilization (NPU). The practical difficulties and poor sensitivity of the nitrogen balance method has led to the adoption of the PDCAAS approach, i.e. the Protein Digestibility-Corrected Amino Acid Score.

PDCAAS, which was introduced by Food and Agriculture Organization of the World Health Organization (FAO/WHO in 1991 (1), is the current international standard for protein quality assessment (2). PDCAAS is based on the combination of an age-related amino acid reference pattern that is representative of human requirements plus estimates of the digestibility of the protein. The amount of potentially limiting amino acids in the test protein is compared to their respective content in the appropriate reference pattern, identifying the single most limiting amino acid which determines the amino acid score. The current consensus is that meeting the minimum requirements for lysine, methionine, and tryptophan, considered the most limiting amino acids in poor quality proteins, determines the amino acid score and will lead to a plateau of nitrogen retention (2). At the plateau of nitrogen balance, any further increase in plasma amino acids would stimulate increased oxidation and elimination of the “excess” amino acids,

implying that protein quality above requirements does not matter. This score is assumed to predict the Biological Value (BV) of a protein, or the anticipated ability of the absorbed test protein to fulfill human amino acid requirements. The score is then corrected for digestibility giving the PDCAAS value, which is assumed to predict NPU. This method of measuring protein quality however does not account for any metabolic value of amino acids above the minimum reference score. These amino acids are simply considered to be in “excess” of needs. The fact that increasing plasma amino acids causes an increase in oxidation does not mean that these amino acids have no physiological value or do not participate in important roles in cell metabolism or intracellular signaling prior to their oxidation or even after oxidation. As understanding of metabolic actions of amino acids expand, the concept of protein quality also expands to incorporate these newly emerging actions of amino acids into a protein quality concept. New research reveals increasingly complex roles for amino acids in regulation of body composition, glucose homeostasis, and cell signaling. More importantly increased dietary protein intake may have enhance health and prevent muscle loss in aging adult populations.

A new concern about dietary recommendations is protein distribution. Current recommendations of protein quantity are daily requirements, assuming that the distribution of protein is unimportant so long as the total daily protein goal is met and data from de Castro et al. indicates that adults consume nearly 65% of their protein at dinner (6). However, there is mounting evidence however that metabolic responses of muscle protein synthesis (MPS) and signaling through the mammalian target of rapamycin (mTOR) are regulated on a meal-to-meal basis (3-5). If true, a logical extension is that multiple stimulations of MPS are superior to a single stimulation of MPS for optimizing maintenance of muscle mass. It is unlikely that other meals contain sufficient leu to saturate the leu signaling mechanism and stimulate MPS. Therefore, many adults may meet total protein requirements on a per day basis, but not optimize individual meal responses of MPS.

### *Dietary protein and muscle mass with aging*

An important example of the need for an expanded understanding of protein quality and amino acid needs is the age-related disease of sarcopenia. Sarcopenia is defined as the loss of skeletal muscle mass, strength, and endurance with age. Whereas muscle wasting and cachexia are rapid losses in muscle mass, sarcopenia is a gradual loss of muscle mass (~0.5-1% /year) beginning approximately at age 40. Advanced sarcopenia is associated with muscle weakness, frailty, increased likelihood of disability, reduced quality of life, and increased falls in the elderly (7). The number of people age 65 and older is expected to increase by ~80% in the next 20 years so this presents a large scale health concern for our aging population.

Sarcopenia is associated with a variety of metabolic and social factors including sedentary lifestyle and suboptimal diet. Many elderly choose to eat less protein due to problems with chewing, digestion, expense of high protein foods, and increased satiety. These lifestyle changes are compounded by a reduced anabolic response to dietary protein intake in the elderly which may be due to decreased intramuscular expression and phosphorylation of signaling proteins (i.e. mTOR, p70 S6 kinase (S6K), and eukaryotic initiation factors 4E-BP1 and 2B) (8-10).

Insufficient protein intake in elderly individuals may cause loss of muscle by reducing MPS and thus promoting net muscle protein catabolism (10, 11). Even moderate increases in protein intake may help prevent muscle loss and improve anabolic signaling in the elderly (12, 13). Interestingly, Arnal et al. has demonstrated that protein distribution and intake at individual meals may be equally important as total daily protein intake in the elderly (5). These data indicate the need to define benefits of high protein diets for adults and elderly and the quantity and quality of protein intake needed to achieve these benefits. Furthermore, if protein needs to maintain muscle mass increase with aging, understanding and acceptance of this change in metabolic requirements requires a clear mechanism with measurable outcomes.

### ***Leucine metabolism and metabolic effects***

A proposed mechanism to explain these phenomena is an age-related shift in regulation of MPS from a hormone-driven growth model to a model of amino acid signaling driven by the branched-chain amino acid (BCAA) leucine (leu) (3, 14). Leu is unique among amino acids for its regulatory roles in metabolism, including translational control of MPS (14, 15) via stimulation of the mTOR signaling pathway. Research from Garlick et al. indicates that the anabolic effects of amino acids can be contributed to leu (16). The metabolic effects of leu are, at least in part, associated with the absence of the branched-chain aminotransferase (BCAT) enzyme in liver (17). Absence of BCAT in liver results in an increased supply of the three BCAAs available to skeletal muscle. Dietary BCAA reach the blood virtually unaltered from their levels in the diet; thus, leu reaches peripheral tissues in direct proportion to its dietary intake.

### ***Translational control of muscle protein synthesis by leucine***

Short-term regulation of MPS is achieved at the level of translation, with primary emphasis on the assembly of the structural machinery for MPS through initiation (15, 18). The basic components for protein synthesis include the large (60S) and small (40S) ribosomal subunits, mRNA coding for individual proteins, transfer RNA (tRNA) for individual amino acids, and more than a dozen catalytic proteins identified as eukaryotic initiation factors (eIFs). These eIFs serve to guide the assembly of the ribosome on the mRNA and provide the cell with sensitivity to environmental factors responding to changes in diet (such as leu availability) and physical activity.

Ribosome assembly on mRNA requires activation of the 40S ribosomal subunit and preparation of the 5'cap end of the mRNA. This is facilitated by binding of the 40S ribosomal subunit with eIF2, which carries a high energy molecule of GTP, and the initiator amino acid methionine (Met-tRNA). This complex of eIF2-GTP-Met-tRNA (known as the ternary complex) binds with the 40S subunit to form the 43S pre-initiation complex. Formation of the ternary complex is sensitive to cellular energy status and intracellular leu concentrations (15, 19).

Binding of the 43S pre-initiation complex to an mRNA is believed to be a rate-controlling step in translation initiation (15, 19, 20). This binding is facilitated by eIF4F, a three-subunit complex consisting of (1) eIF4E, a protein that binds to the 5'cap structure of the mRNA to be translated; (2) eIF4G, a peptide that serves as a scaffold to connect eIF4E and eIF4A with the mRNA and 40S subunit; and (3) eIF4A, a RNA helicase that functions to unwind secondary structure in the 5'-untranslated region of the mRNA. The function of the eIF4F complex is to recognize, unfold, and guide the mRNA to the 43S pre-initiation complex.

Formation of the eIF4F complex is regulated through the availability of eIF4E for binding with eIF4G and the phosphorylation state of eIF4G. Availability of eIF4E for eIF4G is controlled by the eIF4E inhibitory binding protein 4E-BP1. Binding of 4E-BP1 with eIF4E prevents the formation of the eIF4E-eIF4G complex (21). Binding of 4E-BP1 to eIF4E is determined by the phosphorylation state of the binding protein.

Phosphorylation of 4E-BP1 by the protein kinase mTOR reduces the affinity of 4E-BP1 for eIF4E, allowing eIF4E to bind with eIF4G and form the active eIF4F complex. Activity of mTOR is sensitive to leu concentrations and stimulation through the PI3-kinase signal cascade.

Another downstream target of mTOR is the 70-kD ribosomal protein S6 kinase (S6K). S6K is activated by phosphorylation from mTOR and the active S6K in turn activates the ribosomal protein S6 (rpS6) allowing it to bind to the 40S subunit (15, 19). Activation of rpS6 results in preferential translation of mRNAs that encode components of the MPS mechanism, including the ribosomal proteins eIF4G, eukaryotic elongation factors (eEF1 and eEF2), and poly(A) binding protein. All of these proteins are involved in translation of mRNA to protein. Activation of S6K therefore increases the cell's capacity for MPS. Together, the effects of leu concentration through mTOR activity on eIF4E and S6K influence both the rate and capacity of translation in skeletal muscle. Inhibiting mTOR prevents phosphorylation and activation of S6K.

Furthermore, though important, mTOR alone does not fully account for leu stimulation of MPS. For instance, co-administration of leu with the mTOR inhibitor

rapamycin only partially inhibited the leu-induced increase in MPS (22). Interestingly, leu has been shown to increase MPS via an mTOR independent mechanism that increases eIF4G phosphorylation (4, 23). Thus, leu concentration is important for both availability of eIF4E and activation of eIF4G.

### ***In vivo effects of leucine on protein synthesis and mTOR signaling***

Leucine effects on protein synthesis have been shown to be age-dependent with stimulation of MPS requiring greater amounts of leu with increasing age (24). Elderly require a high proportion of leu in an essential amino acid mixture for stimulation of MPS (25). The reduced anabolic response to leu and essential amino acids is associated with a decrease in total mTOR and S6K content and reduced activation of mTOR (10). MPS can be restored in old rats if leu concentrations are raised to supra-physiological concentrations (24). In humans, Katsanos et al. (25) found that MPS can be increased in the elderly using a small dose of essential amino acids (6.7g) containing a high proportion (2.8g) of leu. The same dose of EAA failed to stimulate MPS when the leu content was reduced to 1.7g. These results are in accordance with the research by Arnal et al. (5) that showed the anabolic response of whole body protein turnover to a meal could be normalized in the elderly using a protein-pulse feeding pattern providing a large amount of amino acids at a meal. Furthermore, Garlick et al. demonstrated leu alone will stimulate MPS to the same extent as a solution containing all the amino acids (16). It is likely that the progressive loss of muscle mass during aging may be in part attributed to consumption of meals insufficient in leu to initiate MPS in skeletal muscle multiple times per day.

### **Significance**

These data suggest that there is a threshold of leu that must be achieved within the protein intake at a meal in order to optimize MPS. Current recommendations are based on the assumption that post-prandial increases in plasma amino acids reflect excess dietary intake without addressing their metabolic effects on initiating MPS. We proposed

that the post-prandial increase in plasma leu reflects an important metabolic threshold that is critical to the anabolic response of a meal (3). This concept has only been tested using purified amino acid solutions. In order to test the validity of leu as an indicator of protein quality rather than limiting amino acids we propose using whole protein sources to create different plasma leu concentrations in order to determine if leu content of a protein source is a suitable measure of protein quality and to also determine where various thresholds exist for leu to stimulate MPS.

We also aim to determine the effect of protein distribution throughout the day on maintaining muscle mass. This concept of protein distribution may be a crucial aspect of dietary protein intake that the current recommendations do not address. It is likely that to optimize muscle mass in adults and prevent age-related muscle dysfunction that protein needs should be met by eating multiple high quality protein meals per day with each meal containing sufficient leu to reach the threshold and stimulate MPS. Finally, we will examine long term body composition and muscle weight outcomes in response to chronic consumption of isonitrogenous, isoenergetic meals containing different leu contents and different protein/leu distributions throughout the day to determine if these short term meal responses have measureable long term effects.

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## **CHAPTER 2: The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats<sup>1</sup>**

### **Introduction**

Leucine (Leu) is known to be a unique regulator of muscle protein synthesis (MPS) and translation initiation and has been proposed as a key mechanism translating diet quality into the meal response of MPS (1,2). Leu alone can stimulate MPS to the same extent as a complete protein or complete mixture of amino acids by activating several factors involved with initiating mRNA translation, primarily through the mammalian target of rapamycin (mTOR) signaling pathway, including ribosomal protein S6 kinase (S6K), and eukaryotic initiation factor 4E binding protein-1 (4E-BP1) in a dose dependant fashion (3,4).

While leu's stimulatory effects on MPS and translation initiation have been extensively documented *in vivo* using purified solutions, the impact of leu when consumed as part of complete meals is unknown. Further, neither the relationship of leu in complete meals on the post-prandial time course of MPS nor the impact of meals containing isonitrogenous protein sources with different leu contents on MPS have not been investigated.

The time course of MPS in response to an oral dose of free leu has been previously demonstrated by Anthony et al (4) with synthesis peaking from 30 to 60 min after leu administration and returning to control levels by 120 min. The ability of leu to stimulate MPS suggests that leu content may be an important criterion for defining optimum protein quantity and quality for a meal. Low protein meals (~10 g/meal) with limited leu content produce minimal increases in plasma leu in humans as compared to

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<sup>1</sup> Norton LE, Layman DK, Bunpo P, Anthony TG, Brana DV, Garlick PJ. **The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats.** *J Nutr.* 2009 Jun;**139**(6):1103-9. The American Society for Nutrition grants the rights to the author(s) for any research article published in the Journal of Nutrition to be reprinted in the author(s) thesis.

higher protein meals (~30 g/meal) (5). It is not well understood if feeding isonitrogenous complete meals with different leu contents produces differences in plasma leu, translation initiation, mTOR signaling and MPS.

The goal of this study was two-fold: first, to define the time course of plasma and intracellular leu concentrations following consumption of a complete meal containing a leu-rich protein source designed to produce a large plasma leu response and to correlate these responses with mTOR signaling, translation initiation factors and MPS. Second, to assess how complete meals containing 3 different levels of wheat or whey proteins (10, 20, and 30% of total energy) with different leu contents (6.8% and 10.9% leu respectively) impact plasma amino acid profiles, mTOR signaling and MPS.

## **Materials and Methods**

### ***Animals and diets.***

The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

**Experiment 1** examined the time course of changes in plasma leu, MPS and translation initiation factors after a meal. Male Sprague-Dawley rats (300 ± 15g; Harlan-Teklad, Indianapolis, IN) were maintained at 24°C with a 12-h light:dark cycle and free access to water. Rats were fed diets providing 20/50/30% of energy from whey protein, carbohydrates and fats, respectively (**Table 2.1**).

Rats were trained to meal feed using 3 meals per day consisting of a small (4 g) meal consumed between 07:00 and 07:20 followed by free access to diets from 13:00 to 14:00 and 18:00 to 19:00 (5). Rats consumed ~19 g/d of total diet. After 5 d of meal training, rats were food deprived for 12 h (from 19:00 until 07:00) and then examined at 6 time points: prior to the first meal (time 0) and at 45, 90, 135, 180 and 300 min after the 4g meal. At the indicated times, rats were killed and blood and tissue samples collected. Gastrocnemius, soleus, and plantaris muscles were excised and immediately frozen in liquid nitrogen for later analysis.

**Experiment 2** compared the potential of wheat protein vs. whey protein to stimulate MPS and translation initiation in skeletal muscle at three levels of protein intake (10, 20, and 30% of energy from protein) at 90 min after the test meal representing the peak level of MPS observed in Experiment 1. The two protein sources were selected to provide the same amount of total nitrogen with different levels of leu. Leu accounts for approximately 10.9% of the total amino acids in whey protein and 6.8% in wheat gluten (**Table 2.2**). Male rats ( $250 \pm 12$ g) were maintained similar to Experiment 1 except they were adapted to meal feeding using a control diet providing 14/56/30% of energy from whey protein, carbohydrates and fats, respectively (**Table 2.1**). After 5d of meal feeding rats were randomized into either control (n=7) or one of the 6 treatment groups: 10% wheat protein (n=8), 10% whey protein (n=8), 20% wheat protein (n=8), 20% whey protein (n=8), 30% wheat protein (n=8) and 30% whey protein (n=8) (Tables 1 and 2). Wheat gluten diets were supplemented with lysine to meet NRI requirements and to approximate whey lysine levels (Table 1). Rats were food deprived for 12 h and then provided a single 4g test meal from one of the 6 experimental diets. The test meals contained approximately 0, 29, 47, 60, 94, 89 and 142 mg of leu respectively for the control, 10% wheat, 10% whey, 20% wheat, 20% whey, 30% wheat and 30% whey groups. Rats were killed 90 min after consumption of the meal and blood and tissue samples collected.

#### ***Determination of Muscle Protein Synthesis.***

Protein synthesis was measured in skeletal muscle using the flooding dose method (7). A 40% enriched L-[ $^2\text{H}_5$ ]phenylalanine solution (150mmol/L; Cambridge Isotopes, Andover, MA) was administered at 150  $\mu\text{mol}/100$  g body weight and injected via tail vein (1 mL/100g body weight). After 10 min animals were killed by decapitation and hind limbs quickly removed and immersed in an ice-water mixture. Gastrocnemius, plantaris, and soleus muscles were removed from cooled hind limbs, frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$ .

Frozen muscle tissue was powdered in liquid nitrogen, and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1mL per 50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by gas chromatography mass spectroscopy as described previously (8,9). The enrichment of L-[<sup>2</sup>H<sub>5</sub>]phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies Santa Clara, CA). The samples were run under electron impact ionization and in splitless mode, and phenylethylamine ions at mass-to-charge ratio (m/z) 106 (m + 2) and 109 (m + 5) were monitored for enrichment analysis.

The muscle supernatant was used for determination of intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid phase extraction (SPE) using EZ:faast™ amino acid analysis sample testing kit (Phenomenex Inc. Torrance, CA, USA) and <sup>2</sup>H<sub>5</sub>-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at m/z 206 (m) and 211 (m+5) (10).

Fractional rates of protein synthesis (FSR) were assessed from the rate of incorporation of L-[<sup>2</sup>H<sub>5</sub>]phenylalanine into total mixed muscle protein as described previously (11). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[<sup>2</sup>H<sub>5</sub>]phenylalanine incorporation. FSR, defined as the percentage of tissue protein renewed each day, were calculated according to the formula:  $FSR = (E_b \times 100) / (E_a \times t)$  where t is the time interval between injection and cooling of sampled tissue expressed in days and E<sub>b</sub> and E<sub>a</sub> are the enrichments of [<sup>2</sup>H<sub>5</sub>]Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

### ***Plasma measurements.***

Plasma was obtained from trunk blood by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Linco Research, MO). Plasma glucose (Thermo Fisher Scientific, Middletown,

VA) was determined by the glucose oxidase method. Plasma amino acid concentrations were analyzed by HPLC using a Waters 2475 Fluorescence detector (12).

***Quantitation of eIF4E, 4E-BP1·eIF4E, and eIF4G·eIF4E complexes.***

The association of 4E-BP1 and eIF4G with eIF4E in muscle extracts was examined by protein immunoblot analysis as previously described (6,13). Membranes were blotted using a rabbit polyclonal antibody to either eIF4E (Santa Cruz Biotechnology), eIF4G (Bethyl Labs), or 4E-BP1 (Bethyl Labs).

***Phosphorylation of 4E-BP1 and S6K.***

Muscle supernatants were subjected to protein immunoblot analysis as described previously (6,13) using a rabbit polyclonal antibody to 4E-BP1 (Bethyl Labs, Montgomery, TX) and a rabbit polyclonal S6K antibody (Bethyl Labs, Montgomery, TX).

***Statistical analysis.***

All data were analyzed by SPSS 15.0 software package for windows. A 1-way ANOVA was performed with the post prandial time as the independent variable for experiment 1. A 2-way ANOVA was performed with treatment group as the independent variable for experiment 2. When a significant overall effect was detected, differences among individual means were assessed using Fisher's LSD post hoc test. Separate comparisons to baseline were made using a t-test. All data sets were tested for normal distribution and variance homogeneity using Levene's test. When variances were not homogeneous, means were compared using a Games-Howell test. Correlation between data was determined by linear regression (Pearson's correlation). The level of significance was set at  $P < 0.05$  for all statistical tests. Values in the text are means  $\pm$  SEM.

## Results

### Experiment 1

Time course changes in MPS, plasma leu and the activity of protein factors involved in regulating mRNA translation were examined at 0, 45, 90, 135, 180 and 300 min in rats after consuming a 4g complete meal containing 20% whey protein (providing ~94 mg of leu). MPS increased from 0 to 45 min after the meal and reached peak values at 45 and 90 min post-prandial (**Figure 2.1**). From 90 to 135 minutes MPS values decreased significantly and returned to baseline values at 180 min.

Plasma leu increased at 45 min after the meal and remained elevated through 300 min with peak values from 45 to 180 min (**Figure 2.1A**). Intracellular leu concentrations changed in a pattern similar to plasma leu (**Figure 2.2B**). Plasma isoleucine, valine, lysine and methionine followed similar post-prandial patterns and concentrations returned to baseline by 300 min (**Table 2.3**).

Plasma insulin concentrations increased significantly at 90 min (**Table 2.3**) then returned to baseline by 180 min. Plasma glucose was not different from baseline at any point during the time course (data not shown).

Time course changes in 4E-BP1 and S6K were measured to evaluate the mTOR signaling pathway. Both 4E-BP1 (**Figure 2.3A**) and S6K (**Figure 2.3B**) phosphorylation increased between 45 min and 180 min. At 300 min both 4E-BP1 and S6K phosphorylation declined from peak activation with 4E-BP1 not different from baseline while S6K phosphorylation remained at ~50% of peak activation.

Formation of the eIF4F translation initiation complex was evaluated by determining the association of eIF4E with eIF4G. Association of eIF4E with eIF4G increased between 90 and 180 min ( $P < 0.05$  at 90, 135 min,  $p = 0.055$  at 180 min; **Figure 2.3C**), and returned to baseline at 300 min.

Changes in MPS highly correlated with plasma and intracellular leu levels during the early post-prandial period. From 0 to 90 min after the meal, plasma leu correlated ( $p < 0.001$ ) with activation of S6K ( $r = 0.858$ ), 4E-BP1 ( $r = 0.802$ ) and MPS ( $r = 0.782$ ). The correlation of plasma leu with MPS was less strong from 90 to 300 min post-prandial

( $r=0.492$ ,  $p<0.01$ ). MPS had a weak correlation with plasma insulin over time ( $r=0.424$ ,  $p<0.01$ ).

## Experiment 2

Experiment 2 compared the potential of wheat protein vs. whey protein at three dietary levels of protein intake (10, 20, and 30% of energy from protein) to stimulate MPS and translation initiation in skeletal muscle at 90 min representing the peak level of post-prandial MPS observed in Experiment 1. Plasma leu increased significantly above food-deprived controls in 10% whey but not 10% wheat (**Figure 2.4**). Within the 10%, 20%, and 30% protein treatment groups, whey protein produced higher plasma leu concentrations than wheat except for the 30% group. Plasma concentrations of isoleucine and valine were also greater in the whey groups as compared to the wheat groups except at the 30% protein level (**Table 2.4**). Plasma concentrations of lysine, methionine, and threonine were not different between wheat and whey for any of the groups examined with the exception of methionine which was significantly lower in the 10% wheat group compared to the 10% whey group (**Table 2.4**). Plasma lysine and threonine increased above baseline at the 20 and 30% protein levels ( $P<0.05$ ) with no significant difference between whey and wheat.

Phosphorylation of 4E-BP1 (**Figure 2.5A**) and S6K (**Figure 2.5B**) increased after all test meals. However, whey protein promoted mTOR signaling greater than wheat protein at nearly all protein levels.

MPS increased after all meals except the 10% wheat group (**Figure 2.6**). Consistent with mTOR signaling targets 4E-BP1 and S6K, the main effect was whey protein increased MPS more than wheat protein at equal protein intakes with the greatest individual differences observed in the 10% ( $P<0.05$ ) and 30% ( $P=0.062$ ) protein groups where whey stimulated MPS 52% and 19% more than wheat respectively.

Plasma insulin concentrations were increased after the meals except for 10% whey and 20% wheat ( $P<0.05$ ). Peak values were obtained in the 20% whey and 30% wheat groups (Table 4).

## Discussion

These experiments evaluated time course changes and peak activation in MPS, translation factors, and changes in plasma amino acids in response to nutritionally complete meals with the purpose of determining the contribution of leu to directing postprandial changes in MPS. The major conclusion of this work is that leu is the primary factor directing post-prandial translation factor signaling and peak stimulation of MPS, but that the duration of the meal response in skeletal muscle is influenced by factors in addition to leu concentrations.

Experiment 1 examined the time course of MPS after a meal containing 20% of energy from whey protein. MPS increased above baseline levels by 45 min with a peak value at 90 min and return to baseline at 3 h post-prandially. Previous research using purified amino acids demonstrated a more rapid MPS response that peaked at 45 min and returned to baseline by 2 h (3,4,15). Thus, feeding a complete meal appears to both delay and prolong MPS in skeletal muscle compared to oral intake of leu or EAA (3, 4).

These results provide support for the role of leu as a key trigger for post-prandial stimulation of MPS after a complete meal but indicate that elevated plasma leu is not sufficient to produce sustained elevations of MPS. The cause of MPS becoming refractory to plasma leu could not be explained by the mTOR targets 4E-BP1 and S6K as they remained activated at 180 min post-prandial. Likewise, the rate-limiting step of eIF4F assembly, eIF4E binding to eIF4G is increased above baseline at 135 min, a point at which MPS is decreasing from peak activation.

The mechanism for the post-prandial decline in MPS with elevated leu is unknown (16). Layman and Wisont (17) demonstrated that leu infusion in rats stimulated MPS but prolonged infusion depleted plasma valine and isoleucine in proportion to the rate of substrate use for MPS. Co-infusion of valine and isoleucine prevented plasma depletion. Likewise, Escobar et al (18) reported that leu infusion in neonatal pigs depleted plasma EAA coincident with a decline in MPS. These investigators found that infusion of a complete mixture of EAA maintained MPS. Contrary to these findings, Bohe et. al. (16) documented the refractory nature of skeletal muscle to elevations of

amino acids during a 6 h infusion of a complete mixture of EAA in adults. The infusion produced constant elevations in plasma EAA however, the synthetic response lasted only 2 h and was unable to be further stimulated by infusion of additional EAA during the 6 h period. Likewise, in the current experiment, the refractory response to leu occurred without a parallel decline in other EAA.

Another potential mechanism for the refractory response may include plasma insulin. Insulin is not required to stimulate protein synthesis in adults, but it does optimize the MPS response of muscle to amino acids (4,19). Bohe et al. (16) found that that plasma insulin time course somewhat paralleled the decline in MPS. Based on this, perhaps an elevation in insulin is necessary to sustain the anabolic response of MPS associated with elevated plasma leu. If insulin contributes to the duration of MPS in the current study, the mechanism appears to be independent of insulin's effects on the translation initiation as phosphorylation of 4E-BP1 and S6K remained elevated at 180 min where MPS and plasma insulin declined to baseline. The correlation of plasma insulin with MPS was statistically significant over the time course but less than the correlation between leu and MPS. Insulin is also known to stimulate peptide elongation in skeletal muscle (20). Changes in plasma insulin concentrations may contribute to the refractory period of MPS through changes in peptide elongation. Taken together, current information is insufficient to explain why skeletal muscle becomes refractory to the anabolic effects of elevated amino acids.

Experiment 2 examined responses of translation initiation and MPS to isonitrogenous meals with different leu contents. MPS and translation initiation are known to respond in a dose relationship to leu administration (3,4), but to our knowledge, this was the first experiment to explore dosing leu as a component of different protein sources in complete meals. Overall, whey protein with high leu content was superior to wheat protein in initiating MPS in rats fed complete meals. These findings are consistent with a previous report showing that whey protein fed after exercise stimulated mTOR signaling to a greater extent than soy protein (6).

In accordance with the leu contents of wheat and whey protein, plasma levels of leu were greater at every level of protein intake for animals fed whey vs. wheat protein. Interestingly, feeding rats the 4g meal containing 10% wheat protein (approximately 29mg leu) failed to increase plasma leu levels. Consistent with the plasma data, 10% wheat did not stimulate MPS. Conversely, feeding a meal containing 10% whey (approximately 47mg leucine) almost doubled plasma leu levels and significantly increased MPS suggesting a minimum threshold of leu intake to increase plasma leu concentration and initiate MPS after 90 minutes. Further, at 10% protein intake, 4E-BP1 and S6K were phosphorylated to a greater extent by whey protein than wheat protein. Interestingly, 10% wheat protein stimulated phosphorylation of 4E-BP1 and S6K above baseline values despite the lack of increase of plasma leu. This may be attributed to the rise in plasma insulin in the 10% wheat group which is also known to activate the mTOR pathway but to a reduced level in the absence of leu (19).

A second factor that may contribute to differences in the post prandial rise in plasma amino acids is the rate of gastric emptying and peptide digestion and absorption (21). Whey proteins which are highly soluble empty rapidly from the stomach compared with casein (21); so it is possible that whey and wheat proteins may empty at different rates which could impacted post prandial plasma leu, MPS, and mTOR signaling responses. However, research from Bos et al. (21) demonstrated that plasma amino acids peak at approximately 60 min post prandially in humans which is similar to the reported amino acids peak for whey protein in humans (22). This is consistent with the observed post prandial plasma leu concentrations which are in proportion to the actual leu content of each protein.

The time course of stimulation of MPS after a meal and the refractory nature of the anabolic response at 3 h provokes the question as to whether a second stimulation can be achieved with a new meal. Along these lines, these data suggest that the meal distribution of protein throughout the day is an important factor to consider in future experiments. Current dietary guidelines for protein make no mention of distribution of intake throughout the day. Data from de Castro (23) indicate that Americans consume

over 65% of their daily protein after 18:30 h. It is unlikely that they reach a threshold of protein intake necessary to stimulate MPS at meals prior to 18:30. Research from Arnal et al. (24) also supports the importance of a protein threshold at meals. Furthermore, if the anabolic response to a high protein meal only lasts 3 h, then it is likely that a large portion of the day is spent in a catabolic state. This suggests that optimum protein intakes for adults require proteins rich in leu to be provided in multiple meals throughout the day.

### **Conclusions**

In summary, these data suggest that leu content of various protein sources is an important indicator of protein quality as it relates to acute stimulation of MPS. These data also provide further evidence that a specific threshold of leu intake is needed to initiate mRNA translation and MPS and that low intake of some protein sources may not reach this threshold. Additionally, these data lead us to propose that the distribution of protein and specifically leu intake throughout the day is an important factor to optimize the muscle anabolic response to each meal.

### **Acknowledgements**

We thank Ms. Indu Rupassara for assistance with GCMS analyses.

## Tables and Figures

**Table 2.1** Composition of test diets

Component	Baseline Diet <sup>1</sup>	10% Wheat	10% Whey	20% Wheat	20% Whey <sup>2</sup>	30% Wheat	30% Whey
	<i>g/kg</i>						
Vital Wheat Gluten <sup>3</sup>	0.0	132.0	0.0	256.0	0.0	378.0	0.0
L-Lysine supplement <sup>4</sup>	0.0	7.2	0.0	14.4	0.0	21.6	0.0
Whey Protein Isolate <sup>5</sup>	159.6	0.0	115.2	0.0	228.0	0.0	340.6
Cornstarch	357.4	377.8	401.8	246.6	290.0	117.4	176.4
Maltodextrin	134.1	134.1	134.1	134.1	134.1	134.1	134.1
Sucrose	101.5	101.5	101.5	101.5	101.5	101.5	101.5
Soybean Oil	140.9	140.9	140.9	140.9	140.9	140.9	140.9
Cellulose (Fiber)	53.7	53.7	53.7	53.7	53.7	53.7	53.7
Mineral Mix <sup>6</sup>	37.6	37.6	37.6	37.6	37.6	37.6	37.6
Vitamin Mix <sup>6</sup>	10.7	10.7	10.7	10.7	10.7	10.7	10.7
Choline Bitaurate	2.7	2.7	2.7	2.7	2.7	2.7	2.7

<sup>1</sup>Diet used to adapt animals to meal feeding in experiment 2.

<sup>2</sup>Diet used in experiment 1.

<sup>3</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 75.5% protein, 15.5% carbohydrate, 9% other.

<sup>4</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

<sup>5</sup>Whey Protein provided by Perham, Perham, MN. 92.1% protein, 1.6% carbohydrate, 6.3% other.

<sup>6</sup>Mineral and Vitamin supplements (25) from Harlen-Teklad, Madison, WI.

**Table 2.2** Amino acid compositions of protein sources

Amino Acid	Wheat <sup>1</sup>	Whey <sup>2</sup>
	<i>g/100g protein</i>	
Alanine	3.1	4.9
Arginine	4.7	2.4
Aspartate	4.0	10.6
Cysteine	1.9	2.5
Glutamate/Glutamine	31.7	16.9
Glycine	3.8	1.8
Histidine	1.8	2.0
Isoleucine	3.0	6.2
Leucine	6.8	10.9
Lysine <sup>3</sup>	2.8 (+6.3)	9.1
Methionine	1.9	2.0
Phenylalanine	4.4	3.3
Proline	9.4	5.6
Serine	3.9	4.7
Threonine	2.6	6.4
Tryptophan	1.3	1.7
Tyrosine	2.4	3.0
Valine	4.5	6.0

<sup>1</sup>Vital Wheat Gluten purchased from Harlan Teklad, Madison, WI. 76% protein, 15.2% carbohydrate, 8.8% other.

<sup>2</sup>Whey protein isolate provided by Perham, Perham, MN. 92.1% protein, 1.6% carbohydrate, 6.3% other.

<sup>3</sup>Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match whey protein.

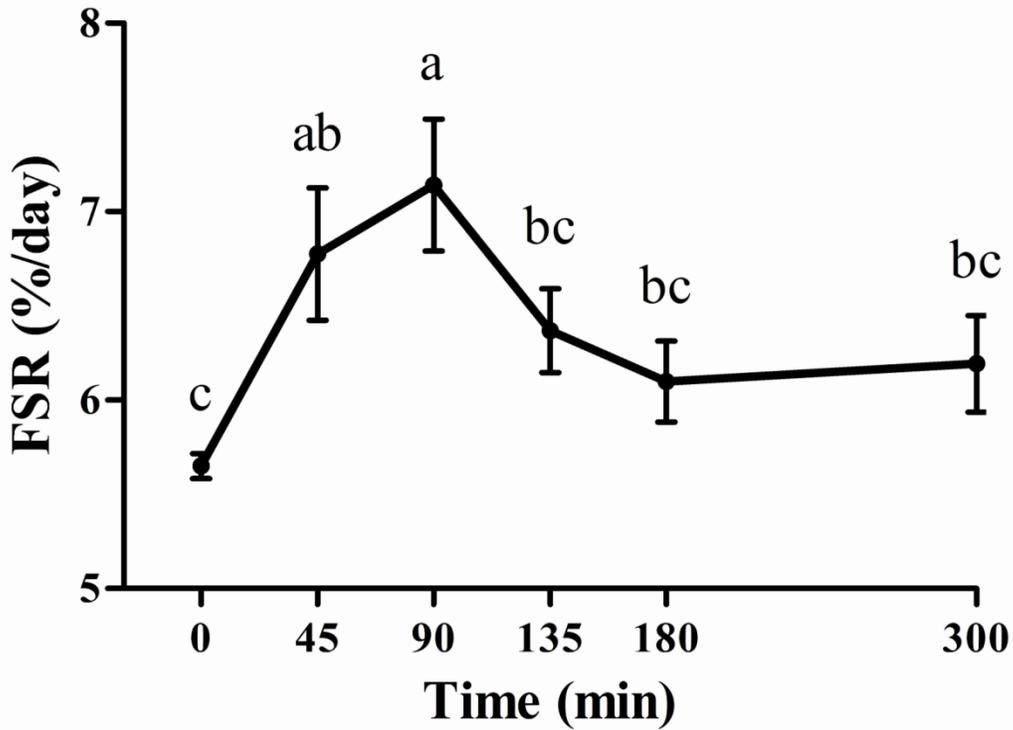


**Table 2.4** Selected plasma essential amino acid and insulin concentrations 90 min after feeding complete meals containing wheat or whey<sup>1</sup>

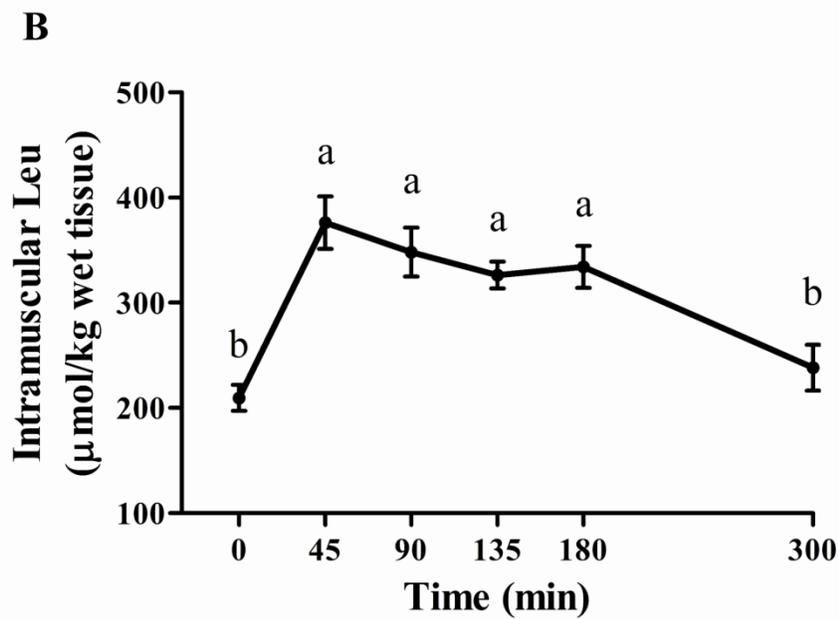
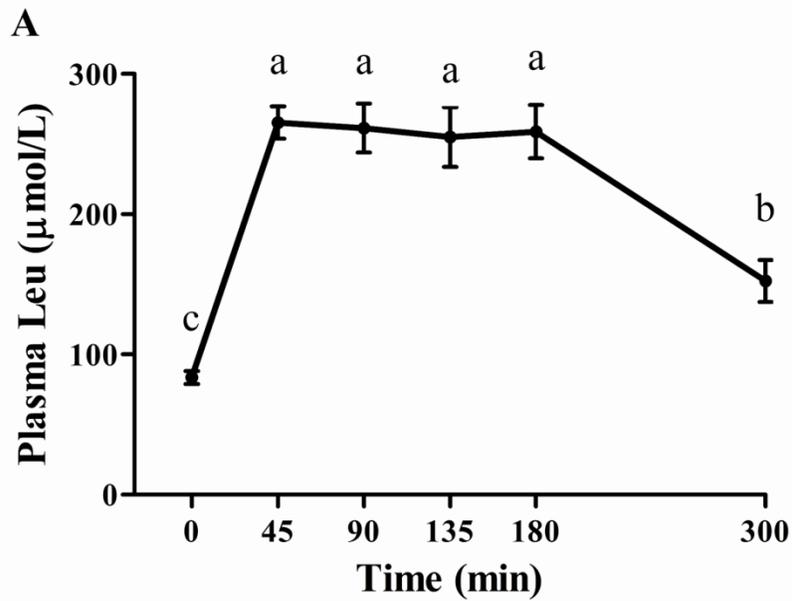
	Baseline <sup>2</sup>	10% Wheat	10% Whey	20% Wheat	20% Whey	30% Wheat	30% Whey
Leucine	83.1 ± 4.8	76.9 ± 3.9 <sup>d</sup>	149.5 ± 11.0 <sup>c</sup>	180.7 ± 10.3 <sup>c</sup>	242.3 ± 12.2 <sup>b</sup>	350.7 ± 27.3 <sup>ab</sup>	442.8 ± 12.2 <sup>a</sup>
Isoleucine	44.5 ± 2.3	50.6 ± 2.5 <sup>d</sup>	101.5 ± 9.3 <sup>c</sup>	121.5 ± 9.8 <sup>bc</sup>	155.1 ± 12.7 <sup>b</sup>	223.7 ± 19.6 <sup>ab</sup>	257.5 ± 17.5 <sup>a</sup>
Valine	118.0 ± 4.6	83.6 ± 4.4 <sup>c</sup>	190.5 ± 18.2 <sup>b</sup>	211.2 ± 13.4 <sup>b</sup>	319.8 ± 35.3 <sup>ab</sup>	430.7 ± 39.1 <sup>a</sup>	505.4 ± 40.1 <sup>a</sup>
∑ BCAA	245.6 ± 11.0	211.1 ± 10.4 <sup>c</sup>	441.5 ± 37.4 <sup>b</sup>	513.4 ± 32.2 <sup>b</sup>	717.3 ± 51.1 <sup>ab</sup>	967.8 ± 77.2 <sup>a</sup>	1177.2 ± 84.2 <sup>a</sup>
Lysine	449.3 ± 39.9	394.5 ± 26.1 <sup>b</sup>	530.1 ± 65.0 <sup>ab</sup>	663.7 ± 69.5 <sup>ab</sup>	821.8 ± 84.4 <sup>a</sup>	705.1 ± 70.1 <sup>ab</sup>	658.2 ± 49.8 <sup>ab</sup>
Methionine	44.1 ± 3.9	31.0 ± 0.9 <sup>c</sup>	46.8 ± 3.8 <sup>ab</sup>	60.2 ± 6.4 <sup>ab</sup>	59.8 ± 4.6 <sup>ab</sup>	70.9 ± 7.0 <sup>a</sup>	77.9 ± 8.8 <sup>a</sup>
Threonine	568.2 ± 53.1	536.2 ± 46.6 <sup>c</sup>	670.6 ± 109.2 <sup>bc</sup>	766.3 ± 74.2 <sup>b</sup>	902.4 ± 85.8 <sup>ab</sup>	1038.3 ± 88.3 <sup>a</sup>	1024.6 ± 71.4 <sup>a</sup>
Insulin <i>pmol/L</i>	76.4 ± 6.5	142.7 ± 15.9 <sup>ab</sup>	119.0 ± 24.3 <sup>b</sup>	118.1 ± 21.1 <sup>b</sup>	178.4 ± 20.2 <sup>a</sup>	171.7 ± 22.5 <sup>ab</sup>	134.6 ± 18.1 <sup>ab</sup>

<sup>1</sup>Values expressed as means ± SEM, n=6-8. Main effects for wheat vs. whey were significant. Labeled means without a common letter differ (P<0.05).

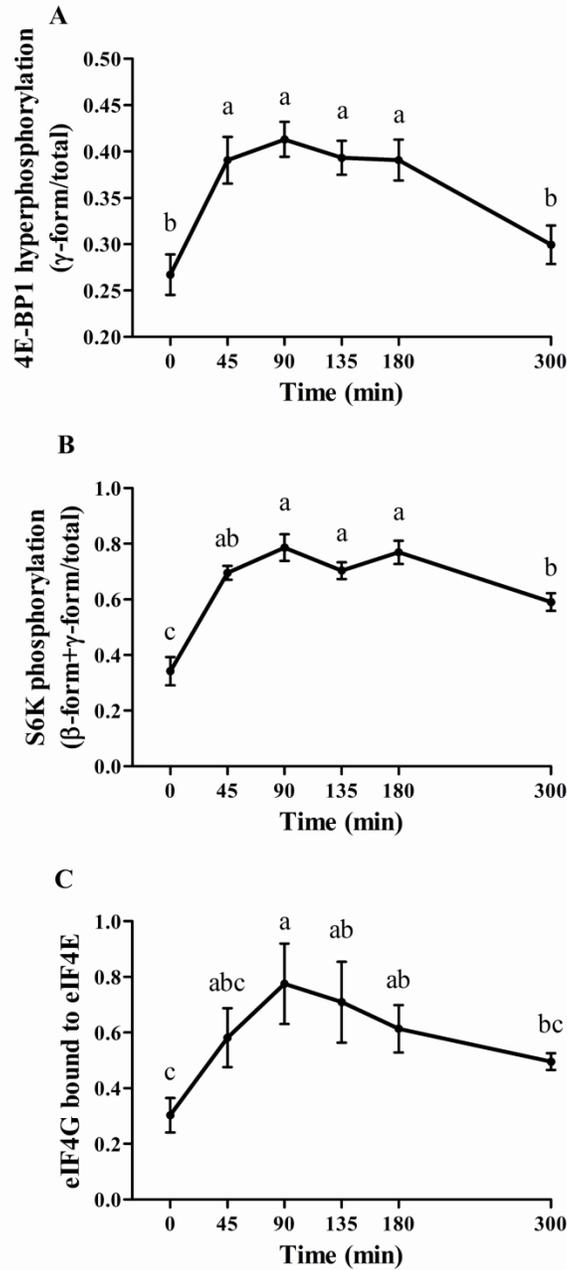
<sup>2</sup>12 hour food-deprived control.



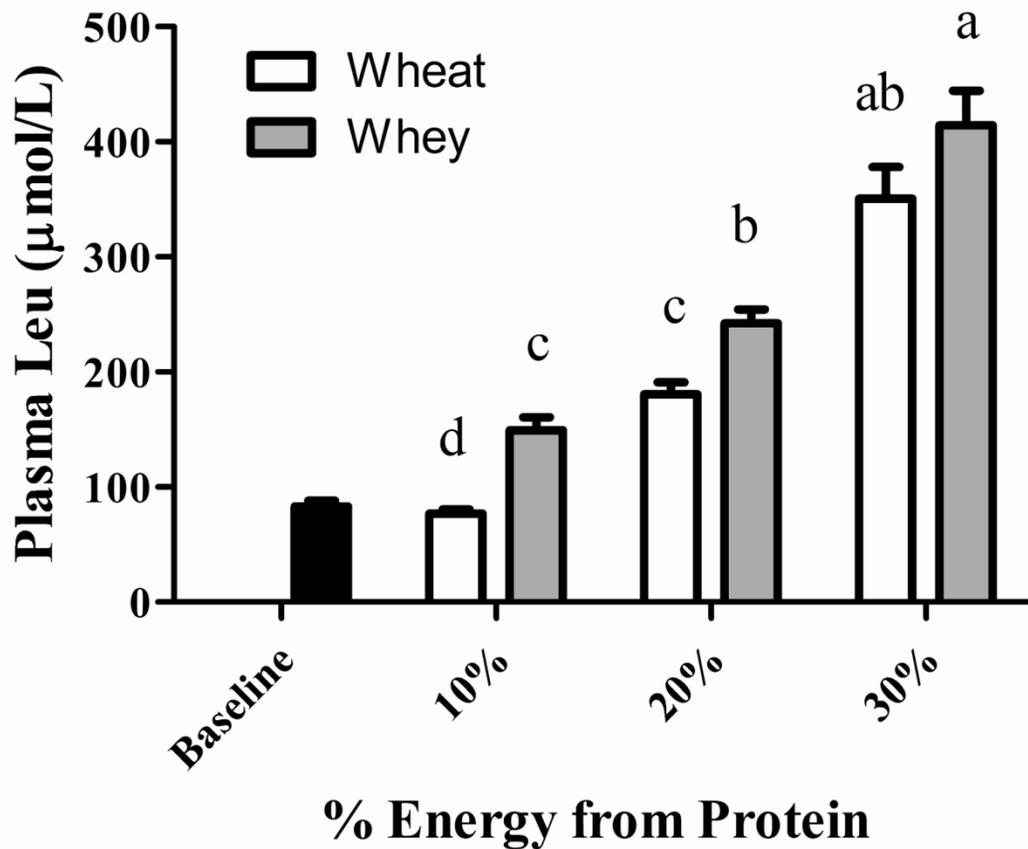
**Figure 2.1** Time course changes in the fractional rate of protein synthesis in gastrocnemius muscle of rats fed a 4g complete meal containing 20%, 50%, and 30% of energy from protein, carbohydrates and fats, respectively. Data are means  $\pm$  SEM; n = 5-6. Labeled means without a common letter differ,  $P < 0.05$ .



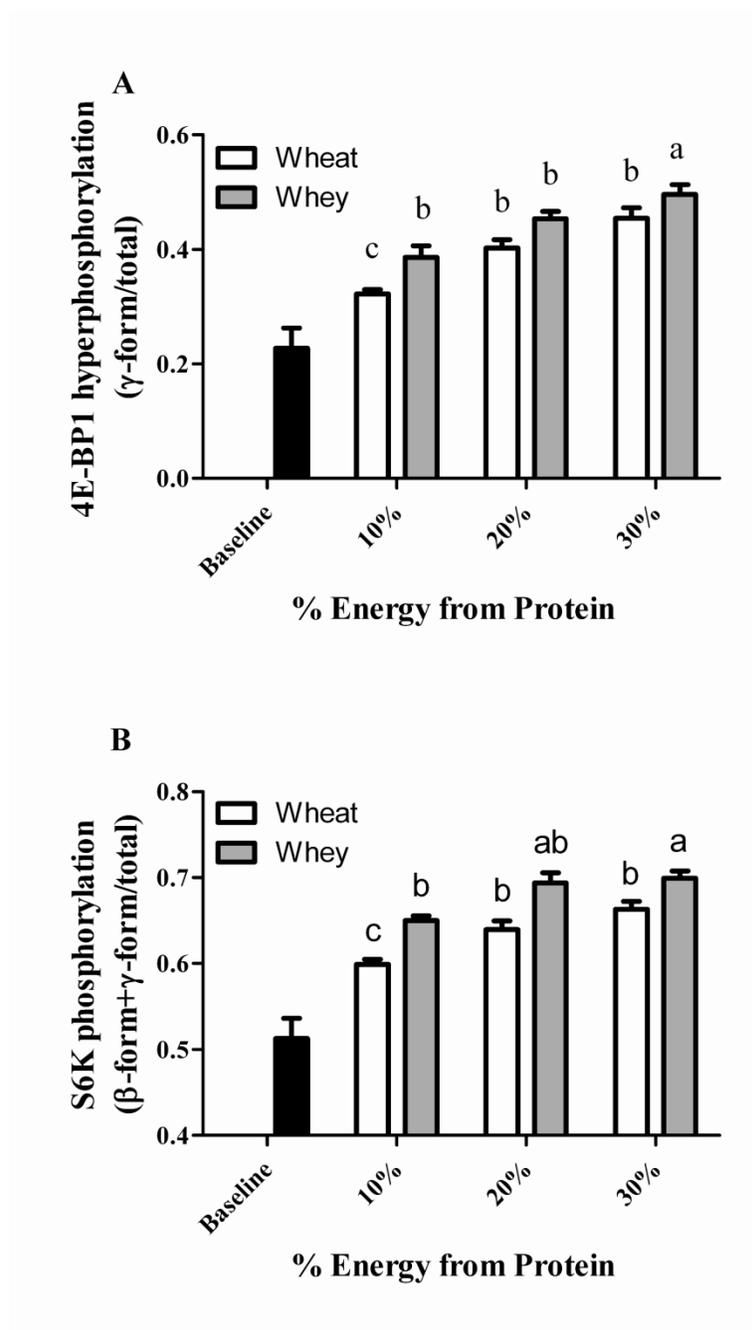
**Figure 2.2** Time course changes in plasma (A) and intramuscular (B) leucine concentrations of rats fed a 4g complete meal containing 20%, 50%, and 30% of energy from protein, carbohydrates and fats, respectively. Data are means  $\pm$  SEM; n = 5-6. Labeled means without a common letter differ, P<0.05.



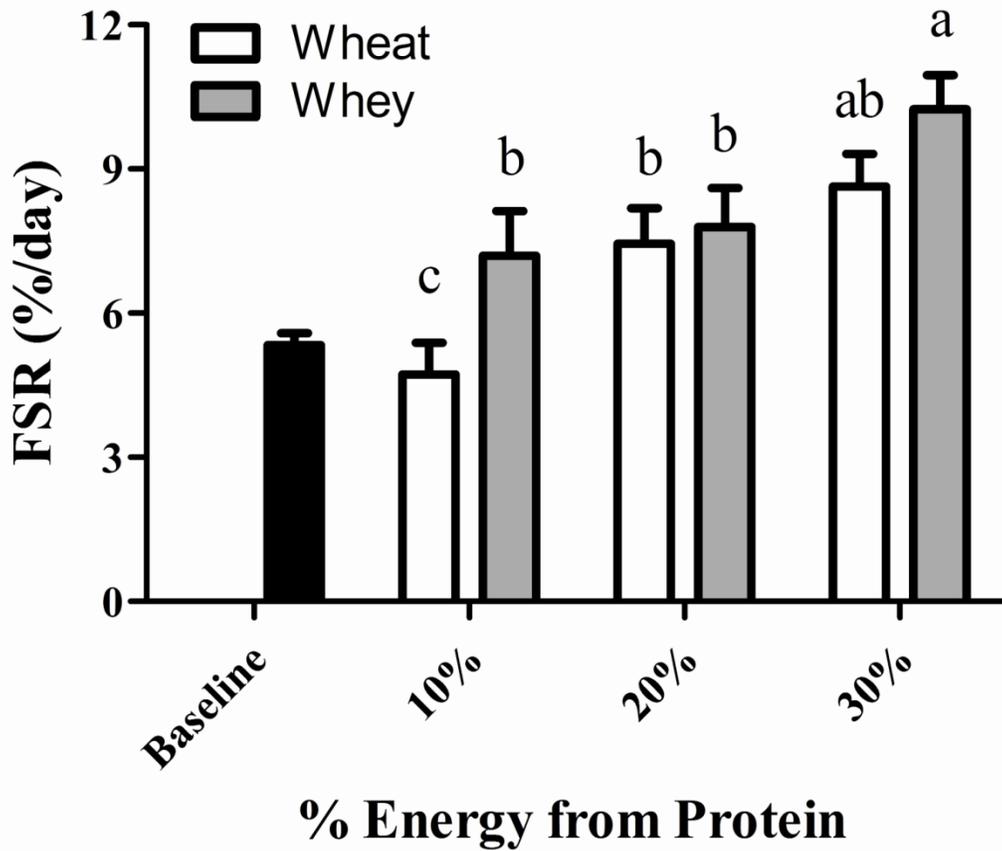
**Figure 2.3** Time course changes in the phosphorylation state of 4E-BP1 (A), p70S6K (B), and binding of eIF4G to eIF4E (C) in gastrocnemius muscle of rats fed a 4g complete meal containing 20%, 50%, and 30% of energy from protein, carbohydrates and fats, respectively. Data are means  $\pm$  SEM; n = 5-6. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 2.4** Plasma leucine concentrations of rats fed complete meals containing either wheat or whey at three different total protein contents (10,20, or 30% of energy). Data are means  $\pm$  SEM; n = 7-8. Labeled means without a common letter differ, P<0.05. All fed groups were different than baseline except 10% wheat.



**Figure 2.5** Phosphorylation states of 4E-BP1 (A) and p70S6K (B) in gastrocnemius muscle of rats fed a complete meal containing either wheat or whey (grey bars) at three different total protein contents (10,20, or 30% of energy). Data are means  $\pm$  SEM; n = 7-8. Labeled means without a common letter differ,  $P < 0.05$ . All fed groups were different than baseline.



**Fig. 2.6** Rates of protein synthesis in gastrocnemius muscle of rats fed a complete meal containing either or whey at three different total protein contents (10, 20, or 30% of energy). Data are means  $\pm$  SEM; n = 7-8. Labeled means without a common letter differ,  $P < 0.05$ . All fed groups were different than baseline except 10% wheat.

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## **CHAPTER 3: Isonitrogenous protein sources differentially stimulate skeletal muscle protein synthesis in adult rats fed complete meals based on their respective leucine contents**

### **Introduction**

Leucine (leu) is unique among amino acids as a modulator of the mTOR (mammalian target of rapamycin) signaling cascade and regulator of muscle protein synthesis (MPS). Numerous *in vivo* studies have demonstrated that oral or intravenous administration of free leu stimulates mTOR activation of translation initiation factors eIF4E-BP1 (4E-BP1), eIF4G, and ribosomal protein S6 kinase (S6K). Titration studies of leu regulation of MPS indicate that leu stimulation of mTOR is dependent on increases in intracellular leu concentrations and that this stimulation requires at least 2- to 3-fold increases in plasma leu above baseline (i.e. food deprived) amino acid values (1, 2). While the threshold response of mTOR and MPS to plasma leu concentrations is established from studies using free leu, the significance of this threshold activation in physiological complete meals is unknown.

Individual proteins differ in many characteristics including amino acid contents. Current measures of protein requirements are based on a daily intake of the minimum protein to maintain nitrogen balance and to prevent increases in plasma amino acids stimulating amino acid oxidation. These measurements focus on the lysine, methionine, and threonine contents of proteins as the most limiting of the indispensable amino acids. Leu is abundant in most proteins and is unlikely to be limiting based on nitrogen balance or amino acid oxidation measurements. However, our previous research demonstrated a clear dietary threshold for leu stimulation of MPS (2). To our knowledge, the significance of leu content of different proteins in complete meals on MPS has not been examined.

This study utilizes a meal-feeding protocol with adult rats to examine the importance of the leu content of a meal on postprandial changes in plasma leu, activation of S6K and 4E-BP1, and MPS. The test meal was designed to represent a small breakfast meal (20% of daily energy) with a complete mixture of macronutrients (16% of energy

from protein, 54% carbohydrates, 30% fats) and consumed after an overnight period of food deprivation (12 h). Four proteins (wheat gluten, soy isolate, egg white, and whey protein isolate) were selected to provide a range of indispensable amino acid concentrations with leu ranging from 6.8% to 10.9% (w/w). Based on studies with free leu, we predict that the postprandial MPS response will be determined by the leu content of the individual meals.

## Materials and Methods

### *Animals and diets.*

Male rats ( $250 \pm 12$ g) were purchased from Harlan-Teklad and maintained at 24°C with a 12-h light:dark cycle and free access to water. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

Rats were trained to consume 3 meals/d consisting of a 4 g meal consumed between 07:00 and 07:20 h followed by free access to food from 13:00 to 14:00 and 18:00 to 19:00 (3). Rats consumed ~17 g/d of total diet. After 2 d of adaptation to meal feeding, rats were randomized into treatment groups based on body weight. All diet treatments provided 16/54/30% of energy from protein, carbohydrates and fats, respectively with the treatments differing in source of protein: wheat (n=10), soy (n=10), egg (n=11), or whey protein (n=11) (**Table 3.1**). Wheat gluten diets were supplemented with lysine to exceed the minimum National Research Council (NRC) requirement (**Table 3.2**) and to equal the lysine content of whey protein (**Table 3.3**). A baseline food-deprived control group was also adapted to meal-feeding using whey protein (n=10). Rats were fed the respective diets for 14 d. On the experiment day rats were food deprived for 12 h and then fed their normal 4g breakfast meal. The food-deprived controls received no breakfast meal. The breakfast meals contained 0, 46, 54, 60, and 74 mg of leu, respectively, for the food-deprived controls, wheat, soy, egg, and whey groups. Rats were killed 90 min after consumption of the meal and blood and tissue samples collected.

### ***Determination of Muscle Protein Synthesis.***

Protein synthesis was measured in skeletal muscle using the flooding dose method (4). A 100% enriched L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine solution (150mmol/L; Cambridge Isotopes, Andover, MA) was administrated at 150 μmol/100 g body weight and injected via tail vein (1 mL/100 g body weight). After 10 min animals were killed by decapitation and hind limbs quickly removed and immersed in an ice-water mixture. Gastrocnemius, plantaris, and soleus muscles were removed from cooled hind limbs, frozen in liquid N<sub>2</sub> and stored at -80°C.

Frozen muscle tissue was powdered in liquid nitrogen, and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1mL per 50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by gas chromatography mass spectroscopy (GC-MS) as described previously (5, 6). The enrichment of L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies Santa Clara, CA). Samples were run under electron impact ionization and in splitless mode, and phenylethylamine ions at mass-to-charge ratio (m/z) 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

The muscle supernatant was used for determination of intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid phase extraction (SPE) using EZ:faast™ amino acid analysis sample testing kit (Phenomenex Inc. Torrance, CA, USA) and <sup>2</sup>H<sub>5</sub>-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at m/z 206 (m) and 211 (m+5) (7).

Fractional rates of protein synthesis (FSR) were determined from the rate of incorporation of L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine into total mixed muscle protein as described previously (8). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine incorporation. FSR, defined as the percentage of tissue protein renewed each day, were calculated according to the formula:  $FSR = (E_b \times 100) / (E_a \times t)$  where t is the time interval between injection and cooling of

sampled tissue expressed in days and  $E_b$  and  $E_a$  are the enrichments of [ $^2\text{H}_5$ ]-Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

### ***Plasma measurements.***

Plasma was obtained from trunk blood by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Linco Research, MO). Plasma glucose (Thermo Fisher Scientific, Middletown, VA) was determined by the glucose oxidase method. Plasma amino acid concentrations were analyzed by HPLC using a Waters 2475 Fluorescence detector (9).

### ***Phosphorylation of 4E-BP1, S6K, Akt, and Raptor.***

Muscle supernatants were subjected to protein immunoblot analysis as described previously (10, 11) using a rabbit polyclonal antibody to 4E-BP1 (Bethyl Labs, Montgomery, TX), a rabbit polyclonal S6K antibody (Bethyl Labs, Montgomery, TX), a rabbit polyclonal phospho-Akt antibody (Cell Signaling, Danvers, MA), a rabbit polyclonal Akt antibody (Cell Signaling, Danvers, MA), a rabbit polyclonal phospho-Raptor antibody (Cell Signaling, Danvers, MA), and a rabbit polyclonal Raptor antibody (Bethyl Labs, Montgomery, TX).

### **Statistical analysis.**

All data were analyzed by SPSS 15.0 software package for windows. A 1-way ANOVA was performed with the treatment group as the independent variable. When a significant overall effect was detected, differences among individual means were assessed using Fisher's LSD post hoc test. All data sets were tested for normal distribution and variance homogeneity using Levene's test. When variances were not homogeneous, means were compared using a Games-Howell test. The level of significance was set at  $P < 0.05$  for all statistical tests. All values are presented as means  $\pm$  SEM.

## Results

This experiment compared isonitrogenous, isoenergetic diets containing wheat, soy, egg, or whey as the protein source on the potential to stimulate translation initiation and MPS in skeletal muscle. After a small meal providing 20% of daily energy and containing 16% of energy as protein, plasma leu increased in animals fed egg and whey proteins but not in wheat and soy groups. The greatest concentration of plasma leu occurred in animals fed whey protein (**Table 3.4**). Similar postprandial patterns of plasma amino acids concentrations (i.e. whey > egg > soy > wheat) were observed for isoleucine and valine with whey producing the highest concentrations at 90 min after the meal (**Table 3.4**). Other plasma amino acids varied among the groups largely in proportion to the amino acid content of the protein source (Table 4). Lysine was different between animals fed soy and whey, with animals fed whey having the greatest concentration of postprandial lysine and animals fed soy having the lowest lysine levels. Plasma methionine concentrations were only increased in animals fed egg with the other groups not being different from food deprived controls (**Table 3.4**). Plasma threonine was increased by feeding egg and whey with peak values achieved in animals fed whey protein.

MPS increased in animals fed egg and whey but not in animals fed wheat and soy with peak values achieved in the whey group (**Figure 3.1**). This was consistent with the phosphorylation of the mTOR signaling targets 4E-BP1 (**Figure 3.2A**) and S6K (**Figure 3.2B**). Phosphorylation of S6K increased after egg and whey meals but not wheat and soy with peak values obtained in the whey protein group. Feeding increased 4E-BP1 phosphorylation in all groups with egg and whey greater than wheat or soy.

Akt activation was determined by phosphorylation of Akt at Ser473. Akt phosphorylation was greatest in animals fed soy compared with animals fed egg, whey, or the food deprived controls (**Figure 3.3A**). Animals fed wheat had intermediate levels of Akt activity and were not different from the other groups.

Phosphorylation of Raptor at Ser792 is a surrogate marker for AMP protein kinase (AMPK) activity and represents inhibition of mTOR (12, 13). Similar to Akt

activity, Raptor phosphorylation was greatest in animals fed soy compared to food deprived controls and animals fed egg or whey, with animals fed wheat having an intermediate level of Raptor phosphorylation compared with soy and the other groups (**Figure 3.3B**).

Plasma glucose and insulin were increased at 90 min after the meal in all groups except soy which was not different from food deprived controls (**Table 3.4**).

### **Discussion**

This experiment examined responses of MPS, translation initiation, and plasma amino acids after feeding nutritionally complete meals with protein sources selected for differences in leu content (wheat, soy, egg, and whey). The major finding of this work is that isonitrogenous, isoenergetic meals with different protein sources stimulate MPS and anabolic signaling in proportion to plasma leu concentrations which relate to the leu content of the respective protein. Previous research demonstrated that MPS and translation initiation respond in a dose dependent manner to administration of free leu (1). We recently demonstrated that a similar effect could be achieved using different amounts of wheat or whey proteins as part of isonitrogenous, isoenergetic complete meals (2). This experiment extends these findings and demonstrates that after a small breakfast meal with limited protein content, whey and egg proteins increased MPS whereas wheat and soy proteins did not. MPS appears to respond to postprandial changes in plasma leu concentrations and reflects a precise dietary threshold consistent with the leu content of the individual proteins. These findings support the theory of a threshold requirement for leu intake to stimulate MPS.

Consistent with plasma leu and MPS data, S6K phosphorylation increased in the groups fed egg and whey by 22% and 58%, respectively, whereas animals fed wheat and soy had no increase in S6K phosphorylation. Phosphorylation of 4E-BP1 increased after the meal in all groups with maximum phosphorylation of 4E-BP1 achieved in the egg and whey groups.

Other factors that may contribute to differences in the postprandial rise in plasma amino acids include the rate of gastric emptying and peptide digestion and absorption (14). Whey proteins which are highly soluble empty rapidly from the stomach compared with casein (15); so it is possible that wheat, soy, egg, and whey proteins may empty at different rates which could impact postprandial plasma leu and mTOR signaling responses. However, Bos et al. (14, 16) reported that plasma amino acids peaked 60-120 min postprandial in humans fed wheat or soy protein which is similar to the reported amino acids peak for whey protein in humans and rats (2, 15). Likewise, Moore et al. (17) demonstrated that plasma amino acid levels peaked at approximately 1 h after feeding egg albumin in humans after exercise. Consistent with these reports, postprandial plasma leu concentrations at 90 min in the present study are in proportion to the leu content of each protein.

Plasma insulin increased after feeding in all groups except soy potentially contributing to the anabolic effect of the meal. Previously, soy protein has been reported to reduce postprandial insulin concentrations but still increase Akt phosphorylation (18). These findings have been interpreted as increased insulin sensitivity with soy protein (16). However, in the present study downstream signaling for S6K activation or stimulation of MPS are reduced.

Failure of the Akt to stimulate downstream signals at S6K or 4E-BP1 may be due in part to limiting levels of leu to stimulate mTOR signaling or to stimulation of Raptor phosphorylation by AMPK, inhibiting mTOR. The leu content of soy is similar to egg (8.0% vs. 8.8%) however postprandial plasma leu is significantly greater after the egg vs. soy meals. This finding suggests a precise threshold for leu intake required to produce a rise in plasma leu after a meal.

Alternatively the lack of mTOR stimulation may be confounded by the isoflavone content of the soy protein which is reported to inhibit mTOR signaling via stimulation of AMPK (18). Increasing AMPK activity stimulates Raptor phosphorylation reported to inhibit mTOR signaling reducing the activity of S6K (19). Further, S6K is known to phosphorylate IRS-1 to provide feedback regulation of insulin signaling (20). In the

absence of downstream S6k activation, the elevated Akt phosphorylation in the soy group may reflect dysregulation of normal feedback from S6K.

Metabolic differences among proteins may also relate to ratios of other amino acids in the protein. For the proteins used in this study only the wheat gluten lysine content was below the NRC requirement for the adult rat. This specific requirement was corrected by supplementing the wheat gluten diet with lysine to the level of the concentration in the whey protein (Table 2). All other EAA exceeded NRC guidelines.

This study supports the hypothesis that protein distribution at individual meals is an important factor for evaluation of adult protein needs (21, 22). Current protein requirements establish a total daily intake but provide no guidance about distribution throughout the day. The leu content of a meal is important to regulation of MPS. These findings highlight that postprandial increases in plasma (or intracellular) leu after a complete meal serve as a nutrient signal for regulation of MPS (23, 24).

The unique regulatory role of leu may in part explain the inconsistencies in comparison of optimal needs for leu compared with current measures of minimum protein requirements for nitrogen balance or indispensable amino acid oxidation (IAAO) measurements. These standard measurements for protein requirement interpret postprandial increases in plasma amino acids as inefficient and excessive protein intake. While increased amino acid oxidation may reflect reduced efficiency of nitrogen retention of dietary protein and may provide an accurate representation of minimum requirements for an EAA such as lysine with no known metabolic signaling or regulatory roles, the extrapolation that protein intakes above the minimum amount for short-term nitrogen balance are physiologically meaningless is not consistent with the regulatory role of leu in MPS. Further support for this postulation is demonstrated by the close association of changes in plasma leu with rates of MPS and leu oxidation (2, 25).

## **Conclusions**

The current study demonstrates the relationship of the meal content of leu to postprandial increases in plasma leu and stimulation of MPS. These findings support the

hypothesis that meal distribution of dietary protein is important to adult protein utilization and highlights the need for longer-term studies of the potential impact of protein distribution on muscle function and body composition.

### **Acknowledgements**

Thanks to Gabe Wilson for his help with meal feeding and weighing animals during this experiment.

## Tables and Figures

**Table 3.1** Composition of animal diets

Component	Wheat Diet	Soy Diet	Egg Diet	Whey Diet
	<i>g/kg</i>			
Vital Wheat Gluten <sup>1</sup>	190.2	0.0	0.0	0.0
Soy Protein Isolate <sup>2</sup>	0.0	185.3	0.0	0.0
Egg White Solids <sup>3</sup>	0.0	0.0	195.6	0.0
Whey Protein Isolate <sup>4</sup>	0.0	0.0	0.0	188.8
L-Lysine <sup>5</sup>	10.1	0.0	0.0	0.0
Cornstarch	316.7	331.7	321.4	328.2
Maltodextrin	134.1	134.1	134.1	134.1
Sucrose	101.5	101.5	101.5	101.5
Soybean Oil	140.9	140.9	140.9	140.9
Cellulose (Fiber)	53.7	53.7	53.7	53.7
Mineral Mix <sup>6</sup>	37.6	37.6	37.6	37.6
Vitamin Mix <sup>6</sup>	10.7	10.7	10.7	10.7
Choline Bitautrate	2.7	2.7	2.7	2.7
Biotin <sup>7</sup> (mg/kg)	0.0	0.0	16.0	0.0

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein Isolate provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

<sup>5</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

<sup>6</sup>Mineral and Vitamin supplements (26) from Harlan-Teklad, Madison, WI.

<sup>7</sup>Egg White Solids supplemented with 16.0 mg biotin/kg diet.

**Table 3.2** Comparison of test diet amino acid compositions with NRC requirements<sup>1,2</sup>

Amino Acid	Wheat Diet	Soy Diet	Egg Diet	Whey Diet	NRC Requirement
	<i>g/kg diet</i>				
Phenylalanine/Tyrosine	11.5	17.0	16.8	10.7	1.9
Histidine	3.1	4.2	3.9	3.4	0.8
Isoleucine	5.1	8.1	9.0	10.5	3.1
Leucine	11.5	13.6	14.9	18.5	1.8
Lysine	15.4	10.7	11.0	15.4	1.1
Methionine/Cysteine	6.5	4.4	13.9	7.6	2.3
Threonine	4.4	6.5	7.6	10.9	1.8
Tryptophan	2.2	2.0	2.7	2.7	0.5
Valine	7.6	8.0	11.5	10.2	2.3

<sup>1</sup>Table 2-2 from Nutrient Requirements of Laboratory Animals Fourth Revised Edition (27).

<sup>2</sup>Values calculated for 300 g rat at maintenance.

**Table 3.3** Amino acid compositions of protein sources

Amino Acid	Vital Wheat Gluten <sup>1</sup>	Soy Protein Isolate <sup>2</sup>	Egg White Solids <sup>3</sup>	Whey Protein Isolate <sup>4</sup>
	<i>g/100g Protein</i>			
Alanine	3.1	4.0	6.1	4.9
Arginine	4.7	7.5	5.8	2.4
Aspartate	4.0	11.5	10.3	10.6
Cysteine	1.9	1.3	4.4	2.5
Glutamate/Glutamine	31.7	19.2	13.1	16.9
Glycine	3.8	4.1	3.5	1.8
Histidine	1.8	2.5	2.3	2.0
Isoleucine	3.0	4.8	5.3	6.2
Leucine	6.8	8.0	8.8	10.9
Lysine <sup>5</sup>	2.8 (+6.3)	6.3	6.5	9.1
Methionine	1.9	1.3	3.8	2.0
Phenylalanine	4.4	5.2	5.9	3.3
Proline	9.4	5.2	3.8	5.6
Serine	3.9	5.4	6.9	4.7
Threonine	2.6	3.8	4.5	6.4
Tryptophan	1.3	1.2	1.6	1.7
Tyrosine	2.4	4.8	4.0	3.0
Valine	4.5	4.7	6.8	6.0

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

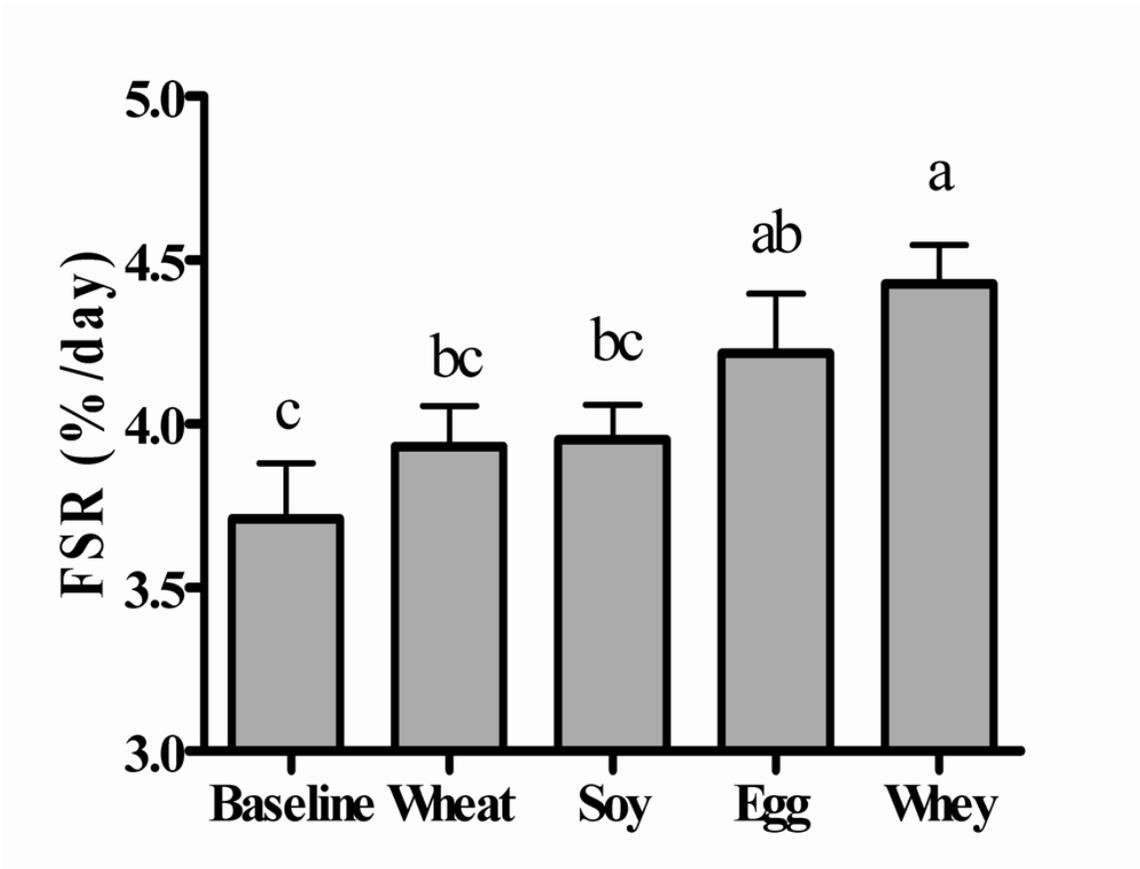
<sup>5</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

**Table 3.4** Selected plasma essential amino acid, insulin, and glucose concentrations 90 min after feeding complete meals containing wheat, soy, egg, or whey protein<sup>1</sup>

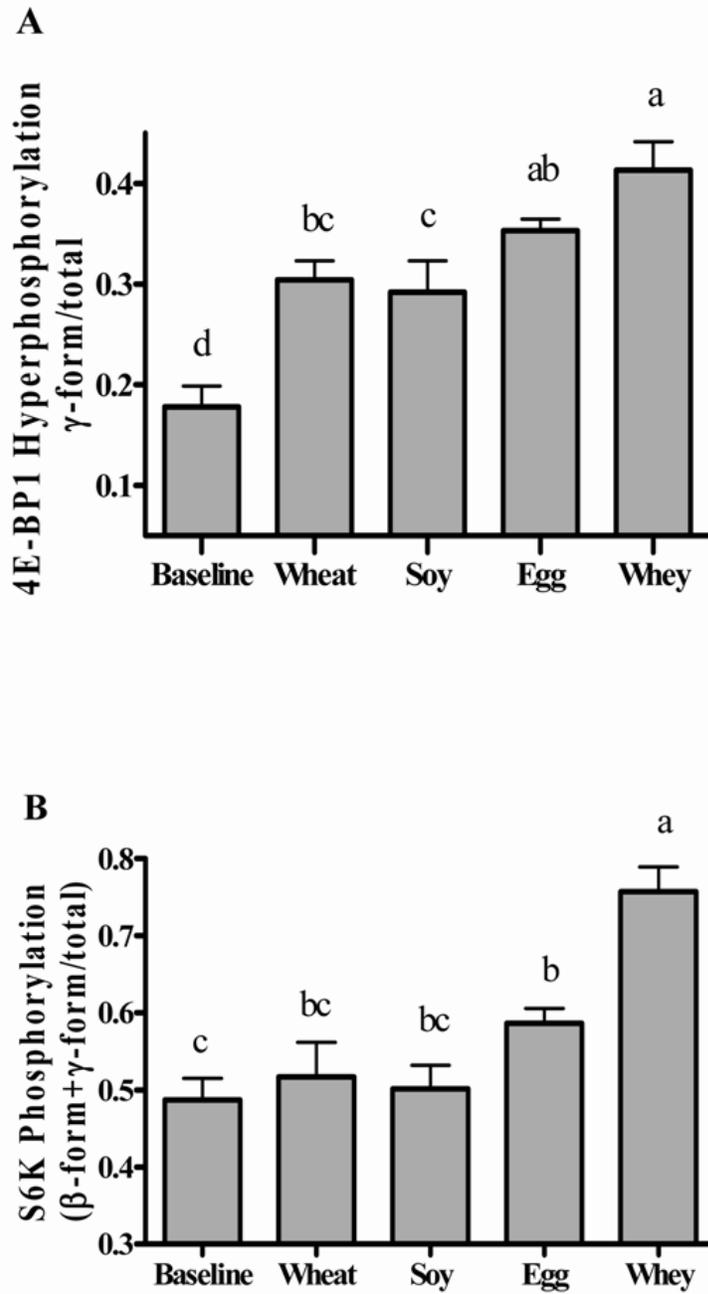
	Baseline <sup>2</sup>	Wheat	Soy	Egg	Whey
	<i>μmol/L</i>				
Leucine	84 ± 4.6 <sup>c</sup>	78 ± 4.3 <sup>c</sup>	84 ± 5.6 <sup>c</sup>	146 ± 8.4 <sup>b</sup>	192 ± 11.4 <sup>a</sup>
Isoleucine	56 ± 3.4 <sup>d</sup>	50 ± 2.9 <sup>d</sup>	74 ± 4.0 <sup>c</sup>	121 ± 6.3 <sup>b</sup>	144 ± 8.2 <sup>a</sup>
Valine	117 ± 8.2 <sup>cd</sup>	95 ± 5.2 <sup>d</sup>	143 ± 8.1 <sup>c</sup>	143 ± 14.2 <sup>a</sup>	248 ± 13.7 <sup>b</sup>
∑ BCAA	257 ± 16.0 <sup>bc</sup>	223 ± 12.1 <sup>c</sup>	301 ± 17.4 <sup>b</sup>	562 ± 28.5 <sup>a</sup>	584 ± 33.0 <sup>a</sup>
Lysine	510 ± 29.8 <sup>ab</sup>	527 ± 23.8 <sup>ab</sup>	419 ± 37.2 <sup>b</sup>	495 ± 35.6 <sup>ab</sup>	549 ± 29.3 <sup>a</sup>
Methionine	51 ± 2.9 <sup>bc</sup>	46 ± 2.8 <sup>bc</sup>	38 ± 4.1 <sup>c</sup>	86 ± 6.8 <sup>a</sup>	52 ± 5.2 <sup>b</sup>
Threonine	252 ± 13.7 <sup>c</sup>	269 ± 30.4 <sup>bc</sup>	349 ± 40.6 <sup>bc</sup>	357 ± 28.3 <sup>ab</sup>	538 ± 51.2 <sup>a</sup>
Insulin <i>pmol/L</i>	140 ± 10.5 <sup>b</sup>	178 ± 9.0 <sup>a</sup>	161 ± 11.0 <sup>ab</sup>	184 ± 12.0 <sup>a</sup>	170 ± 7.2 <sup>a</sup>
Glucose <i>mg/dl</i>	128 ± 15.4 <sup>b</sup>	190 ± 37.7 <sup>ab</sup>	116 ± 21.1 <sup>b</sup>	236 ± 33.6 <sup>a</sup>	171 ± 20.1 <sup>ab</sup>

<sup>1</sup>Values expressed as means ± SEM, n=8-10. Labeled means without a common letter differ (P<0.05).

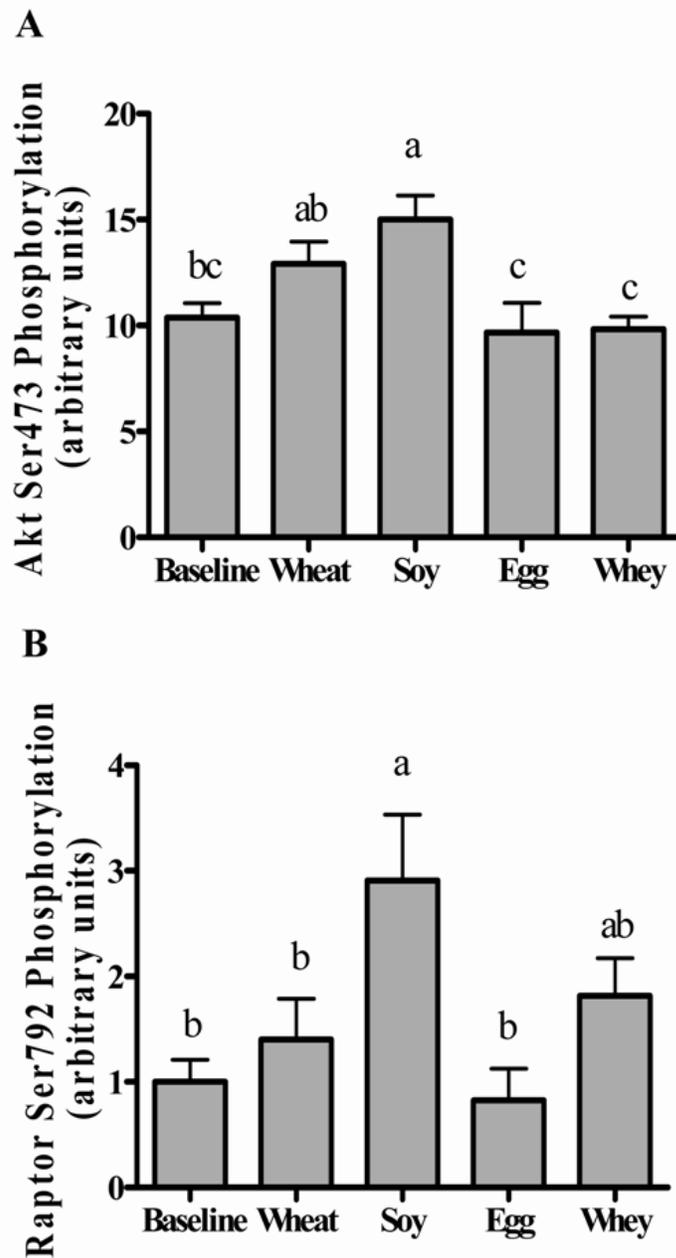
<sup>2</sup>12 h food-deprived control.



**Figure 3.1** Rates of protein synthesis in gastrocnemius muscle of rats fed a complete meal containing wheat, soy, egg, or whey proteins. Data are means  $\pm$  SEM; n = 9-10. Labeled means without a common letter differ, P<0.05.



**Figure 3.2** Phosphorylation states of 4E-BP1 (A) and p70S6K (B) in gastrocnemius muscle of rats fed a complete meal containing wheat, soy, egg, or whey proteins. Data are means  $\pm$  SEM; n = 9-10. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 3.3** Phosphorylation of Akt at Ser473 (A) and Raptor at Ser792 (B) in gastrocnemius muscle of rats fed a complete meal containing wheat, soy, egg, or whey proteins. Data are means  $\pm$  SEM; n = 9-10. Labeled means without a common letter differ,  $P < 0.05$ .

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## **CHAPTER 4: Leucine content of isonitrogenous and isoenergetic protein sources positively influences long term body composition and muscle mass in rats**

### **Introduction**

Maintenance of muscle mass requires a balance between the rates of protein synthesis and breakdown. Together the processes of synthesis and breakdown to called protein turnover and the balance ultimately determines muscle mass. Factors such as aging, disease, or energy restriction can decrease MPS and reduce muscle mass by disrupting this balance (1-5). Progressive loss of muscle mass over time can cause a host of problems including reduced mobility, quality of life, strength, and the ability to perform everyday tasks (6). It is therefore important to identify dietary factors which may positively influence protein synthesis and help maintain muscle mass. Leu has been proposed as a key amino acid regulator of MPS. Indeed, several studies have demonstrated leu's unique anabolic signaling effects on MPS using purified amino acid solutions (7, 8).

Our research group has previously demonstrated that the leu content of isonitrogenous meals predict the postprandial response of MPS (9). Interestingly, further research demonstrated that a specific threshold amount of leu at a meal is required to increase plasma leu and increase MPS. Previously we demonstrated that small breakfast meals containing wheat and soy with leu contents of 47 mg and 54 mg of leu, respectively, were insufficient to increase plasma leu or MPS, whereas isonitrogenous meals containing egg and whey with 60 mg and 74 mg of leu, respectively, significantly increased plasma leu, initiated mTOR signaling, and triggered MPS. This is in contrast to current recommendations that are based on methods of nitrogen balance, direct amino acid oxidation (DAAO), or indispensable amino acid oxidation (IAAO) which consider increases in plasma amino acids that result in increases of amino acid oxidation as excess and inefficient. Evidence now exists that increases in plasma amino acids are an essential part of the nutritional signal for skeletal muscle to recognize adequate dietary protein to initiate MPS.

While the experimental data in support of a dietary leu threshold are compelling their significance is unclear without long term changes in muscle mass or body composition. Postprandial changes in plasma amino acids, mTOR signaling, and MPS in feeding studies have only been shown in short-term studies and it is unclear if these effects are maintained long term and if they produce differences in muscle weights. It is also unclear if the daily distribution of protein (i.e. leu) makes a difference on these outcomes. If meal responses are important in determining body composition outcomes, then it would be logical to suggest subjects consuming sufficient protein to increase plasma leu and MPS multiple times per day would be superior to a single meal stimulation.

The purpose of this experiment was to determine if feeding isonitrogenous protein sources with different leu contents can differentially impact long term body composition and protein synthesis outcomes and if these outcomes are related to the meal responses of plasma leu and MPS.

## **Materials and Methods**

### ***Animals and diets.***

Male Sprague-Dawley rats ( $275 \pm 10$ g; Harlan-Teklad) will be maintained at 24°C with a 12-h light:dark cycle and free access to water. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

Rats were trained to consume 3 meals/d consisting of a 4 g meal consumed between 07:00 and 07:20 followed by free access to food from 13:00 to 14:00 and 18:00 to 19:00 for 6 days. Rats consumed  $\sim 18$  g/d of total diet. On day 7, rats were randomized into the following diet groups (n=21-25): wheat, soy, egg, and whey and food-deprived controls (**Table 4.1 and Table 4.2**). Wheat protein was supplemented with lysine to meet NRC requirements and approximate whey lysine levels (**Table 4.2 and Table 4.3**). The diets were fed at 80% of ad libitum (14 g/day) intake to produce changes in body composition. In order to study meal responses and control for between animal differences in food intake, rats were fed defined amounts at each meal: a small (4 g) first meal consumed between 07:00 and 07:20 followed by a 4 g meal from 13:00 to

14:00 and an 6 g meal from 18:00 to 19:00, representing a meal pattern designed to mimic human eating behavior (10). These meals were designed to only increase plasma leu & MPS 1x/day for animals fed wheat and soy, but 3x/day for animals fed egg and whey based on data from Chapter 3 (summarized in **Table 4.4**). This was designed to test the theory the multiple stimulations of MPS are superior to a single stimulation. Each of the diets provided 16% of energy from protein. Total daily protein, carbohydrate, and lipid intakes were the same for all groups (**Table 4.3**).

After 2 wk of meal feeding treatment diets approximately half of the rats in each treatment group were set aside for determination of body composition by DXA (Dual energy X-ray absorptiometry). These animals were then allowed to recover and continue meal feeding for the remainder of the study.

Also at 2 wk, the remaining animals in each group that did not undergo DXA were used for measurement of MPS. Animals were food deprived for 12 h and then MPS determined before the first meal (i.e. food-deprived group) or 90 min after completion of the normal breakfast meal. The meals contained approximately 46, 54, 60, and 74 mg leu in the wheat, soy, egg, and whey groups respectively (**Table 4.4**). 90 min after consumption of the meal rats were killed and blood and tissues harvested. Tissues weighed and collected included the gastrocnemius, soleus, and plantaris muscles, and liver. The animals used for DXA at 2 wk continued on the diet treatments for 9 more weeks and then underwent DXA and were sacrificed at wk 11 for the same measurements as the 2 wk time point.

#### ***Determination of Muscle Protein Synthesis.***

Protein synthesis was measured in skeletal muscle 90 minutes after the breakfast meals at 2 wk and 11 wk using the flooding dose method (11). A 100% enriched L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine solution (150mmol/L; Cambridge Isotopes, Andover, MA) was administered at 150 μmol/100 g body weight and injected via tail vein (1 mL/100g body weight) 80 minutes after completion of the meals. 10 min later (90 minutes post meal) animals were killed by decapitation and hind limbs quickly removed and immersed in an

ice-water mixture. Gastrocnemius, plantaris, and soleus muscles were removed from cooled hind limbs, frozen in liquid N<sub>2</sub> and stored at -80°C.

Frozen muscle tissue was powdered in liquid nitrogen, and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1mL per 50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by gas chromatography mass spectroscopy as described previously (12, 13). The enrichment of L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies Santa Clara, CA). Samples were run under electron impact ionization and in splitless mode, and phenylethylamine ions at mass-to-charge ratio (m/z) 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

The muscle supernatant was used for determination of intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid phase extraction (SPE) using EZ:faast™ amino acid analysis sample testing kit (Phenomenex Inc. Torrance, CA, USA) and <sup>2</sup>H<sub>5</sub>-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at m/z 206 (m) and 211 (m+5) (14).

Fractional rates of protein synthesis (FSR) were determined from the rate of incorporation of L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine into total mixed muscle protein as described previously (15). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine incorporation. FSR, defined as the percentage of tissue protein renewed each day, were calculated according to the formula:  $FSR = (E_b \times 100) / (E_a \times t)$  where t is the time interval between injection and cooling of sampled tissue expressed in days and E<sub>b</sub> and E<sub>a</sub> are the enrichments of [<sup>2</sup>H<sub>5</sub>]Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

### ***Plasma measurements.***

Plasma was obtained from trunk blood at 2 wk and 11 wk by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were analyzed using a

commercial RIA kit for rat insulin (Linco Research, MO). Plasma amino acid concentrations were analyzed by HPLC using a Waters 2475 Fluorescence detector (16).

***Phosphorylation of 4E-BP1, S6K, Akt, and Raptor.***

Muscle supernatants were subjected to protein immunoblot analysis as described previously (8, 17) using a rabbit polyclonal antibody to 4E-BP1 (Bethyl Labs, Montgomery, TX), a rabbit polyclonal S6K antibody (Bethyl Labs, Montgomery, TX), a rabbit polyclonal phospho-Akt antibody (Cell Signaling, Danvers, MA), a rabbit polyclonal Akt antibody (Cell Signaling, Danvers, MA), a rabbit polyclonal phospho-Raptor antibody (Cell Signaling, Danvers, MA), and a rabbit polyclonal Raptor antibody (Bethyl Labs, Montgomery, TX).

***Body Composition.***

Prior to the administration of the diet treatments and 2 and 11 weeks after the diet treatments began, body composition was assessed using dual energy X-ray absorptiometry (DXA) analysis. Rats received an anesthetic by intraperitoneal injection of a 0.3mg/kg dexmedetomidine (commercial name Dexdomitor) for sedation during testing. Before testing, the DXA (Hologic QDR 4500A, software version 11.1:3) instrument was be calibrated as per the manufacturer's guidelines. Rats were then transferred to the DXA and whole body (WB) scans were performed. All scans for a given rat were be performed on the same day and analyzed by the same research technician.

***Statistical analysis.***

All data were analyzed by SPSS 15.0 software package for windows. A 1-way ANOVA was performed with the treatment group as the independent variable. When a significant overall effect was detected, differences among individual means were assessed using Fisher's LSD post hoc test. All data sets were tested for normal distribution and variance homogeneity using Levene's test. When variances were not homogeneous, means were compared using a Games-Howell test. Correlations were

determined by linear regression (Pearson's correlation). The level of significance was set at  $P < 0.05$  for all statistical tests. Values are presented as means  $\pm$  SEM.

## Results

This experiment compared long term feeding of isonitrogenous, isoenergetic diets containing wheat, soy, egg, or whey as the protein source on the potential to produce body composition and skeletal muscle mass differences based on postprandial MPS outcomes. Animal weights between groups were the same at the beginning of the experiment; however after 6 days of feeding the respective diets the soy group weighed significantly less than the other groups and remained significantly lighter for the duration of the experiment (**Figure 4.1**). At 23 days of feeding the wheat group was significantly heavier than all the other groups and remained heavier for the duration of the experiment. Egg and whey groups were intermediate between soy and wheat groups for the entire experiment and their weights did not differ.

Gastrocnemius weights were greater in animals fed egg and whey compared to animals fed wheat at 2 weeks (**Figure 4.2**). At 11 weeks, the gastrocnemius muscles were larger in animals fed egg and whey with animals fed soy having the smallest gastrocnemius muscles. Animals fed whey also had significantly larger gastrocnemius muscles compared to animals fed wheat at 11 weeks.

Animals fed wheat protein had significantly greater percentage of body fat than animals fed egg and whey at 2 weeks (**Figure 4.3**). At 11 weeks these results were maintained with the soy group also having significantly lower body fat levels than animals fed wheat protein.

Consistent with body weight, body fat, and muscle weight data, animals fed soy had significantly less lean body mass than animals fed wheat, egg, or whey at 2 weeks and 11 weeks with no differences in lean body mass between wheat, egg, and whey groups at either time point (**Figure 4.4**).

At 2 weeks, after a breakfast meal providing ~30% of daily energy and containing 16% of energy as protein, plasma leu increased in animals fed egg and whey proteins but

not in wheat and soy groups. The greatest concentration of plasma leu occurred in animals fed whey and egg protein (**Table 4.6**). Similar postprandial patterns of plasma amino acids concentrations (i.e. whey > egg > soy > wheat) were observed for isoleucine and valine with whey producing the highest concentrations at 90 min after the meal (**Table 4.6**). Other plasma amino acids varied among the groups largely in proportion to the amino acid content of the protein source (**Table 4.6**). Lysine was different between animals fed soy and whey, with animals fed whey having the greatest concentration of postprandial lysine and animals fed soy having the lowest lysine levels. Plasma methionine concentrations were only increased in animals fed egg with the other groups not being different from food deprived controls (**Table 4.6**). Plasma threonine was only increased by feeding whey with the other groups being no different from baseline. At 11 weeks this meal feeding and measurements were repeated with very similar results (**Table 4.7**). No essential amino acid was significantly below baseline post-prandially at 2 or 11 weeks.

Postprandial MPS was significantly greater in animals fed egg and whey than animals fed wheat and soy (**Figure 4.5**) at both the 2 and 11 week time points with no significant difference between the egg and whey groups. This was consistent with the post-prandial phosphorylation of the mTOR signaling targets 4E-BP1 (**Figure 4.6A**) and S6K (**Figure 4.6B**) at 2 weeks. Phosphorylation of S6K increased after egg and whey meals but not wheat and soy with peak values obtained in the whey protein group. Feeding increased 4E-BP1 phosphorylation in all groups with egg and whey greater than wheat or soy.

Akt activation was determined by phosphorylation of Akt at Ser473. Post-prandial Akt phosphorylation was significantly greater in animals fed soy compared to all other groups and food deprived controls at 2 weeks (**Figure 4.7A**).

Phosphorylation of Raptor at Ser792 is a surrogate marker for AMPK activity and represents inhibition of mTOR (18, 19). Similar to Akt activity, post-prandial Raptor phosphorylation was significantly increased in animals fed soy compared to all other groups and food deprived controls at 2 weeks (**Figure 4.7B**).

Post-prandial insulin concentrations were not different between any groups or different from baseline at 2 weeks (data not shown). At 11 weeks however, animals fed wheat had significantly greater post-prandial insulin increases at 90 minutes after feeding than animals fed egg and whey (**Figure 4.8**). Animals fed egg and whey exhibited a reduction in insulin. Soy insulin concentrations were unchanged 90 minutes after feeding and not different from any other group.

### **Discussion**

This experiment examined the potential of long term meal feeding of isonitrogenous, isoenergetic meals that contained different protein sources to alter muscle weight and body composition in adult rats. The major finding of this work was that meals containing different proteins that are considered nutritionally adequate can produce differential effects on MPS and ultimately different muscle weights in relation to the leu content of the protein. Previous research has demonstrated that MPS and translation initiation responds in a dose dependent manner to administration of free leu (20). We recently demonstrated that a similar effect could be achieved using whole proteins (wheat, soy, egg, and whey) as a part of complete meals. That study showed that meals containing 47 mg or 54 mg leu from wheat and soy respectively were unable to increase plasma leu or MPS in adult rats, while isonitrogenous meals containing 60 mg or 74 mg of leu from egg or whey proteins, respectively, increased plasma leu and MPS. These data suggested that a narrow and highly responsive threshold exists for the relationship of plasma leu serving as a trigger for initiation of MPS. This experiment extends these findings by demonstrating that the post prandial MPS responses to meals containing sufficient leu to reach this threshold are maintained for at least 11 wks and these meal responses produce differences in muscle mass and body composition. To our knowledge, no previous studies have demonstrated the leu thresholds at meals is important to adult muscle mass and body composition.

The differences in gastrocnemius muscle weights were consistent with post-prandial MPS and translation initiation factors. Animals with the largest gastrocnemius

muscles had greater rates of postprandial MPS and increased mTOR signaling. These metabolic responses were associated with increases in post-prandial plasma leu and the leu content of the respective diets. Animals consuming the small meals containing egg and whey proteins with greater leu density received sufficient dietary leu to reach the threshold required to increase plasma leu and trigger metabolic responses. The mTOR targets S6K and 4E-BP1 were both correlated with MPS ( $r=0.482$  and  $r=0.452$ ,  $P<0.05$ ) at 2 weeks. Corresponding to this, MPS increased only in animals fed egg and whey. Likewise, plasma leu correlated with post-prandial increases in MPS at 2 ( $r=0.589$ ,  $P<0.01$ ) and 11 weeks ( $r=0.693$ ,  $P<0.01$ ). These short term post-meal anabolic responses produced greater gastrocnemius muscle weights based on the leu content of the meals which were correlated with muscle weights at 2 ( $r=0.451$ ,  $P<0.01$ ) and 11 weeks ( $r=0.400$ ,  $P=0.01$ ). Not surprisingly, the postprandial MPS changes were correlated with gastrocnemius weights at 11 weeks ( $r=0.471$ ,  $P<0.01$ ). This demonstrates that short term rises in postprandial leu are not only crucial for increasing MPS, but for long term optimization of muscle mass.

In addition to muscle weights, lean body mass and body fat related to the protein quality of the meals. Leu content of the diet as well as post-prandial plasma leu, mTOR signaling, and MPS were associated with differences in lean body mass and body fat. Post-prandial MPS was correlated with lean body mass at 2 ( $r=0.551$ ,  $P<0.001$ ) and 11 weeks ( $r=0.491$ ,  $P<0.01$ ). Furthermore, lean body mass was also correlated with increases in plasma leu at 11 weeks ( $r=0.425$ ,  $P<0.01$ ) with a trend for significance at 2 weeks ( $r=0.322$ ,  $P=0.08$ ). In contrast, plasma leu was negatively correlated with body fat at 2 weeks ( $r=-0.417$ ,  $P<0.05$ ) and 11 weeks ( $r=-0.542$ ,  $P<0.01$ ). These data support the hypothesis that increases in post-prandial plasma leu and MPS are critical for maintaining optimal LBM. Additionally, these data suggest that increasing MPS may positively impact body fat. These findings are consistent with the report by She et al. who demonstrated that chronic elevations in protein turnover inhibited diet induced obesity in a BCAT knockout model (21). It appears that increasing plasma leu and MPS increases energy expenditure and positively influences body composition. She et al (21) suggested

that protein turnover was a futile cycle that may explain the increased thermogenesis associated with higher protein diets.

Consistent with the findings in Chapter 3, this study also found that soy protein increased Raptor and Akt phosphorylation but failed to stimulate S6K or 4E-BP1 phosphorylation. Although the leu content of the breakfast & lunch meals containing soy do not reach the threshold required to increase plasma leu and MPS, this is compounded by the isoflavones content of soy, which has been shown previously to increase AMPK activity leading to inhibition of mTOR signaling (22). Increased phosphorylation of Raptor may explain why increased Akt activity in animals fed soy is unable to increase mTOR signaling. Further, S6K is known to phosphorylate IRS-1 to provide feedback regulation of insulin signaling (23). In the absence of downstream S6k activation, the elevated Akt phosphorylation in the soy group may reflect dysregulation of normal feedback from S6K. Interestingly, both Akt and Raptor phosphorylation were negatively correlated with lean body mass ( $r=-0.733$  and  $r=-0.410$ ,  $P<0.05$ ). This is consistent with the soy animals having the lowest bodyweights, lean body mass, and gastrocnemius weights. It is likely that reduced anabolic signaling from low leu coupled with the effects of soy isoflavones may account for the reduced mass of animals fed soy. Furthermore, activation of AMPK may at least partially explain why animals fed soy had lower percentage of bodyfat compared to animals fed wheat as both groups failed to increase plasma leu and MPS after the breakfast meal as activation of AMPK by soy protein has been shown to inhibit fat gain in mice (24).

Insulin concentrations, while not different between any group and food deprived controls at 2 weeks, did show interesting differences at 11 weeks. Only animals fed wheat had increased levels of plasma insulin at 90 minutes post-prandial while levels of egg and whey dropped by 88 and 149 pmol/L respectively with soy remaining unchanged from food-deprived levels. A possible explanation for these differences could be that the 90 minute post-prandial measurement does not represent the peak of insulin response, but rather could be a point at which insulin levels are falling. This may suggest that animals fed wheat had impaired clearance of insulin or prolonged duration of insulin release with

better clearance or shorter duration release of insulin in animals fed egg and whey. Soy was not different from baseline food deprived controls which we have observed our prior experiment and may be related to the isoflavones content of the soy preventing increases in insulin concentrations (25). These data suggest that feeding different isonitrogenous diets with different protein sources may potentially differentially affect insulin and glucose metabolism.

This study supports the hypothesis that protein distribution at individual meals is an important factor for evaluation of adult protein needs (26, 27). Current protein requirements establish a total daily intake but provide no guidance about distribution throughout the day. The current study was designed to include 2 protein sources (wheat and soy) that would not reach the leu threshold at the breakfast and lunch meals to increase plasma leu and MPS and only likely receive one postprandial increase in plasma leu and MPS per day at the dinner meal. In contrast to that, this study also included 2 protein sources (egg and whey) that would contain sufficient leu at every meal to increase plasma leu and MPS. This experiment demonstrates that increases in plasma (or intracellular) leu after meals serve as a nutrient signal for regulation of MPS (28, 29) and multiple stimulations per day are superior to a single response for optimizing body composition and muscle mass. The leu content and distribution of meals is important to regulation of MPS and these findings highlight that postprandial and that these meal responses are critical for body composition and muscle mass outcomes.

The unique regulatory role of leu may in part explain the inconsistencies in comparison of optimal needs for leu compared with current measures of minimum protein requirements for nitrogen balance or indispensable amino acid oxidation (IAAO) measurements. These standard measurements for protein requirement interpret postprandial increases in plasma amino acids as inefficient and excessive protein intake. While increased amino acid oxidation may reflect reduced efficiency of nitrogen retention of dietary protein and may provide an accurate representation of minimum requirements for an EAA such as lysine with no known metabolic signaling or regulatory roles, the extrapolation that protein intakes above the minimum amount for short-term

nitrogen balance are physiologically meaningless is not consistent with the regulatory role of leu in MPS. Further support for this postulation is demonstrated by the close association of changes in plasma leu with rates of MPS and leu oxidation (9, 30).

### **Conclusions**

The current study demonstrates the relationship of the meal content of leu to postprandial increases in plasma leu and MPS and demonstrates that these short term postprandial responses relate to long term body composition and muscle mass outcomes. These findings support the hypothesis that meal content and distribution of dietary protein and leu is important to adult protein utilization and highlights the ineffectiveness of current measures of protein quality for assessment of optimal muscle health.

### **Acknowledgements**

Thanks to Gabe Wilson, Chris Moulton, and Adam Schiltz for their help with meal feeding and weighing animals during this experiment.

## Tables and Figures

**Table 4.1** Composition of animal diets

Component	Wheat Diet	Soy Diet	Egg Diet	Whey Diet
	<i>g/kg</i>			
Vital Wheat Gluten <sup>1</sup>	190.2	0.0	0.0	0.0
Soy Protein Isolate <sup>2</sup>	0.0	185.3	0.0	0.0
Egg White Solids <sup>3</sup>	0.0	0.0	195.6	0.0
Whey Protein Isolate <sup>4</sup>	0.0	0.0	0.0	188.8
L-Lysine <sup>5</sup>	10.1	0.0	0.0	0.0
Cornstarch	316.7	331.7	321.4	328.2
Maltodextrin	134.1	134.1	134.1	134.1
Sucrose	101.5	101.5	101.5	101.5
Soybean Oil	140.9	140.9	140.9	140.9
Cellulose (Fiber)	53.7	53.7	53.7	53.7
Mineral Mix <sup>6</sup>	37.6	37.6	37.6	37.6
Vitamin Mix <sup>6</sup>	10.7	10.7	10.7	10.7
Choline Bitaurate	2.7	2.7	2.7	2.7
Biotin <sup>7</sup> (mg/kg)	0.0	0.0	16.0	0.0

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein Isolate provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

<sup>5</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

<sup>6</sup>Mineral and Vitamin supplements (31) from Harlan-Teklad, Madison, WI.

<sup>7</sup>Egg White Solids supplemented with 16.0 mg biotin/kg diet.

**Table 4.2** Comparison of test diet amino acid compositions with NRC requirements<sup>1,2</sup>

Amino Acid	Wheat Diet	Soy Diet	Egg Diet	Whey Diet	NRC Requirement
	<i>g/kg diet</i>				
Phenylalanine/Tyrosine	11.5	17.0	16.8	10.7	1.9
Histidine	3.1	4.2	3.9	3.4	0.8
Isoleucine	5.1	8.1	9.0	10.5	3.1
Leucine	11.5	13.6	14.9	18.5	1.8
Lysine	15.4	10.7	11.0	15.4	1.1
Methionine/Cysteine	6.5	4.4	13.9	7.6	2.3
Threonine	4.4	6.5	7.6	10.9	1.8
Tryptophan	2.2	2.0	2.7	2.7	0.5
Valine	7.6	8.0	11.5	10.2	2.3

<sup>1</sup>Table 2-2 from Nutrient Requirements of Laboratory Animals Fourth Revised Edition (32).

<sup>2</sup>Values calculated for 300 g rat at maintenance.

**Table 4.3** Amino acid compositions of protein sources

Amino Acid	Vital Wheat Gluten <sup>1</sup>	Soy Protein Isolate <sup>2</sup>	Egg White Solids <sup>3</sup>	Whey Protein Isolate <sup>4</sup>
	<i>g/100g Protein</i>			
Alanine	3.1	4.0	6.1	4.9
Arginine	4.7	7.5	5.8	2.4
Aspartate	4.0	11.5	10.3	10.6
Cysteine	1.9	1.3	4.4	2.5
Glutamate/Glutamine	31.7	19.2	13.1	16.9
Glycine	3.8	4.1	3.5	1.8
Histidine	1.8	2.5	2.3	2.0
Isoleucine	3.0	4.8	5.3	6.2
Leucine	6.8	8.0	8.8	10.9
Lysine <sup>5</sup>	2.8 (+6.3)	6.3	6.5	9.1
Methionine	1.9	1.3	3.8	2.0
Phenylalanine	4.4	5.2	5.9	3.3
Proline	9.4	5.2	3.8	5.6
Serine	3.9	5.4	6.9	4.7
Threonine	2.6	3.8	4.5	6.4
Tryptophan	1.3	1.2	1.6	1.7
Tyrosine	2.4	4.8	4.0	3.0
Valine	4.5	4.7	6.8	6.0

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

<sup>5</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

**Table 4.4** Protein distribution

	Meal	Breakfast	Lunch	Dinner	Total intake
Diet Group	<i>Protein intake (g)</i>				
Wheat <sup>1</sup>		0.68	0.68	1.02	2.38
Soy <sup>2</sup>		0.68	0.68	1.02	2.38
Egg <sup>3</sup>		0.68	0.68	1.02	2.38
Whey <sup>4</sup>		0.68	0.68	1.02	2.38

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

**Table 4.5** Leucine distribution

	Meal	Breakfast	Lunch	Dinner	Total intake
Diet Group		<i>Leucine intake (mg)</i>			
Wheat <sup>1</sup>		46	46	69	161
Soy <sup>2</sup>		54	54	76	184
Egg <sup>3</sup>		60	60	90	210
Whey <sup>4</sup>		74	74	111	259

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

**Table 4.6** Postprandial Plasma amino acid concentrations at 2 weeks<sup>1</sup>

	Baseline <sup>2</sup>	Wheat	Soy	Egg	Whey
	<i>μmol/L</i>				
Leucine	91 ± 4.4 <sup>b</sup>	78 ± 8.1 <sup>b</sup>	78 ± 7.6 <sup>b</sup>	130 ± 18.5 <sup>ab</sup>	146 ± 28.4 <sup>a</sup>
Isoleucine	74 ± 6.5 <sup>b</sup>	65 ± 4.3 <sup>b</sup>	74 ± 7.1 <sup>b</sup>	117 ± 15.0 <sup>ab</sup>	121 ± 24.0 <sup>a</sup>
Valine	130 ± 3.4 <sup>b</sup>	97 ± 10.2 <sup>bc</sup>	118 ± 12.1 <sup>b</sup>	251 ± 32.9 <sup>a</sup>	182 ± 49.0 <sup>ab</sup>
∑ BCAA	295 ± 12.0 <sup>b</sup>	245 ± 16.3 <sup>c</sup>	270 ± 26.5 <sup>bc</sup>	498 ± 66.3 <sup>a</sup>	457 ± 96.9 <sup>a</sup>
Lysine	543 ± 15.3 <sup>ab</sup>	549 ± 79.9 <sup>ab</sup>	435 ± 62.1 <sup>b</sup>	492 ± 64.6 <sup>ab</sup>	626 ± 46.2 <sup>a</sup>
Methionine	53 ± 2.6 <sup>ab</sup>	41 ± 4.8 <sup>bc</sup>	38 ± 2.1 <sup>bc</sup>	83 ± 12.0 <sup>a</sup>	60 ± 19.4 <sup>ab</sup>
Threonine	389 ± 54.2 <sup>b</sup>	452 ± 17.5 <sup>ab</sup>	518 ± 67.0 <sup>ab</sup>	537 ± 73.2 <sup>ab</sup>	860 ± 209.2 <sup>a</sup>

<sup>1</sup>Values expressed as means ± SEM, n=5-8. Labeled means without a common letter differ (P<0.05).

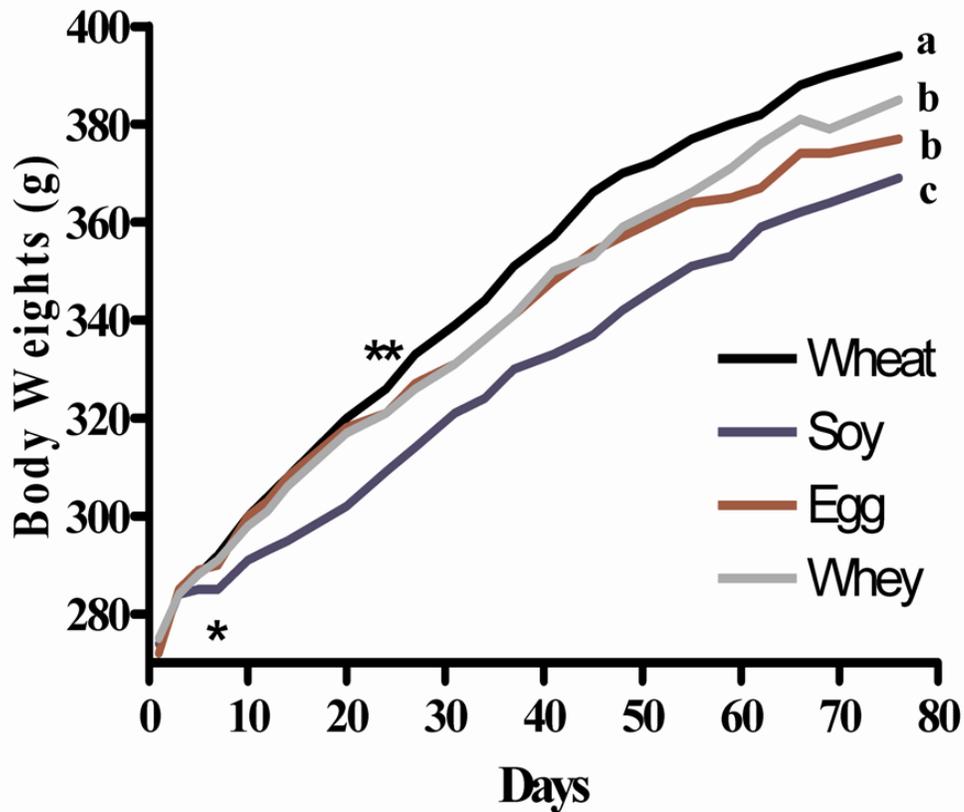
<sup>2</sup>12 hr food-deprived control.

**Table 4.7** Plasma amino acid concentrations at 11 weeks<sup>1</sup>

	Wheat		Soy		Egg		Whey	
	FD <sup>2</sup>	Fed	FD	Fed	FD	Fed	FD	Fed
Leucine	84 ± 5.9 <sup>bc</sup>	69 ± 3.8 <sup>bc</sup>	78 ± 4.4 <sup>bc</sup>	78 ± 3.7 <sup>bc</sup>	77 ± 3.8 <sup>bc</sup>	135 ± 2.7 <sup>a</sup>	86 ± 5.4	143 ± 7.8 <sup>a</sup>
Isoleucine	53 ± 4.7 <sup>bc</sup>	51 ± 2.4 <sup>c</sup>	57 ± 3.2 <sup>bc</sup>	57 ± 2.7 <sup>bc</sup>	53 ± 2.6 <sup>bc</sup>	89 ± 4.5 <sup>a</sup>	52 ± 3.3 <sup>c</sup>	89 ± 6.1 <sup>a</sup>
Valine	118 ± 4.7 <sup>cd</sup>	112 ± 11.4 <sup>cd</sup>	111 ± 6.6 <sup>cd</sup>	130 ± 3.8 <sup>c</sup>	119 ± 6.1 <sup>cd</sup>	239 ± 10.2 <sup>a</sup>	125 ± 5.6 <sup>cd</sup>	182 ± 10.5 <sup>b</sup>
∑ BCAA	255 ± 14.0 <sup>bc</sup>	232 ± 11.8 <sup>c</sup>	247 ± 14.1 <sup>bc</sup>	265 ± 9.5 <sup>bc</sup>	250 ± 12.0 <sup>bc</sup>	462 ± 21.0 <sup>a</sup>	263 ± 14.0 <sup>bc</sup>	413 ± 23.7 <sup>a</sup>
Lysine	493 ± 29.2 <sup>b</sup>	594 ± 48.3 <sup>a</sup>	452 ± 26.9 <sup>bc</sup>	438 ± 27.4 <sup>bc</sup>	448 ± 24.1 <sup>bc</sup>	443 ± 13.8 <sup>bc</sup>	476 ± 30.7 <sup>bc</sup>	540 ± 43 <sup>ab</sup>
Methionine	45 ± 1.5 <sup>bc</sup>	37 ± 1.0 <sup>c</sup>	40 ± 3.0 <sup>bc</sup>	34 ± 0.9 <sup>c</sup>	39 ± 1.9 <sup>c</sup>	81 ± 5.2 <sup>a</sup>	45 ± 2.2 <sup>bc</sup>	55 ± 2.1 <sup>b</sup>
Threonine	290 ± 12.9 <sup>cd</sup>	241 ± 15.1 <sup>d</sup>	284 ± 21.6 <sup>cd</sup>	288 ± 15.5 <sup>cd</sup>	279 ± 21.9 <sup>cd</sup>	324 ± 32.2 <sup>bc</sup>	374 ± 16.5 <sup>b</sup>	510 ± 33.2 <sup>a</sup>

<sup>1</sup>Values expressed as means ± SEM, n=5-8. Labeled means without a common letter differ (P<0.05).

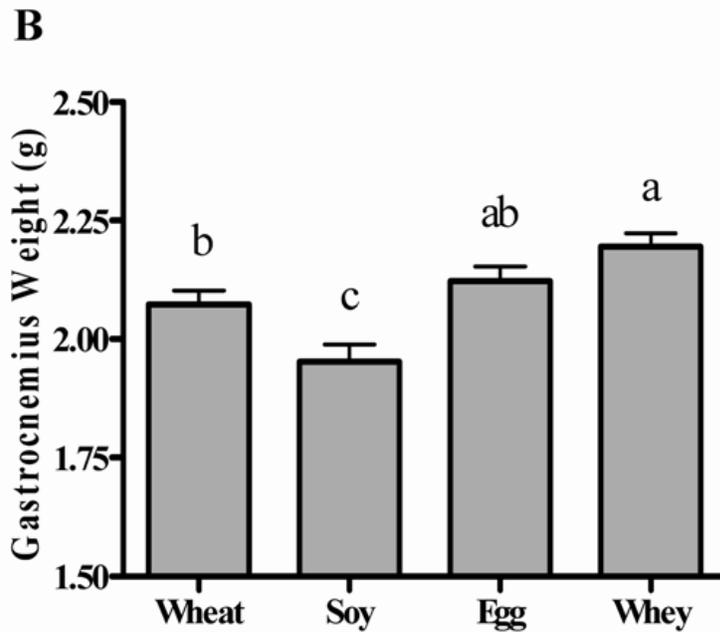
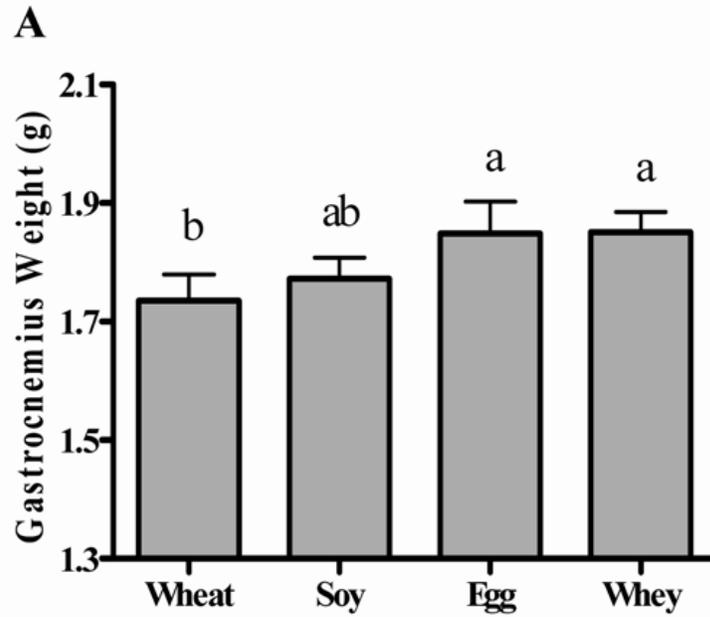
<sup>2</sup>12 hr food-deprived control.



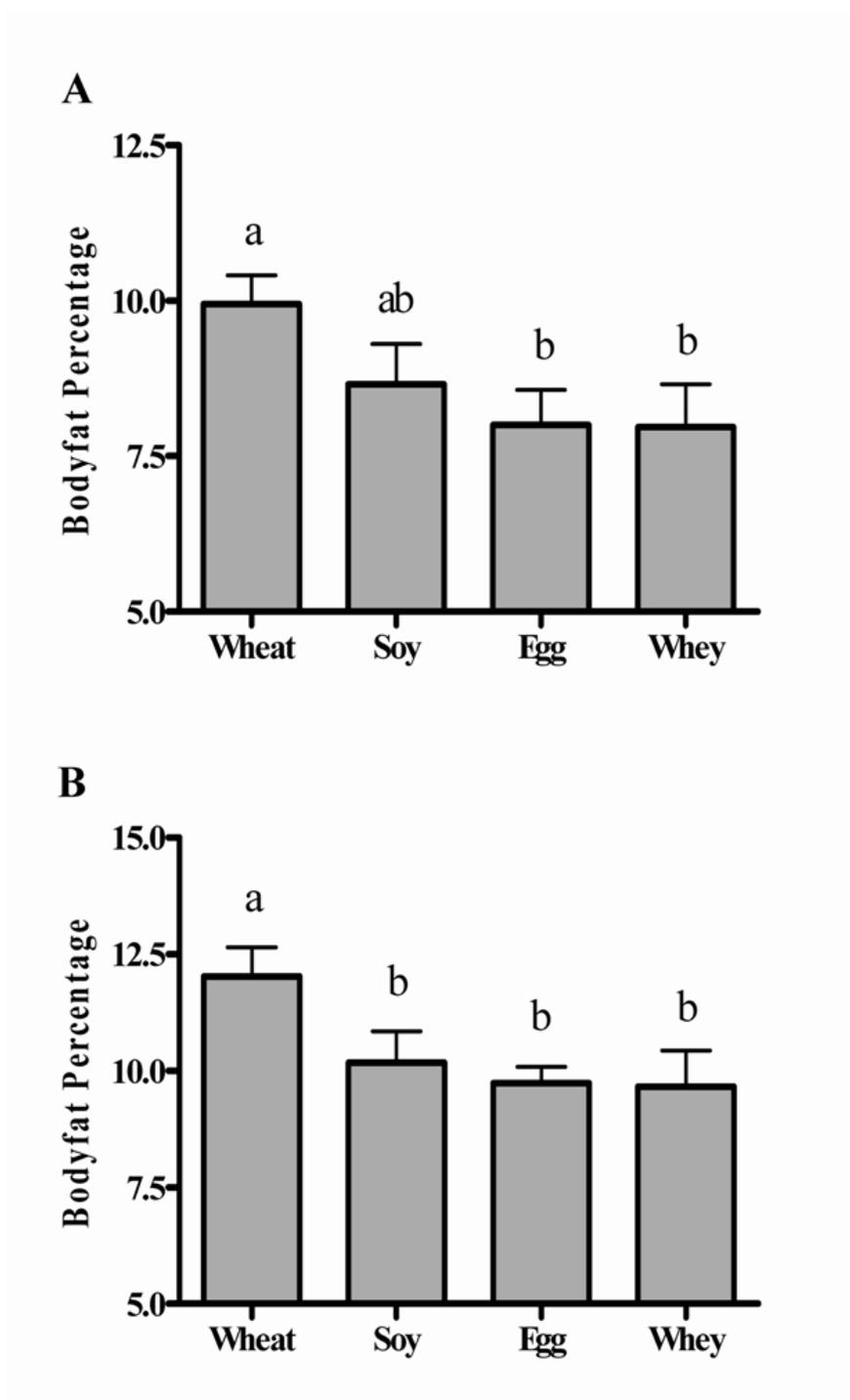
**Figure 4.1** Body weights of rats fed complete meals containing wheat, soy, egg, or whey proteins for 11 weeks. Data are means; n = 13-25. Labeled means without a common letter differ, P<0.05.

\*Time point (day 6) at which bodyweights of animals fed soy were significantly less than all other groups.

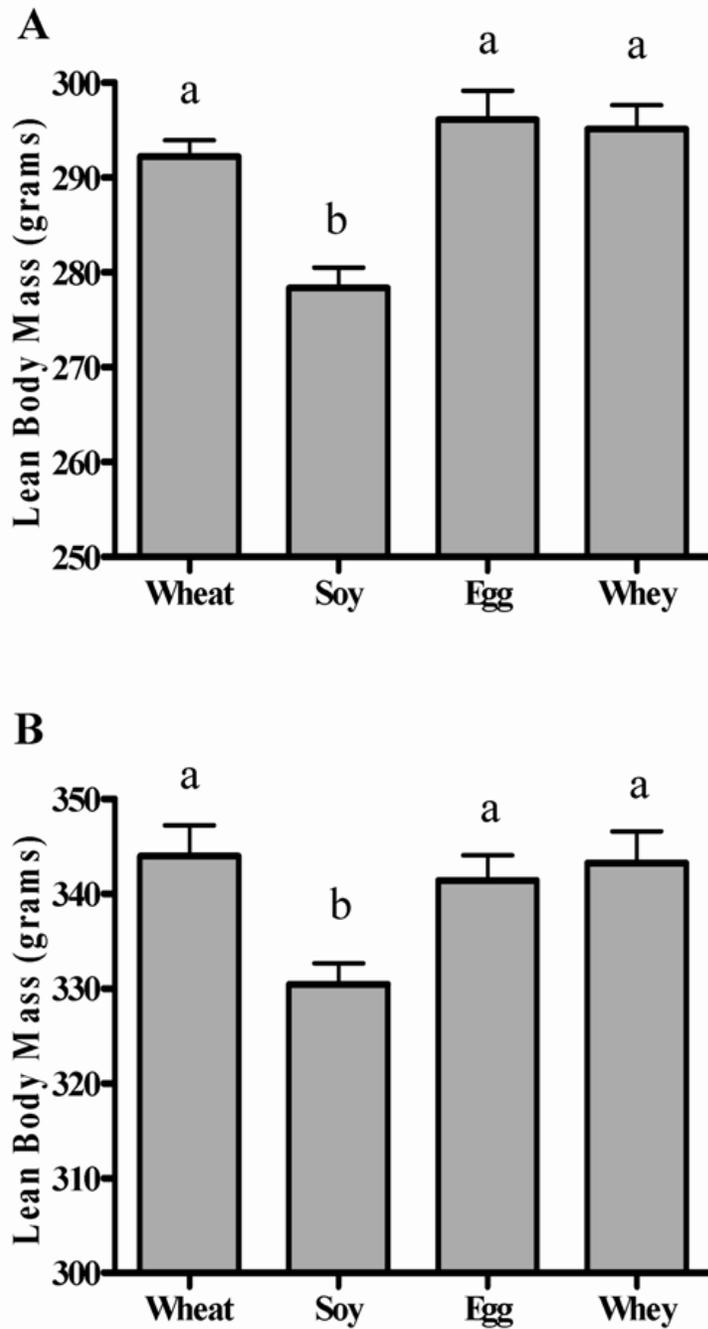
\*\*Time point (day 23) at which bodyweights of animals fed wheat were significantly larger than all other groups.



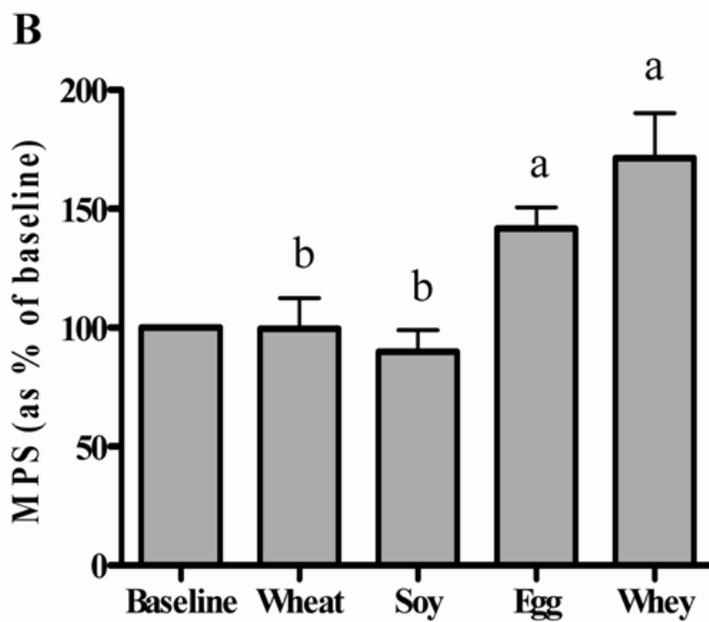
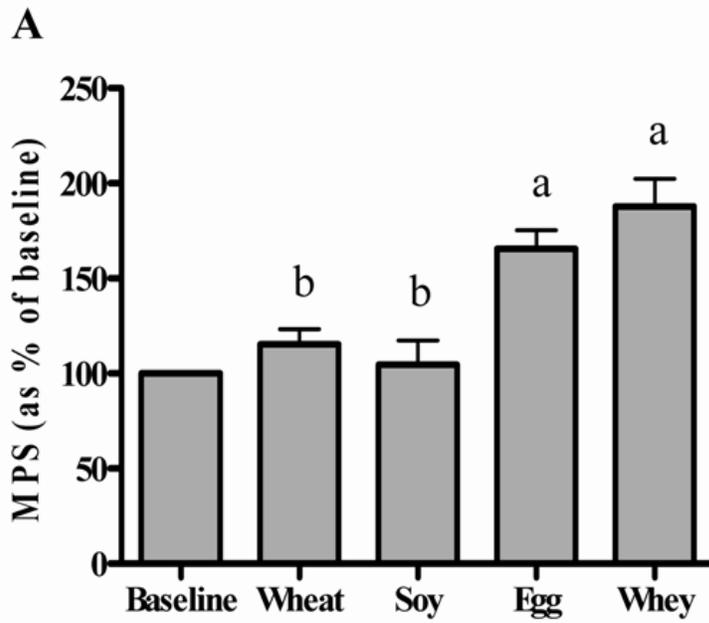
**Figure 4.2** Gastrocnemius muscle weights of rats fed complete meals containing wheat, soy, egg, or whey proteins for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 8-13. Labeled means without a common letter differ,  $P < 0.05$ .



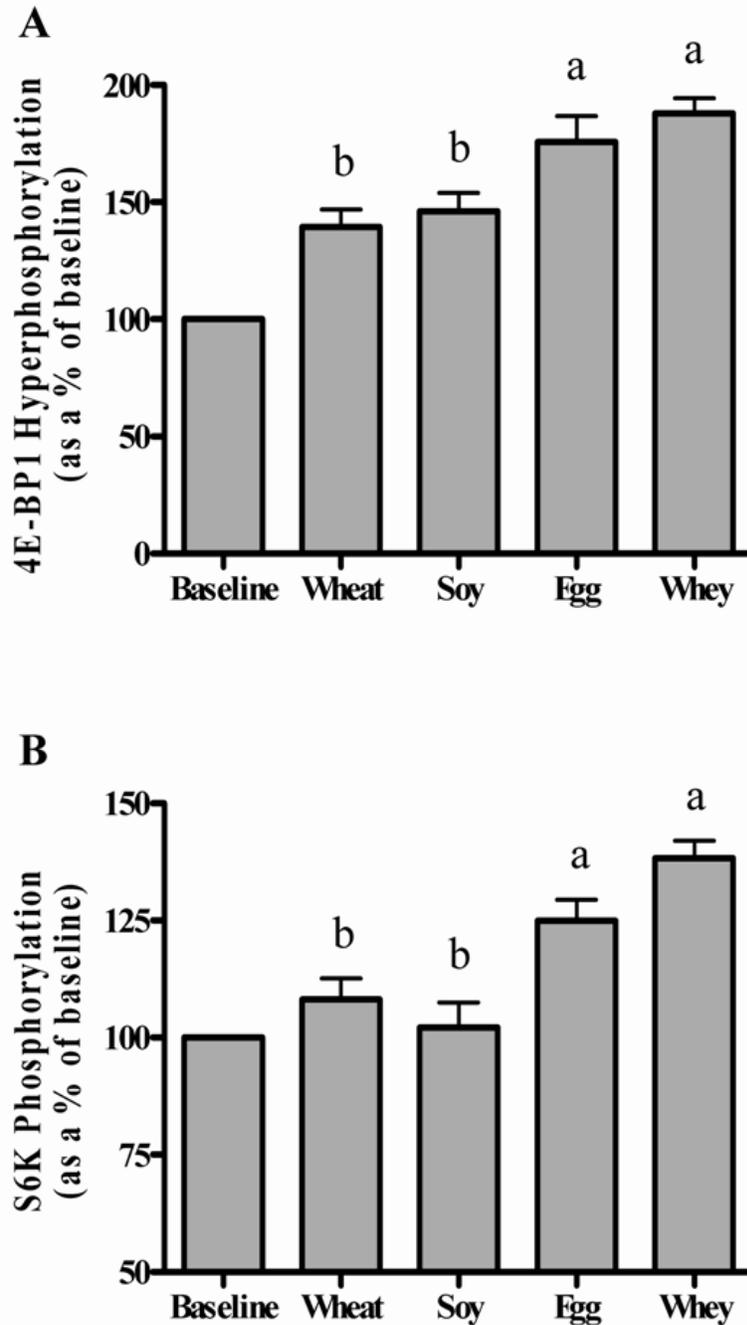
**Figure 4.3** Bodyfat percentages of rats fed complete meals containing wheat, soy, egg, or whey proteins for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 8-13. Labeled means without a common letter differ,  $P < 0.05$ .



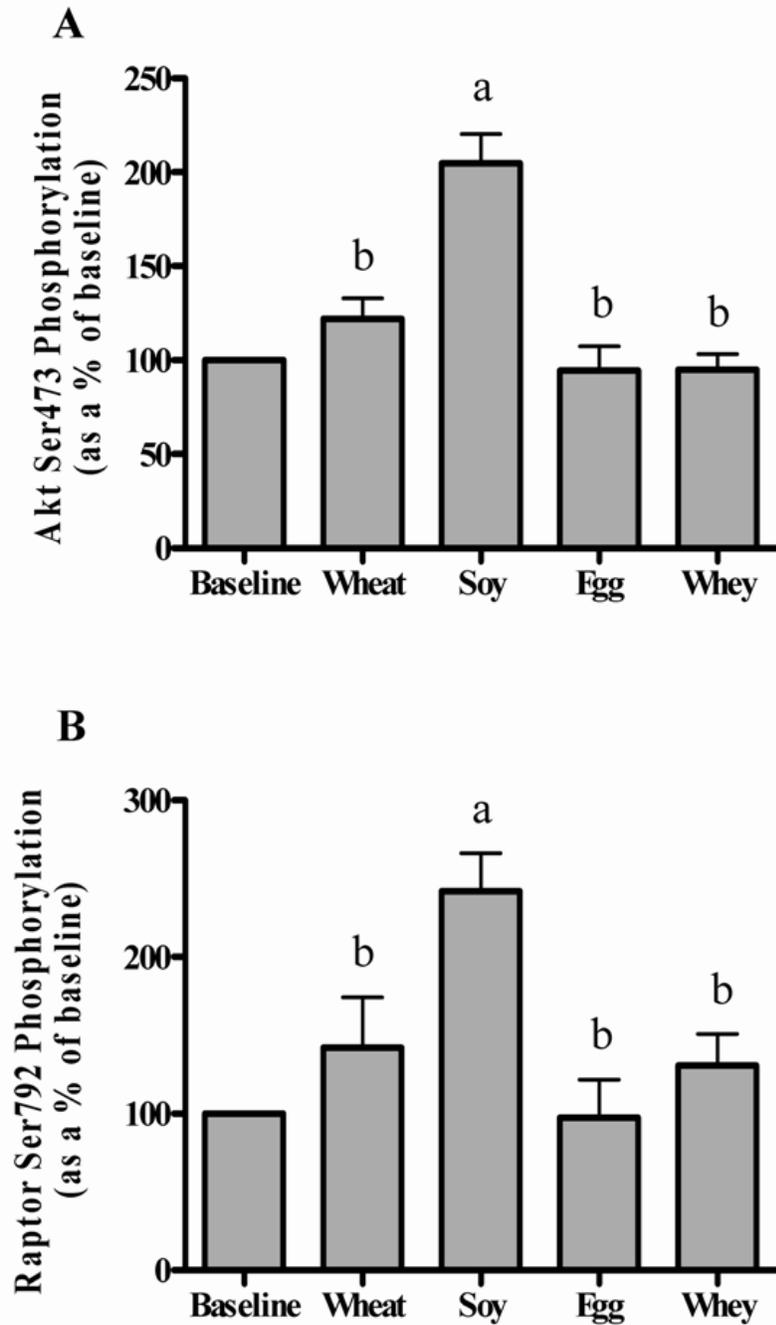
**Figure 4.4** Lean body masses of rats fed complete meals containing wheat, soy, egg, or whey proteins for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 8-13. Labeled means without a common letter differ,  $P < 0.05$ .



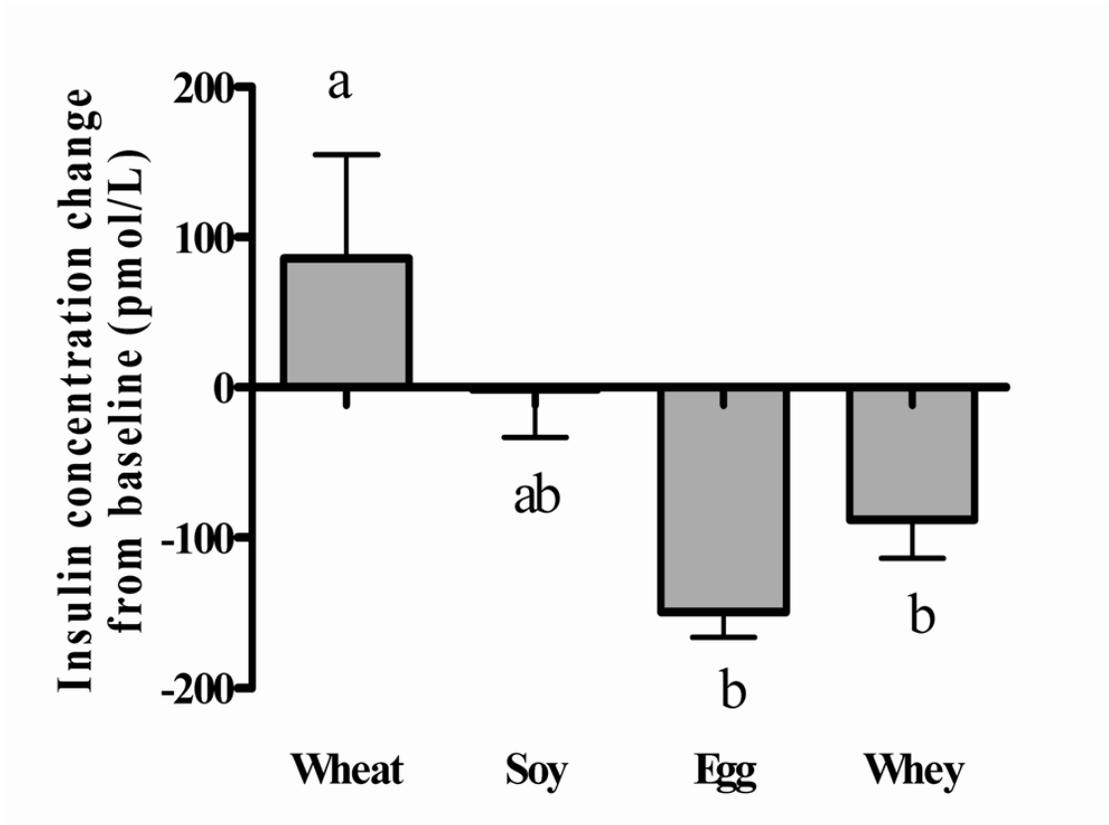
**Figure 4.5** Postprandial rates of muscle protein synthesis in rats fed complete meals containing wheat, soy, egg, or whey proteins for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 5-8. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 4.6** Postprandial phosphorylation of (A) 4E-BP1 and (B) S6K in rats fed complete meals containing wheat, soy, egg, or whey proteins for 2 weeks. Data are means  $\pm$  SEM; n = 7-8. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 4.7** Postprandial phosphorylation of (A) Akt and (B) Raptor in rats fed complete meals containing wheat, soy, egg, or whey proteins for 2 weeks. Data are means  $\pm$  SEM; n = 7-8. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 4.8** Post-prandial changes in insulin concentrations at 90 minutes after a complete meal containing either wheat, soy, egg, or whey proteins compared to the pre-meal baseline. Rats had consumed the individual diet treatments for 11 wk. Data are means  $\pm$  SEM; n = 5-8. Labeled means without a common letter differ,  $P < 0.05$ .

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## **CHAPTER 5: Protein distribution affects muscle mass based on differences in postprandial muscle protein synthesis and plasma leucine in rats**

### **Introduction**

Current recommendations for protein intakes are expressed as a daily requirement. This recommendation is based on a substrate concept for amino acid requirements that assumes the protein distribution throughout the day is not important so long as the overall amino acid needs are met. Current research however suggests protein distribution does matter as changes in protein turnover in muscle are regulated on a meal-to-meal basis largely associated with the leucine (leu) content of the meal (1-3). We have demonstrated previously that the anabolic response to a meal peaks between 45-90 minutes post-meal and returns to baseline after 3 hours.

Data from de Castro indicate that Americans consume over 65% of their daily protein after 18:30 h (4). With the average daily protein intake for elderly at 66 g/day, it is unlikely that they reach a threshold of protein intake necessary to stimulate MPS at meals prior to 18:30 (5). It is likely then that a large number of people only consume sufficient protein/leu to induce an anabolic response at dinner and this response may only last for 3 hours. Research from Arnal et al. also supports the importance of a meal protein threshold for adults (1). Furthermore, if the anabolic response to a high protein meal only lasts 3 hr, then it is likely that a large portion of the day is spent in a catabolic state.

In Chapter 4, we found that isonitrogenous, isocaloric diets that induce multiple increases in postprandial plasma leu and MPS are superior for long term body composition and muscle mass as compared to diets that provide fewer postprandial anabolic responses. These findings suggest that optimum protein intakes for adults require proteins rich in leu to be provided in multiple meals throughout the day. A logical extrapolation is that a person may reach their daily protein recommendation, but still not optimize the meal responses of MPS for optimal muscle mass. The purpose of this experiment was to examine whether distribution of protein and specifically leu

throughout the day can have an impact on MPS, body composition, and muscle mass in rats fed isonitrogenous, isocaloric meals from the same protein source with the same leu content.

## **Materials and Methods**

### ***Animals and diets.***

Male Sprague-Dawley rats ( $275 \pm 10$ g; Harlan-Teklad) will be maintained at 24°C with a 12-h light:dark cycle and free access to water. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

Rats were trained to consume 3 meals/d consisting of a 4 g meal consumed between 07:00 and 07:20 followed by free access to food from 13:00 to 14:00 and 18:00 to 19:00 for 6 days. Rats consumed ~18 g/d of total diet. On day 7, rats were randomized into the following diet groups (n=21-25): ED-Whey (evenly distributed whey protein) and UD-Whey (unevenly distributed whey protein) and food-deprived controls (**Table 5.1 and Table 5.2**). The diets were fed at 80% of ad libitum (14 g/day) intake to produce changes in body composition. In order to study meal responses and control for between animal differences in food intake, rats were fed defined amounts at each meal with 4 g meals at 07:00 and 13:00 and a 6 g meal at 18:00, representing a meal pattern designed to mimic human eating behavior (6). ED-Whey provided 16% of energy from protein at each meal, whereas UD-Whey provided 8%, 8%, and 27.5% of energy from protein at breakfast, lunch, and dinner, respectively. Total daily leu, protein, carbohydrate, and lipid intakes were the same for all groups (**Table 5.3 and Table 5.4**). The protein and leu distributions were designed to provide 3 stimulations of MPS for animals fed ED-Whey vs. only a single threshold stimulation at dinner for animals fed UD-Whey based on previous research (Chapter 3 and 4). After 2 wk of meal feeding treatment diets approximately half of the rats in each treatment group were set aside for determination of body composition by DXA (Dual energy X-ray absorptiometry). These animals were then allowed to recover and continue meal feeding for the remainder of the

study. The other animals that did not undergo DXA were food deprived for 12 h and then either sacrificed (food-deprived group) or fed their normal first meal at the 2 wk time point. The meals contained approximately 37 and 74 mg leu in the UD-Whey and ED-Whey meals respectively.

Either before the breakfast meal (i.e. baseline) or 90 min after consumption of the meal rats were killed and blood and tissues harvested. Tissues weighed and collected included the gastrocnemius, soleus, and plantaris muscles, and liver. The remaining animals continued to consume the diet treatments for 9 more weeks and then underwent DXA and sacrificed at wk 11 for the same measurements as the 2 wk time point.

#### ***Determination of Muscle Protein Synthesis.***

Protein synthesis was measured in skeletal muscle 90 minutes after the breakfast meals at 2 wk and 11 wk using the flooding dose method (7). A 100% enriched L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine solution (150mmol/L; Cambridge Isotopes, Andover, MA) was administered at 150 μmol/100 g body weight and injected via tail vein (1 mL/100g body weight) 80 minutes after completion of the meals. 10 min later (90 minutes post meal) animals were killed by decapitation and hind limbs quickly removed and immersed in an ice-water mixture. Gastrocnemius, plantaris, and soleus muscles were removed from cooled hind limbs, frozen in liquid N<sub>2</sub> and stored at -80°C.

Frozen muscle tissue was powdered in liquid nitrogen, and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1mL per 50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by gas chromatography mass spectroscopy as described previously (8, 9). The enrichment of L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies Santa Clara, CA). Samples were run under electron impact ionization and in splitless mode, and phenylethylamine ions at mass-to-charge ratio (m/z) 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

The muscle supernatant was used for determination of intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid

phase extraction (SPE) using EZ:faast™ amino acid analysis sample testing kit (Phenomenex Inc. Torrance, CA, USA) and <sup>2</sup>H<sub>5</sub>-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at m/z 206 (m) and 211 (m+5) (10).

Fractional rates of protein synthesis (FSR) were determined from the rate of incorporation of L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine into total mixed muscle protein as described previously (11). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[<sup>2</sup>H<sub>5</sub>]-phenylalanine incorporation. FSR, defined as the percentage of tissue protein renewed each day, were calculated according to the formula:  $FSR = (E_b \times 100) / (E_a \times t)$  where t is the time interval between injection and cooling of sampled tissue expressed in days and E<sub>b</sub> and E<sub>a</sub> are the enrichments of [<sup>2</sup>H<sub>5</sub>]Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

#### ***Plasma measurements.***

Plasma was obtained from trunk blood by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Linco Research, MO). Plasma amino acid concentrations were analyzed by HPLC using a Waters 2475 Fluorescence detector (12).

#### ***Phosphorylation of 4E-BP1, S6K, Akt, and Raptor.***

Muscle supernatants were subjected to protein immunoblot analysis as described previously (13, 14) using a rabbit polyclonal antibody to 4E-BP1 (Bethyl Labs, Montgomery, TX), and a rabbit polyclonal S6K antibody (Bethyl Labs, Montgomery, TX).

#### ***Body Composition.***

Prior to the administration of the diet treatments and 2 and 11 weeks after the diet treatments began, body composition was assessed using dual energy X-ray absorptiometry (DXA) analysis. Rats received an anesthetic by intraperitoneal injection

of a 0.3mg/kg dexmedetomidine (commercial name Dexdomitor) for sedation during testing. Before testing, the DXA (Hologic QDR 4500A, software version 11.1:3) instrument was be calibrated as per the manufacturer's guidelines. Rats were then transferred to the DXA and whole body (WB) scans were performed. All scans for a given rat were be performed on the same day and analyzed by the same research technician.

### *Statistical analysis.*

All data were analyzed by SPSS 15.0 software package for windows. A 1-way ANOVA was performed with the treatment group as the independent variable. When a significant overall effect was detected, differences among individual means were assessed using Fisher's LSD post hoc test. All data sets were tested for normal distribution and variance homogeneity using Levene's test. When variances were not homogeneous, means were compared using a Games-Howell test. Correlations were determined by linear regression (Pearson's correlation). The level of significance was set at  $P < 0.05$  for all statistical tests. All values are presented as means  $\pm$  SEM.

## **Results**

This experiment examined whether long term feeding of isonitrogenous, isoenergetic diets containing whey protein distributed evenly (ED-Whey) or unevenly (UD-Whey) could produce body composition and skeletal muscle mass differences based on postprandial MPS outcomes. Animal weights were not different between treatment groups at the beginning of the experiment and through 59 days of feeding. The ED-Whey group was significantly heavier than the UD-Whey group after 59 days (**Figure 5.1**).

Gastrocnemius muscle weights were greater in animals fed ED-Whey compared with animals fed UD-Whey at 2 weeks (**Figure 5.2**) and at 11 weeks (**Figure 5.2**). UD-Whey had a trend ( $p=0.09$ ) for bigger liver weights at 2 weeks compared to ED-Whey (**Figure 5.3**). This trend became significant at 11 weeks (**Figure 5.3**).

Body fat percentage was not different between the two groups at 2 or 11 weeks (**Figure 5.4**). Consistent with these data, lean body mass was not different between the two groups at either time point (data not shown).

At 2 weeks, after a breakfast meal providing ~30% of daily energy and containing 16% of energy as protein, plasma leu increased in animals ED-Whey but not in animals fed UD-Whey (**Table 5.5**). Similar postprandial patterns of plasma amino acids concentrations were observed for valine and isoleucine, though the difference in isoleucine concentrations was not significant, with whey producing the highest concentrations at 90 min after the meal (**Table 5.5**). Other plasma amino acids varied among the groups largely in proportion to the amino acid content of the meal consumed (**Table 5.5**). Lysine was greater in the ED-Whey group compared to the UD-Whey group and was significantly lower than baseline levels in animals fed UD-Whey. Plasma methionine concentrations were not significantly different between treatment groups however methionine was significantly below baseline concentrations in the UD-Whey group (**Table 5.5**). Plasma threonine was only increased in both treatment groups compared to food-deprived baseline control concentrations. At 11 weeks this meal feeding and measurements were repeated with very similar results with animals fed ED-Whey having significantly greater postprandial plasma concentrations of all amino acids measured except for methionine as the difference was not significant (**Table 5.6**). At 11 weeks, plasma concentrations of all BCAA were reduced postprandially compared to baseline food-deprived values (**Table 5.6**). Additionally, baseline food-deprived concentrations were not different between ED-Whey and UD-Whey, except isoleucine which was greater in UD-Whey at 11 weeks (**Table 5.6**).

Post-prandial MPS after a breakfast meal was significantly greater in animals fed ED-Whey compared with animals fed UD-Whey (**Figure 5.5**) at both the 2 and 11 week time points. This was consistent with the post-prandial phosphorylation of the mTOR signaling targets 4E-BP1 (**Figure 5.6A**) and S6K (**Figure 5.6B**) at 2 weeks. Phosphorylation of S6K increased after the breakfast meal in animals fed ED-Whey but not UD-Whey. Feeding increased 4E-BP1 phosphorylation in both groups however, ED-

Whey had significantly greater levels of 4E-BP1 phosphorylation compared with animals fed UD-Whey.

Post-prandial insulin concentrations were not different between the treatment groups or different from baseline at 2 weeks (data not shown). At 11 weeks however, animals fed UD-Whey had increased postprandial insulin concentrations compared with baseline controls whereas animals fed ED-Whey had decreased postprandial insulin concentrations compared with baseline controls (**Figure 5.7**).

### **Discussion**

This experiment was performed to determine if long term feeding of isonitrogenous, isoenergetic diets with the same leu contents but different meal distribution of protein and leu could impact long term body composition and muscle mass based on their ability to stimulate increase postprandial leu and MPS. The major finding of this work is that protein and specifically leu distribution is crucial to optimizing muscle mass and that multiple increases in post-prandial plasma leu and MPS are superior to a single increase in plasma leu and MPS. Our previous research demonstrated that rats fed egg and whey producing multiple increases in plasma leu and MPS had better body composition and muscle mass outcomes compared to animals fed wheat and soy which did not produce increases in MPS at small 4g meals (Chapter 4).

The difference in post-prandial increases in leu and MPS were consistent with the differences in gastrocnemius weight. Animals fed ED-Whey had greater postprandial leu and MPS increases than animals fed UD-Whey after small meals. Also closely associated with plasma leu and MPS were the phosphorylation of the mTOR targets 4E-BP1 and S6K. We hypothesize that long term daily feeding of meals producing different postprandial meal responses in plasma leu and MPS lead to greater tissue deposition in muscle over time.

Contrary to our previous experiment, body composition and lean body mass were not different between the groups. This was somewhat surprising considering the body fat outcomes of animals receiving multiple stimulations of MPS per day vs. animals

receiving less stimulation in the previous experiment. A possible explanation was that the uneven distribution of protein intake shifted tissue weights from skeletal muscle to other lean body tissues like the gut and liver. Indeed, animals fed UD-Whey had significantly bigger livers at 11 weeks than animals fed ED-Whey. A possible mechanism for these differences could be that MPS is maximized well below the level of protein intake fed to UD-Whey animals at dinner and but that large protein meal stimulates greater deposition in liver compared to the moderate protein meal consumed by ED-Whey. Therefore, we hypothesize that consuming enough total leu, regardless of distribution will optimize bodyfat, but optimization of muscle mass requires proper distribution of dietary protein and leu intake.

Also surprising was the post-prandial insulin concentrations at 11 weeks. At 90 minutes post meal, animals fed ED-Whey had actually decreased insulin concentrations below baseline by 88 pmol/L whereas animals fed UD-Whey had increased insulin concentrations by 86 pmol/L above baseline. This may be due to the increased carbohydrate content of the breakfast meal in animals fed UD-Whey compared to ED-Whey (62% CHO vs. 54% CHO). The increased carbohydrate content may have caused a greater insulin response which had not been cleared at 90 minutes post meal.

Current protein recommendations are daily amounts based on short term nitrogen balance and amino acid oxidation studies in a steady state. Humans however, eat in discreet meals producing non-steady state outcomes. de Castro et al. reported that Americans consume approximately ~65% of their total protein at dinner (4). By current dietary guidelines, this is acceptable so long as total daily protein requirement is met. However, if such a large amount of protein is consumed at dinner, it is likely that the other meals contain insufficient protein or leu to increase plasma leu and MPS. In the current study, animals consuming UD-Whey with ~70% of their total protein intake at dinner had smaller gastrocnemius muscles than animals fed ED-Whey. Therefore, it may be possible to meet one's daily protein requirement, but not optimize MPS and muscle mass.

## **Conclusions**

The current study confirms the hypothesis that protein/leu distribution are important and can have differential effects on postprandial leu, mTOR signaling, and MPS and produce differential deposition of lean tissue mass in skeletal muscle and liver. Protein quantity, quality, and distribution are all important factors for producing optimal muscle mass and current protein recommendations may be insufficient to fully encapsulate the postprandial meal response effects of dietary protein and leu.

## **Acknowledgements**

Thanks to Gabe Wilson, Chris Moulton, and Adam Schiltz for their help with meal feeding and weighing animals during this experiment.

## Tables and Figures

**Table 5.1** Compositions of animal diets

Component	ED-Whey (16% Protein)	UD-Whey (8% Protein)	UD-Whey (27.5% Protein)
	<i>g/kg</i>		
Whey Protein Isolate <sup>1</sup>	188.8	94.4	324.5
Cornstarch	328.2	422.6	192.5
Maltodextrin	134.1	134.1	134.1
Sucrose	101.5	101.5	101.5
Soybean Oil	140.9	140.9	140.9
Cellulose (Fiber)	53.7	53.7	53.7
Mineral Mix <sup>2</sup>	37.6	37.6	37.6
Vitamin Mix <sup>2</sup>	10.7	10.7	10.7
Choline Bitaurate	2.7	2.7	2.7

<sup>1</sup>Whey Protein Isolate provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

<sup>2</sup>Mineral and Vitamin supplement (15) from Harlan Teklad, Madison, WI.

**Table 5.2** Amino acid composition of Whey Protein Isolate

Amino Acid	Whey Protein Isolate <sup>1</sup>
Alanine	4.9
Arginine	2.4
Aspartate	10.6
Cysteine	2.5
Glutamate/Glutamine	16.9
Glycine	1.8
Histidine	2.0
Isoleucine	6.2
Leucine	10.9
Lysine <sup>5</sup>	9.1
Methionine	2.0
Phenylalanine	3.3
Proline	5.6
Serine	4.7
Threonine	6.4
Tryptophan	1.7
Tyrosine	3.0
Valine	6.0

<sup>1</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

**Table 5.3** Protein distribution

	Meal	Breakfast	Lunch	Dinner	Total intake
Diet Group					
		<i>Protein intake (g)</i>			
ED-Whey <sup>1</sup>		0.68	0.68	1.02	2.38
UD-Whey <sup>1</sup>		0.35	0.35	1.68	2.38

<sup>1</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

**Table 5.4** Leucine distribution

	Meal	Breakfast	Lunch	Dinner	Total intake
Diet Group					
		<i>Leucine intake (mg)</i>			
ED-Whey <sup>1</sup>		74	74	111	259
UD-Whey <sup>1</sup>		38	38	184	260

<sup>1</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

**Table 5.5** Postprandial Plasma amino acid concentrations at 2 weeks<sup>1</sup>

	Baseline <sup>2</sup>	UD-Whey	ED-Whey
		μmol/L	
Leucine	91 ± 4.4 <sup>b</sup>	78 ± 6.4 <sup>b</sup>	146 ± 28.4 <sup>a</sup>
Isoleucine	74 ± 6.5 <sup>a</sup>	72 ± 7.3 <sup>a</sup>	121 ± 24.0 <sup>a</sup>
Valine	130 ± 3.4 <sup>a</sup>	94 ± 5.0 <sup>b</sup>	182 ± 49.0 <sup>a</sup>
∑ BCAA	295 ± 12.0 <sup>a</sup>	233 ± 17.4 <sup>b</sup>	457 ± 96.9 <sup>a</sup>
Lysine	543 ± 15.3 <sup>a</sup>	420 ± 27.4 <sup>b</sup>	626 ± 46.2 <sup>a</sup>
Methionine	53 ± 2.6 <sup>a</sup>	41 ± 3.1 <sup>b</sup>	60 ± 19.4 <sup>ab</sup>
Threonine	389 ± 54.2 <sup>b</sup>	546 ± 41.1 <sup>a</sup>	860 ± 209.2 <sup>a</sup>

<sup>1</sup>Values expressed as means ± SEM, n=5-8. Labeled means without a common letter differ (P<0.05).

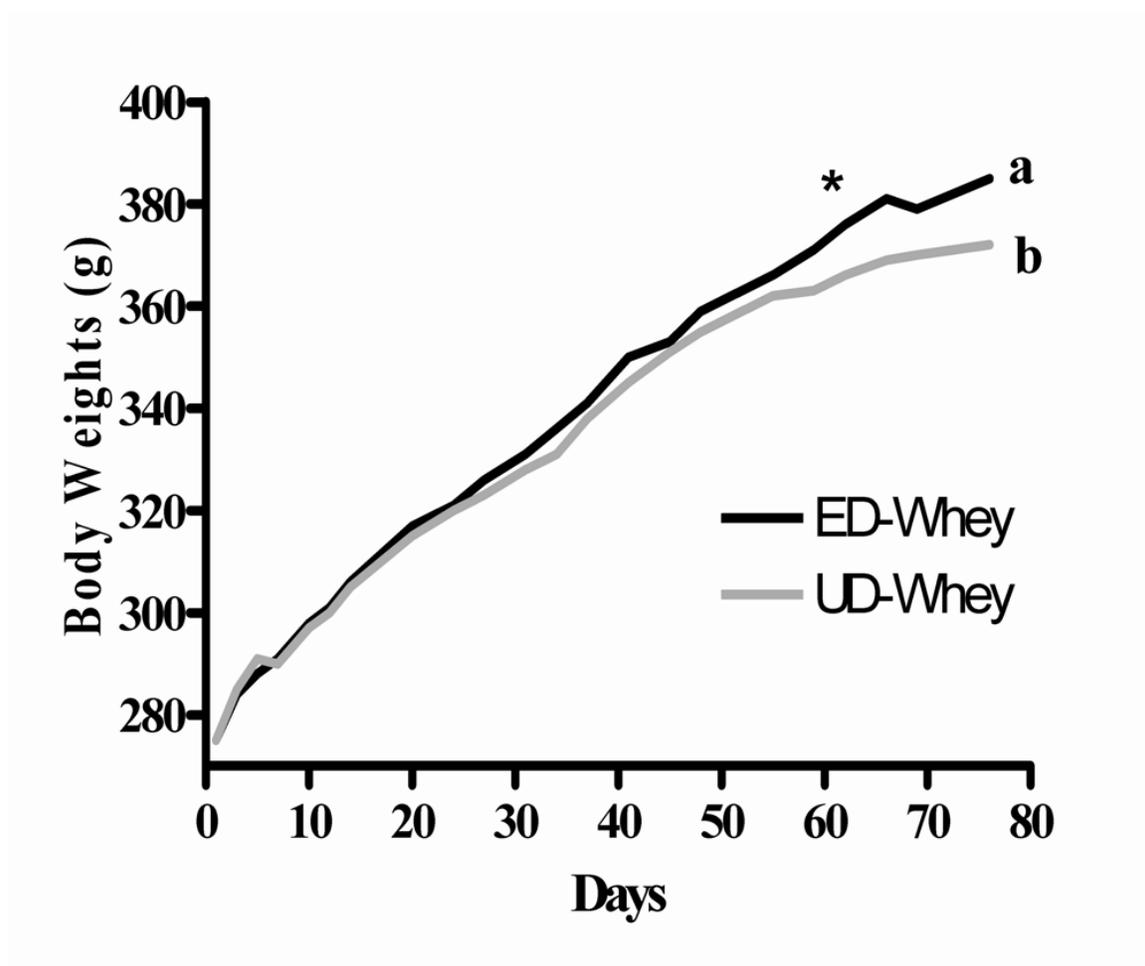
<sup>2</sup>12 hr food-deprived control.

**Table 5.6** Plasma amino acid concentrations at 11 weeks<sup>1</sup>

	UD-Whey		ED-Whey	
	FD <sup>2</sup>	Fed	FD <sup>2</sup>	Fed
Leucine	97 ± 6.8 <sup>b</sup>	75 ± 3.7 <sup>c</sup>	86 ± 5.4 <sup>bc</sup>	143 ± 7.8 <sup>a</sup>
Isoleucine	68 ± 4.9 <sup>b</sup>	53 ± 2.6 <sup>c</sup>	52 ± 3.3 <sup>c</sup>	89 ± 6.1 <sup>a</sup>
Valine	141 ± 8.5 <sup>bc</sup>	99 ± 2.7 <sup>d</sup>	125 ± 5.6 <sup>cd</sup>	182 ± 10.5 <sup>b</sup>
∑ BCAA	307 ± 19.9 <sup>b</sup>	227 ± 8.5 <sup>c</sup>	263 ± 14.0 <sup>bc</sup>	413 ± 23.7 <sup>a</sup>
Lysine	528 ± 39.5 <sup>bc</sup>	428 ± 25.3 <sup>c</sup>	476 ± 30.7 <sup>bc</sup>	540 ± 43 <sup>ab</sup>
Methionine	48 ± 2.0 <sup>b</sup>	41 ± 1.5 <sup>bc</sup>	45 ± 2.2 <sup>bc</sup>	55 ± 2.1 <sup>b</sup>
Threonine	382 ± 23.5 <sup>b</sup>	322 ± 16.8 <sup>bc</sup>	374 ± 16.5 <sup>b</sup>	510 ± 33.2 <sup>a</sup>

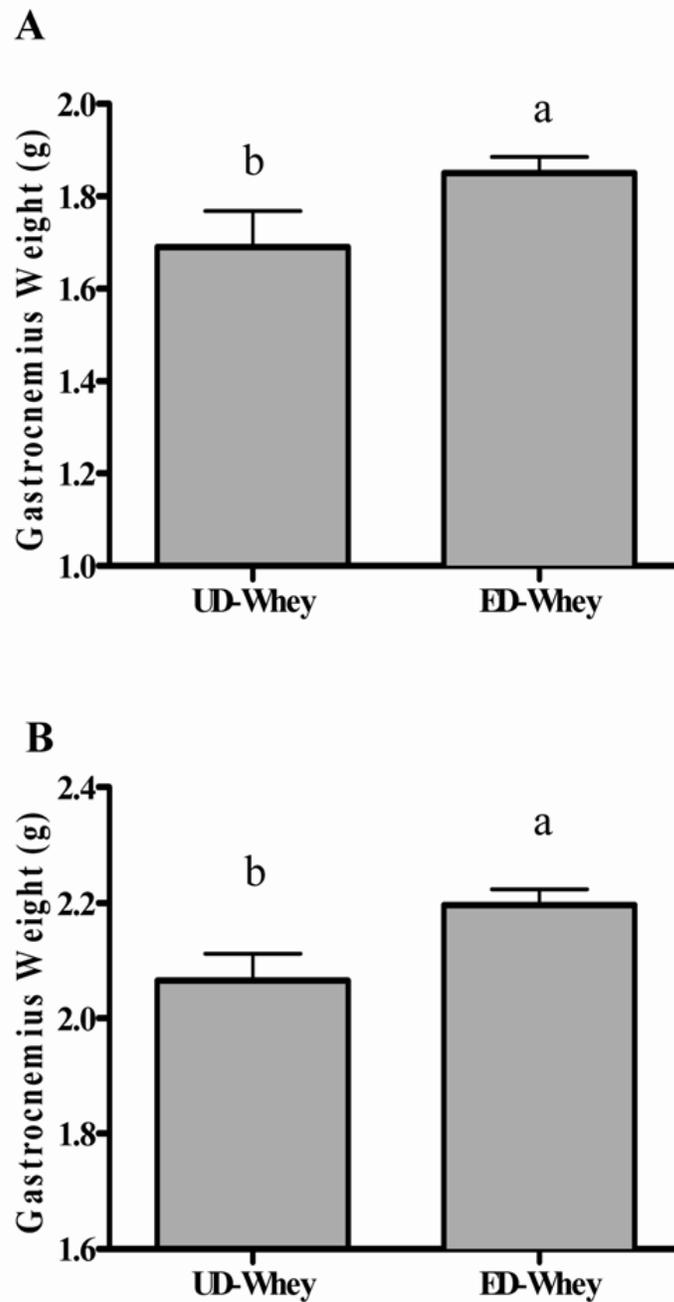
<sup>1</sup>Values expressed as means ± SEM, n=5-8. Labeled means without a common letter differ (P<0.05).

<sup>2</sup>12 hr food-deprived control.

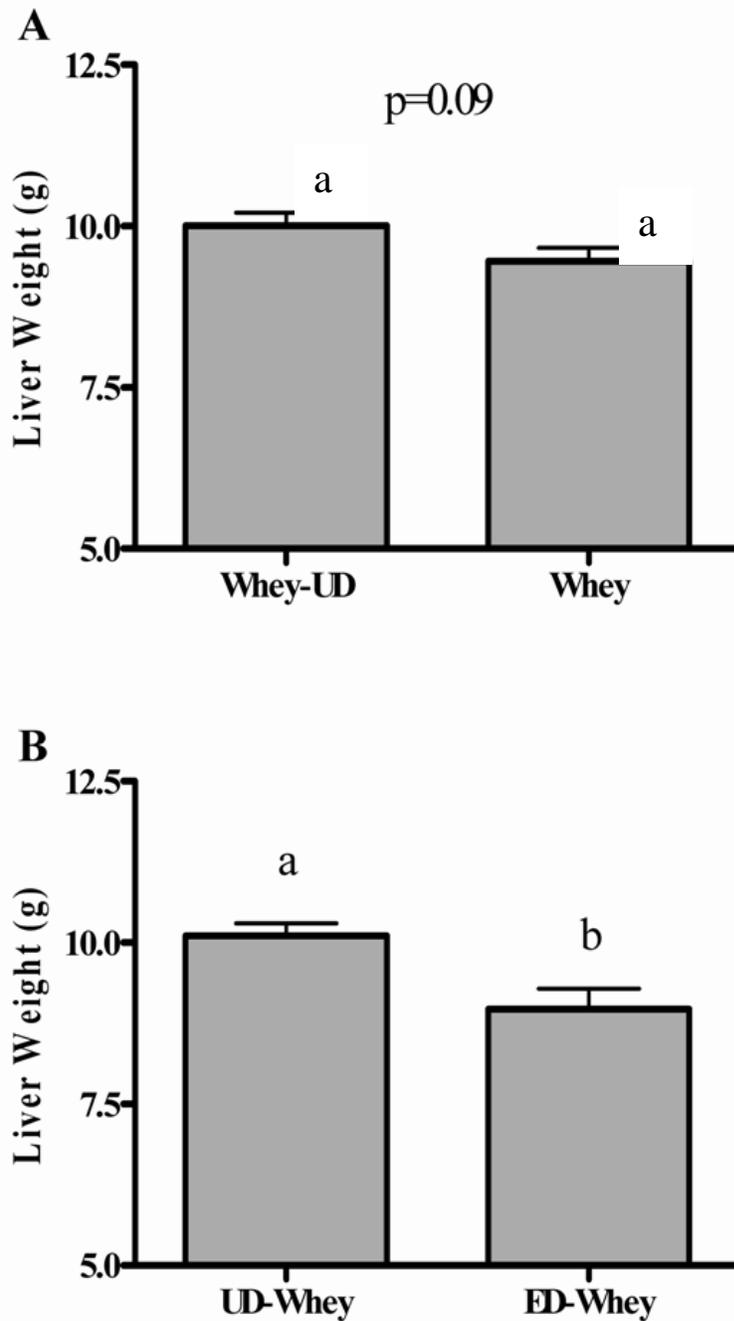


**Figure 5.1** Body weights of rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein for 11 weeks. Data are means; n = 13-25. Labeled means without a common letter differ, P<0.05.

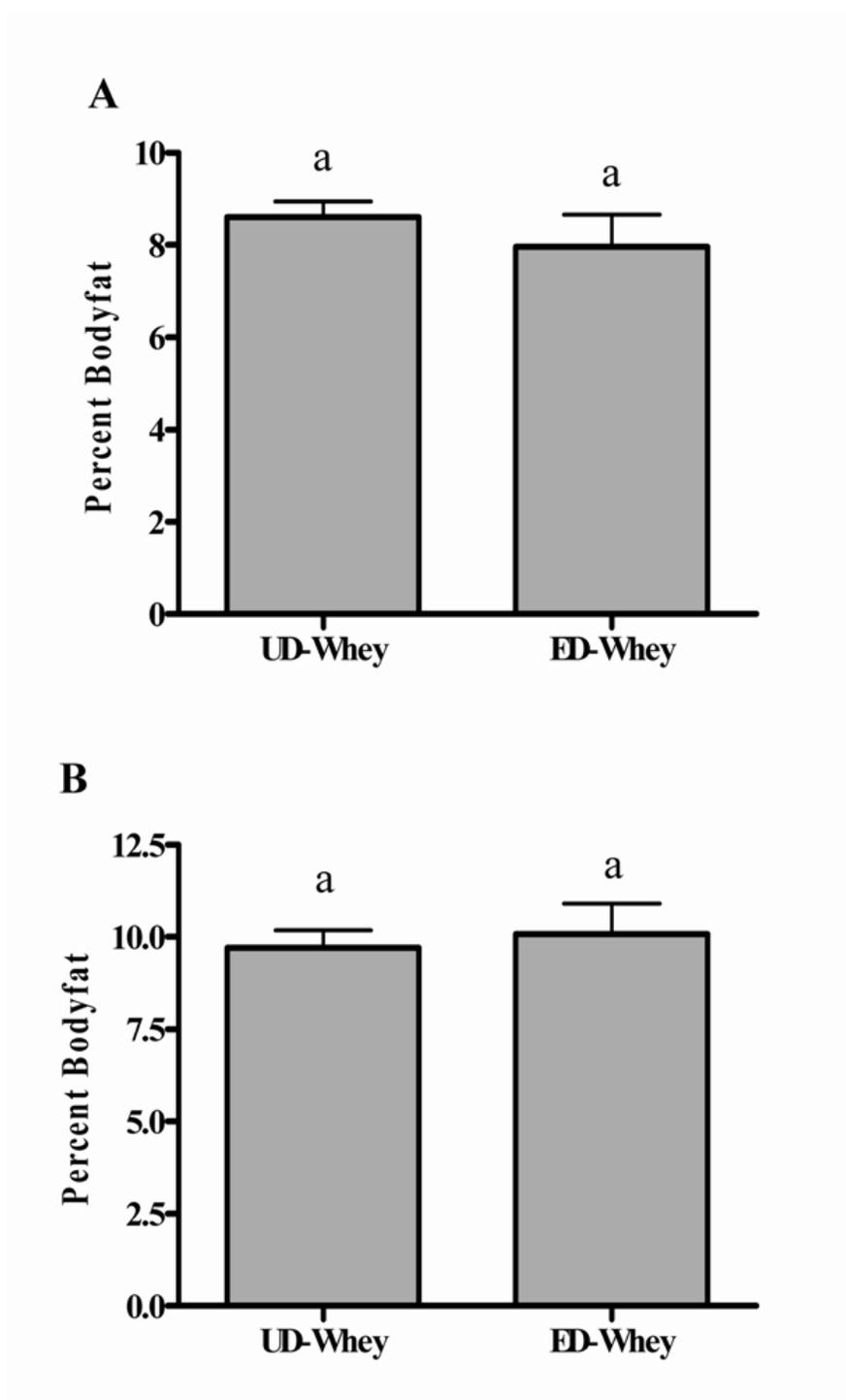
\*Time point (day 59) at which bodyweights of animals were significantly different.



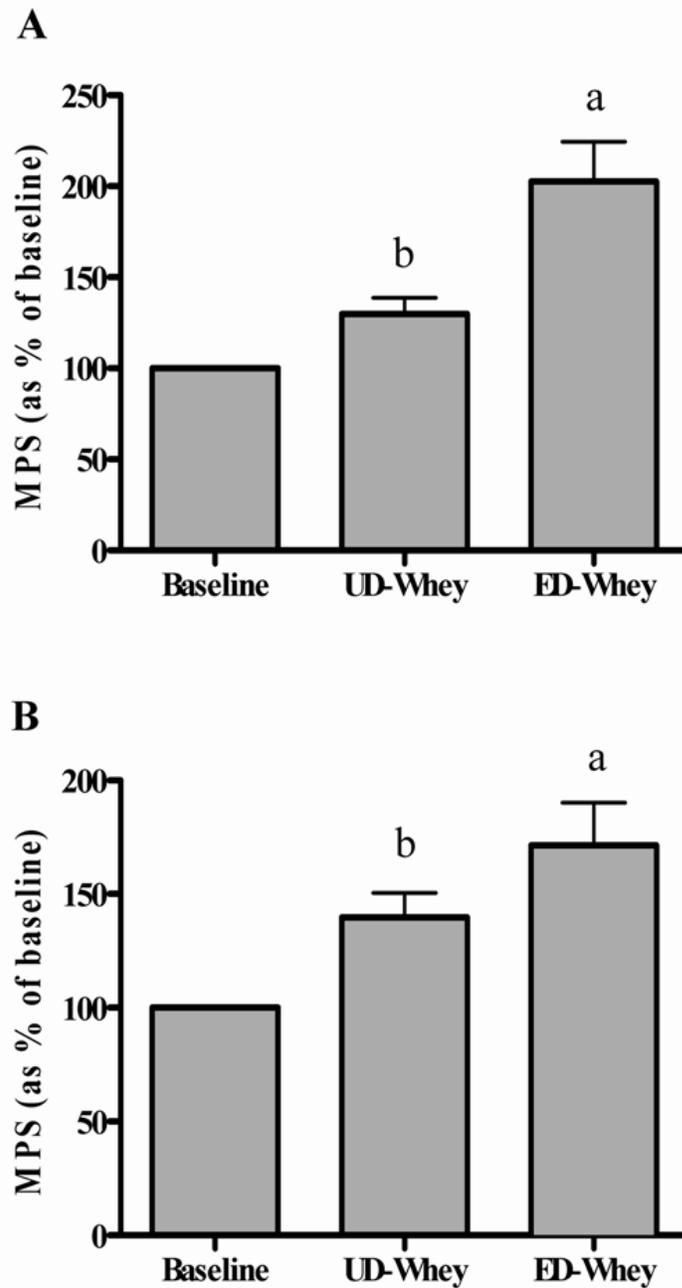
**Figure 5.2** Gastrocnemius muscle weights of rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 8-13. Labeled means without a common letter differ,  $P < 0.05$ .



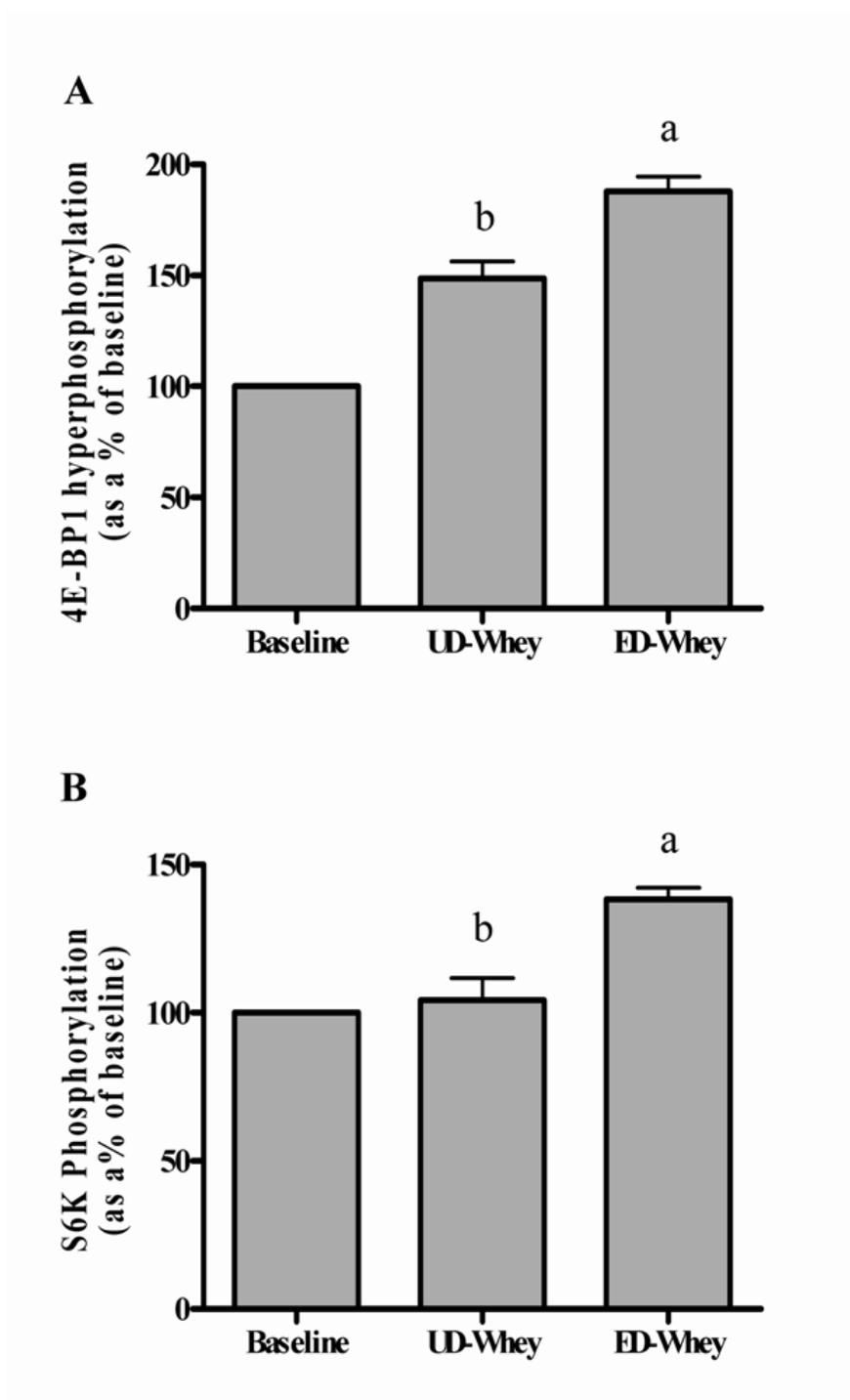
**Figure 5.3** Liver weights of rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 8-12. Labeled means without a common letter differ,  $P < 0.05$ .



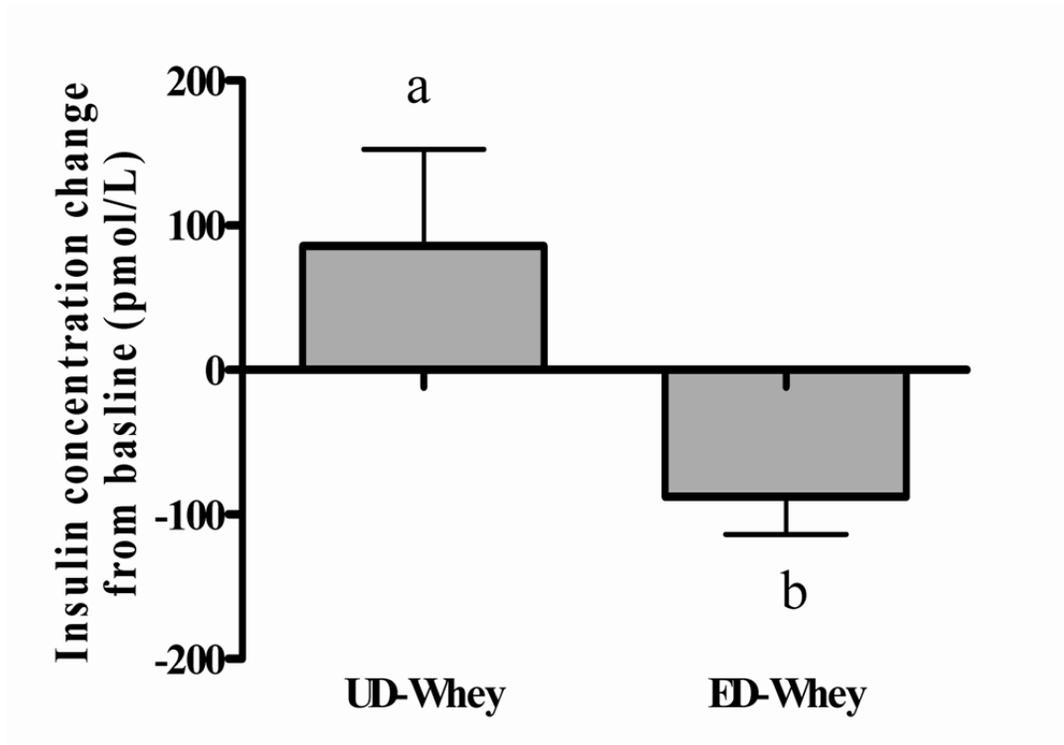
**Figure 5.4** Bodyfat percentages of rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 8-13. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 5.5** Postprandial rates of muscle protein synthesis in rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein for (A) 2 weeks and (B) 11 weeks. Data are means  $\pm$  SEM; n = 5-8. Labeled means without a common letter differ,  $P < 0.05$ .



**Figure 5.6** Postprandial phosphorylation of (A) 4E-BP1 and (B) S6K in rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein. Data are means  $\pm$  SEM; n = 7-8. Labeled means without a common letter differ, P<0.05.



**Figure 5.7** Changes in insulin concentrations from baseline in rats fed diets with unevenly distributed (UD-Whey) or evenly distributed (ED-Whey) whey protein for 11 weeks at 90 minutes post meal. Data are means  $\pm$  SEM; n = 5-8. Labeled means without a common letter differ,  $P < 0.05$ .

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## CHAPTER 6: Summary, conclusions, and speculations

Current recommendations for protein quantity and quality are based on preventing deficiencies in limiting amino acids and are assessed with measurements focused on optimizing efficiency of protein intake such as nitrogen balance, DAAO, and IAAO. These methods interpret increases in plasma amino acids as inefficient utilization because of increased amino acid oxidation. These methods view amino acids only as substrates for protein synthesis and fail to account for metabolic actions of amino acids such as leu on mTOR signaling and MPS. The overall goal of this research was to determine if feeding different protein sources which met limiting amino acids requirements based on current standards for protein quality could produce differences in mTOR signaling, MPS, body composition, and muscle mass based on their leu content. This dissertation provides a fundamentally new concept for protein quantity and quality based on optimizing protein synthesis in skeletal muscle and the leu content of proteins. This new approach is applicable defining protein needs for adult health and maintaining muscle mass and body composition.

The first objective of this dissertation (Chapter 2) was to determine the role leu plays in complete meals by 1) characterizing the time course of MPS response to a meal and the associated changes in plasma leu, mTOR signaling, and insulin and 2) determining peak activation of MPS and mTOR signaling in response to complete meals containing isonitrogenous protein sources with different leu contents. To test these parameters we fed test meals that were balanced for protein, carbohydrates, fat and fiber but contained 10, 20, or 30% of total energy from wheat or whey proteins (6.8 and 10.9% leu), respectively. We determined that whey increased plasma leu, mTOR signaling, and MPS more than wheat at all protein intakes. The time course of MPS after meals lasted for 3 hours with a peak at 90 minutes; however plasma leu and mTOR signaling remained at a plateau for 3 hours before decreasing. Meal leu content and the increases in post-prandial plasma leu were closely associated with peak activation of mTOR signaling and MPS. The meal response of MPS lasted for approximately 3 hours and returned to baseline while plasma leu and translation initiation factors remained elevated.

We termed this point at the end of the post-prandial periods when MPS was not related to plasma leu concentration to be a “refractory response” period.

Our second objective (Chapter 3) was to examine the potential to use leu to predict the quality of individual proteins to stimulate mTOR signaling and MPS at individual meals and determine if a threshold for increasing plasma leu and MPS exists. Adult rats were adapted for 2 weeks to a meal-feeding protocol with complete diets containing 16% protein but with different leu contents using wheat, soy, egg, or whey proteins (6.8, 8.0, 8.8, and 10.9% leu). Animals fed egg and whey significantly increased plasma leu and MPS whereas animals fed wheat and soy and receiving the same amount of total protein failed to stimulate MPS after the meal. The leu content of the meals predicted mTOR signaling and peak MPS responses and were closely related to changes in plasma leu. These findings supported the hypothesis that there is a meal leu threshold for increasing plasma leu.

The third objective (Chapter 4) was to further evaluate the findings of Chapter 3 and to test the hypothesis that long-term meal-feeding of isonitrogenous/isocaloric diets with different protein sources that produced different meal responses for MPS would result in different muscle mass. This study used diets with 16/54/30% of energy from protein/carbohydrates/lipids, respectively, using protein sources (wheat, soy, egg, whey) with different leu contents to produce post-prandial changes in plasma leu and MPS and muscle mass and body composition were evaluated after 2 and 11 weeks of meal-feeding. Based on the leu threshold observed in Chapter 3, the diets were designed such that animals fed egg and whey proteins would receive sufficient leu during each of the three meals to achieve three stimulations of MPS per day as opposed to animals fed wheat and soy proteins with lower leu contents that would achieve the leu threshold only at the larger dinner meal. After 11 weeks of meal-feeding, gastrocnemius muscle weight, lean body mass, and body composition were positively related to the leu content of the diet and reflected the ability of the respective diets to increase post-prandial plasma leu and MPS.

Finally, the fourth objective (Chapter 5) was to determine if long-term differences in distribution of dietary protein and specifically the leu content of meals could make a difference in body composition. We tested this hypothesis by feeding isocaloric/isonitrogenous meals containing whey protein evenly distributed to achieve the leu thresholds at each of 3 meals (ED-Whey) daily or unevenly distributed over 3 meals (UD-Whey) with only the dinner meal exceeding the leu threshold. The ED-Whey treatment with evenly distributed leu produced multiple stimulations of MPS throughout the day and larger gastrocnemius muscle weights compared to the UD-whey that only achieved stimulation of MPS at a single meal. While muscle mass was larger in the ED-Whey treatment, while liver weight was greater in the UD-Whey group. Total body composition was not different between the groups. These findings suggest that the ED-Whey treatment resulted in more efficient use of dietary protein for maintaining functional skeletal mass while the UD-Whey group required larger visceral mass to manage the protein and/or nitrogen influx at the single large protein meal.

This research establishes that the leu content of a meal is an important predictor of post-meal MPS response and ultimately impacts adult skeletal muscle mass. These findings raise additional questions for future research including: examining meal frequency to determine the optimal number of MPS stimulations that can be achieved per day to optimize muscle mass. Based on data showing a refractory response to constant elevations in plasma leu, it is unclear when feeding another meal may produce another response or if the refractory response could be overcome by feeding a meal or free form leu or carbohydrate. Additional questions include determining the impact of exercise on the meal responses or the leu threshold. These are important questions that will require further examination.

In summary, while current protein recommendations of quality and quantity are sufficient to establish requirements for limiting amino acids, they do not fully encapsulate the metabolic benefits of amino acids such as leu. These studies demonstrated that there is a threshold of leu intake at a meal required to increase plasma leu and stimulate mTOR signaling and MPS. Furthermore, leu content of complete meals is an important regulator

of MPS and produces different body composition outcomes with long term feeding of diets differing in leu content and distribution.

## **AUTHOR'S BIOGRAPHY**

Layne Norton was born in Evansville, IN on December 15, 1981 to Kim and Ellen Norton. Layne took interest in science at a young age and was particularly fond of Marine Biology. Layne graduated from F.J. Reitz High School in Evansville, IN in 2000 with honors. He attended Eckerd College in St. Petersburg, FL from 2000-2004 with the original intent to earn his BS in Marine Biology. While pursuing his degree in Marine Biology at Eckerd he became very passionate about bodybuilding and in turn, nutrition and metabolism. He elected to pursue his BS in Biochemistry and graduated with honors in May 2004.

During his time at Eckerd Layne's thirst for knowledge and passion for bodybuilding and nutrition continued to grow and he sought to attend graduate school for Nutritional Science research and in particular, protein metabolism. Layne was accepted to the University of Illinois' Nutritional Sciences division in 2004 and began his research under one of the world's foremost experts on protein metabolism, Dr. Donald Layman. He published several peer review articles and original research during his time at the University of Illinois and also gave presentations at symposiums such as Experimental Biology and The International Society of Sports Nutrition. He married his soul mate, Isabel Lago in 2006 while attending Illinois.

Layne won several awards including 1<sup>st</sup> place at the University of Illinois Nutritional Science Symposium poster competition in 2007 and 2008 and was a winner of the ASN/NSC Graduate Student Research abstract competition in 2010. Layne maintained a GPA of 3.8 at the University of Illinois and graduated with his PhD in Nutritional Science in May of 2010.

A big believer in practical application of his research, Layne competed in various bodybuilding and powerlifting competitions during his time at Eckerd College and the University of Illinois, eventually earning pro status in natural bodybuilding and powerlifting.