Targeted Leucine Supplementation and Dietary Protein Distribution Strategies: Applications for Recovery from Exercise in Trained Men, and Supporting Adaptations to Exercise Training in Older Adults

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, with the exception of assistance from colleagues with data collection and data analysis of sections 7.2.4-7.2.6 and 9.3.4-9.3.6. I certify that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

The regulation of skeletal muscle mass and function by nutrition and exercise is of fundamental interest in both athletic performance and healthy aging paradigms. The amino acid leucine has received much interest in recent years due its potential anabolic properties in athletes, and has been described as a pharmaconutrient for the preservation of skeletal muscle health in older adults. While it is well accepted that elevating plasma leucinemia is a potent stimulator of muscle protein synthesis (MPS), less is understood about how supplementation and dietary strategies can be manipulated to create a plasma aminoacidemia most optimal for MPS. Furthermore, the role of leucine-rich interventions on recovery from exercise induced muscle damage in young athletes, and longer term changes in muscle mass and function when administered over time in older adults, is inconclusive to date, and warrants further investigation. Consequently, the aim of this thesis is to contribute to our current understanding of these areas.

Chapter 4 describes the habitual protein intake, distribution and dietary patterns in young and older adults in Ireland. Chapters 5 and 6 describe the plasma leucine kinetics after ingestion of microencapsulated leucine and free leucine, alone and in combination with a low leucine-containing meal in young, healthy males. Chapter 7 demonstrates that leucine supplementation in the 14 hour recovery period after intense resistance exercise exhibits no beneficial effect on markers of muscle damage and recovery of muscle function. Chapter 8 describes a dietary intervention targeting three leucine-rich meals per day can increase protein intake and improve protein distribution in older adults. Finally, chapter 9 establishes that the same dietary intervention in combination with concurrent aerobic and resistance exercise training is an effective strategy to augment increases in lean body mass and lower limb strength in older adults over 12 weeks compared to training alone.

Abbreviations and Definitions

AA – Amino acid
AUC – Area under the curve
BCAA – Branched-chain amino acid
BM - Body mass
CHO – Carbohydrate
CK – Creatine kinase
CON – Control group
DXA – Dual X-Ray Absorptiometry (Method for assessing body composition)
EAA - Essential amino acid
EIMD – Exercise induced muscle damage
FM – Fat mass
FSR – Fractional synthetic rate (A proxy measurement of muscle protein synthesis)
g – Grams
g/kg – Grams per kilogram of body mass (Generally used to quantify amino acid/macronutrient quantity)
g/kg/d – Grams per kilogram of body mass per day (Generally used to quantify daily body mass relative macronutrient intake)
h – Hour/hours
LBM – Lean body mass
LDH – Lactate dehydrogenase
LEU – Leucine
Min – Minutes
mg – Milligrams
MPB – Muscle protein breakdown
MPS – Muscle protein synthesis
mTOR – Mechanistic target of rapamycin (master upstream regulator of the signalling cascade which ultimately results in an increase in muscle protein synthesis)
NEAA – Non-essential amino acid
PLA – Placebo
PRI – Population reference intake (Recommended intake of a given nutrient)
PRT – Progressive resistance training
PS – Protein synthesis
RCT – Randomised control trial
RET – Resistance exercise training
REX – Resistance exercise (acute)
RM – Repetition max
SD – Standard deviation
y – Years
μM – Micromolar (micromole per litre; Used for quantifying amino acid concentration in plasma)

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Chapter 1

1.0 Introduction

1.1 The Regulation of Skeletal Muscle Mass

Skeletal muscle, which constitutes approximately 40% of the body's total weight, is vital for locomotion, physical exertion and metabolic health (Brook *et al.,* 2016; Petersen *et al.,* 2007). In healthy, active adults, skeletal muscle protein turnover occurs at an average rate of 1.2% per day, and the potential maintenance, growth or loss of muscle mass occurs in response to the dynamic balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Atherton and Smith, 2012). After an overnight fast, MPB is elevated above MPS, resulting in an overall negative net protein balance, termed a catabolic state. Once adequate energy and amino acids (AAs) are consumed, MPB decreases and MPS increases, resulting in an overall positive net protein balance, termed an anabolic state (Kreider and Campbell, 2009). When positive net protein balance is achieved over a prolonged period (i.e. several weeks) this results in muscle accretion over time (McGlory and Phillips, 2014; Churchward-Venne, Burd and Phillips, 2012).

1.2 Dietary Protein as an Anabolic Stimulus

Ingestion of dietary protein and AAs act as an anabolic stimulus, increasing rates of MPS above resting values, in a dose-dependent manner in the several hours post-ingestion (Witard *et al.*, 2014; Moore *et al.*, 2009). This characteristic has led to huge interest in the optimisation of protein intake across populations; in particular, for its application in recovery from exercise and support of muscle hypertrophy in athletes, and for mitigating the age-related decline in skeletal muscle mass in older adults. The branched chain amino acid (BCAA), leucine, has been identified as unique in its ability to stimulate the activation of intracellular pathways that mediate MPS and protein translation (Atherton et al., 2010a). Hyperleucinemia, the increase of plasma leucine concentration, after the ingestion of exogenous protein/AAs, is considered a key driver of MPS (Atherton, *et al.*, 2010b; Pasiakos, 2012). As a consequence, leucine is a nutrient that has generated much interest in recent years. In athletic populations, leucine has received attention for its role in the accretion of skeletal muscle, and its potential use in recovery from exercise (Mobley *et al.*, 2017; Stock *et al.,* 2010). Leucine also has application in healthy ageing, and has been described as a pharmaconutrient in the preservation of skeletal muscle health in older adults (Leenders and van Loon, 2011). The role of plasma leucine kinetics in the muscle protein synthetic response remains unclear, with some studies challenge the hypothesis that the kinetics of plasma leucine modulated MPS (Van Vliet *et al.*, 2017; Gorissen *et al.*, 2016; Burd *et al.,* 2015), suggesting that the regulation of MPS by protein ingestion is more complex than solely leucine plasma kinetics. Nonetheless, few studies have investigated the plasma leucine kinetics after the ingestion of leucine in its free form and after varying temporal feeding strategies of leucine.

The ingestion of animal-based proteins has been shown to elicit a robust muscle protein synthetic response when compared to a plant-based protein (Gorissen *et al.*, 2016; Wilkinson *et al.*, 2007; Yang *et al.*, 2012a; 2012b; Tang *et al.*, 2009). However, this does not imply a blanket recommendation for the reliance on animal-based foods for promoting muscle growth and/or maintenance and meeting nutritional needs. The consumption of some processed animal-based foods has been associated with an increased risk of heart disease, stroke, diabetes mellitus and cancers (Chan *et al.*, 2011; Micha, Wallace and Mozaffarian, 2010). Furthermore, an overreliance on animal-based foods poses a threat to environmental sustainability (de Vries and de Boer, 2010). These are some of the reasons that a shift towards plant-based foods in the diet is apparent (Leitzmann, 2014). Plant-based proteins are inherently low in leucine and other essential amino acids (EAAs) and have been shown to exhibit an inferior postprandial muscle protein synthetic response when compared to animal-based proteins. This makes plant-based proteins an appropriate candidate for leucine-enrichment to potentially increase the postprandial muscle protein synthetic response (Gorissen and Witard, 2018; van Vliet, Burd and van Loon, 2015). Despite the ever-growing research interest of enrichment of plant-based foods with leucine, the plasma AA kinetics after the ingestion of plant-based food in combination with leucine has not been investigated.

1.3 Amino Acids in the Recovery from Exercise Induced Muscle Damage

Intense exercise can result in dull aching pain, tenderness and stiffness and strength loss in the days following an exercise bout. This is often termed delayed-onset muscle soreness (DOMS) and is in large part a consequence of exercise-induced muscle damage (EIMD) (Cheung, Hume and Maxwell, 2003; Connolly, Sayers and Mchugh, 2003) . A nutritional strategy that is efficacious in ameliorating the deleterious effect of EIMD may be advantageous in minimising the compromised quality of exercise sessions in the subsequent days following this muscle-damaging exercise bout. The use of protein and essential amino acids (EAAs) supplemented in the post-exercise window is emerging as one such potential strategy (Howatson *et al.*, 2012; Cockburn *et al.*, 2008; Greer *et al.*, 2007; Matsumoto *et al.*, 2007). The exact mechanism that underpins this enhancement in recovery with AA ingestion is not fully understood. It has been suggested that an increase in post-exercise MPS after protein feeding elicits an increase in muscle recovery. However, since the remodelling of myofibrillar proteins is a lengthy process, it is unlikely that this remodelling process would result in significant benefits on recovery in the several days following exercise. Nonetheless, protein, and in particular, BCAAs have been shown to elicit a benefit on recovery from EIMD (Howatson *et al.*, 2012; Cockburn *et al.*, 2008; Greer *et al.*, 2007; Matsumoto *et al.*, 2007). The efficacy of supplementary leucine alone, or the

enrichment of meals, in ameliorating the negative effects on an intense exercise bout, is underexplored, and warrants further investigation.

1.4 Adjunct Nutrition Strategies for Maximising Muscle Protein Synthesis

While current recommendations on protein intake are given on a gram per body mass (g/kg) per day basis, there is an increased emphasis on the importance of per meal protein recommendations (Layman *et al.*, 2015). The cumulative magnitude and duration of postprandial MPS after the ingestion of each protein meal dictates the time spent in a positive net protein balance over the course of a day (Layman *et al.*, 2015; Areta *et al.*, 2013; Paddon-Jones, 2004). For that reason, it is accepted that maximising the MPS response to each meal results in a greater cumulative daytime stimulation of MPS, and therefore more time spent in a positive net protein balance, which is favourable for muscle accretion. A minimum of 20-30g per meal, or 0.24g/kg per meal in young, and 0.4g/kg in old, has been shown to maximise MPS after a single meal (Witard *et al.*, 2014; Moore *et al.*, 2015; 2009; Symons *et al.*, 2009). This has led to the hypothesis of distributing daily protein evenly over ~4 meals, ensuring each meal reaches this purported meal threshold, is optimal for supporting recovery and adaptation to athletic training, and the preservation of muscle health with advancing age (Morton, McGlory and Phillips, 2015; Breen and Phillips, 2011). Indeed, a more 'even' distribution of protein (e.g. 4 x 20g) throughout the day can result in a greater cumulative anabolic response compared to smaller and frequent meals, or larger and less-frequent protein meals in young (Areta *et al.*, 2013; Moore *et al.*, 2012). The habitual 'skewed' distribution of protein intake in which breakfast and lunch often fall under this meal threshold, while dinner significantly surpasses it (Cardon-Thomas *et al.*, 2017; Tieland *et al.*, 2015) is therefore seen as suboptimal for maximising MPS (Paddon-Jones *et al.*, 2015; Mamerow *et al.*, 2014). Moreover, while protein dose plays an important role in stimulating postprandial MPS response, the source of that protein also plays a role in the magnitude and duration of that response. Indeed, it is well supported that animal protein, which is inherently higher in leucine and other EAAs, stimulates a robust MPS response when compared to and equivalent dose of plant-based protein (Gorissen *et al.*, 2016; Wilkinson *et al.*, 2007; Yang *et al.*, 2012a; 2012b; Tang *et al.*, 2009). Along with total daily protein intake, the protein dose per meal, protein distribution throughout the day and protein source are additional factors that need to be considered when aiming to refine protein recommendations to optimise athletic performance and manage the age-related decline of muscle mass in elderly.

1.5 Minimum is Not the Same as Optimum

The regulation of muscle mass by nutrition is a fundamental consideration in both athletic performance and healthy aging paradigms (Burd, Gorissen and Van Loon, 2013). Nutrition interventions that maximise MPS, thereby facilitating this shift into a positive net protein balance, have potential application in recovery and athletic performance and in the preservation of muscle mass and function in elderly populations (Kreider and Campbell, 2009; Paddon-Jones and Rasmussen, 2009). Protein recommendations in public health terms are currently given on 'one size fits all' basis, with young adults, older adults and athletes considered to have the same protein requirements (European Food Safety Authority, 2012). However, there is a wealth of research, particularly from acute metabolic studies, to suggest that these recommendations are insufficient, for both athletes and older adults (Thomas, Erdman and Burke, 2016; Bauer *et al.*, 2013). When we consider the population reference intake (PRI) of protein, we should be cognisant that these are minimum requirements, and 'minimum' is not the same as 'optimum' in many circumstances. This is particularly relevant for athletes, whose primary goals are optimal adaptation to training and ultimately, maximal athletic performance; and also older adults whose aim is to age well (i.e. by attenuating the decline in functional capacity).

1.6 Age-Related Decline in Muscle Mass

The age-related decline in skeletal muscle mass and function in older adults is a fundamental threat to ageing well and maintaining independence and quality of life throughout the lifespan (Fielding *et al.*, 2011; Janssen, Heymsfield and Ross, 2002). However, the regulation of muscle mass is a process which is modifiable, particularly through exercise and nutrition strategies (Norton *et al.*, 2016; Daly *et al.*, 2014; Liu and Latham, 2010; Onambélé-Pearson, Breen and Stewart, 2010). Therefore, appropriately-designed recommendations around physical activity and diet show promise in ameliorating the loss in muscle mass and function associated with advancing age. While resistance exercise is well-accepted as a means to increase lean body mass (LBM) and strength in older adults (Onambélé-Pearson, Breen and Stewart, 2010), the optimal nutrition strategy for the growth and/or preservation of skeletal muscle in older adults remains to be established. There is a wealth of research, particularly from acute metabolic studies, which suggests the current protein recommendations are insufficient for older adults (Traylor, Gorissen and Phillips, 2018; Bauer *et al.*, 2013), particularly because they exhibit a dampened muscle protein synthetic response to the ingestion of EAAs, also termed 'anabolic resistance' (Wall *et al.,* 2015; Katsanos *et al.,* 2006; Katsanos *et al.,* 2005; Cuthbertson, 2004; Volpi *et al.*, 2000). Nutrition strategies to overcome this anabolic resistance include an increase in per meal protein intake, to optimising daily protein distribution, favouring higher leucine animal-based food over plant-based foods, and the enrichment of lower leucine meals with supplementary leucine to increase the anabolic

potential of that meal (Gorissen and Witard, 2018; van Vliet, Burd and van Loon, 2015). Despite these efforts, an appropriate nutrition strategy in ameliorating the age-related decline in muscle mass, particularly one that is pragmatic for older adults in a real-world setting, remain unclear. Moreover, intervention studies, investigating the effect of supplementary protein on changes in LBM and physical function, have traditionally focused on powdered supplements, as opposed to whole food. There is evidence to suggest that protein its whole-form may exhibit superior anabolic properties; such is the case in young, for whole egg vs. egg whites (Van Vliet *et al.*, 2017), and whole-milk vs. skimmed-milk (Elliot *et al.*, 2006). A whole food-based intervention may therefore show promise in augmenting exercise-mediated changes in LBM and physical function in older adults, but this approach remains underexplored to date.

In summary, while it is well-accepted that leucine plays a key role in the regulation of MPS in the hours after ingestion, its effect on postprandial plasma AA kinetics, recovery from EIMD in young athletes, and longer term changes in muscle mass and function when administered over time in older adults, is underdeveloped, and these gaps in knowledge warrant further investigation.

1.7 Thesis Overview

The central theme of this thesis is supplementation with the branched chain amino acid leucine, as well as dietary protein distribution strategies, and their applications in recovery from exercise, and supporting adaptations to exercise training in older adults. The main research questions of this thesis are: (1) What is the current habitual dietary protein intake and protein distribution in Irish adults? (2) How do different leucine supplementation strategies affect postprandial plasma leucinemia? (3) What is the application of these strategies in recovery from intense resistance exercise in young healthy males? (5) Can a high protein diet, targeting leucine-rich meals, augment exercise-mediated effect on LBM and function over a 12-week period in older adults?

1.7.1 Chapter 4: Habitual protein intake, protein distribution patterns and protein source across the lifespan in Irish adults between 2008 and 2010

Aim: The aim of this current study was to determine age and gender patterns for overall protein intakes, protein distribution and protein sources across the lifespan in Irish adults. **Overview:** The National Adult Nutrition Survey (NANS) investigated habitual food and beverage consumption in 1500 adults aged 18-90 years, in the Republic of Ireland, between 2008 and 2010. This current study was secondary analysis of the

NANS database. Using SPSS statistical software package, patterns for total, body mass relative and per meal protein intake, between genders and across age categories were determined. Protein distribution was assessed, in which the number of meals reaching ≥20g and ≥30g protein per day; and ≥0.24, ≥0.3 and ≥0.4g/kg body mass protein per day was calculated. The source of protein intake across 16 food groups, and between animal- and plant-based proteins were also determined.

1.7.2 Chapter 5: The plasma leucine kinetics after an oral load using micro-encapsulated leucine in young, healthy males

Aim: The aim of this study was to investigate the plasma kinetics of leucine in response to consuming leucine in either its free form or in a novel microencapsulated form, in comparison to that of a maltodextrin control. **Overview:** This study was a randomised control trial with a cross-over design, with five experimental conditions; A bolus of 3g free leucine (BOLUS), a bolus of 1.5g free leucine, and a bolus of 1.5g free leucine consumed 2 hours later (PULSE), 1.5g microencapsulated targeted-release leucine + 1.5g free leucine with (ME LEU+LEU), 3g microencapsulated, targeted-release leucine (ME LEU), 3g maltodextrin (CONTROL). Ten healthy active males, (aged 26.4±4.1 years) visited the laboratory on five separate occasions, consuming one of each condition. Blood samples were taken at 15, 30, 45 and 60 min, and every 30 min thereafter for the subsequent 3 hours. Blood samples were later analysed for plasma AA concentrations using high performance liquid chromatography.

1.7.3 Chapter 6: The plasma leucine kinetics after oral loading using leucine-enrichment of a low-leucine mixed meal, in young, healthy males

Aim: The aim of this study was to investigate the plasma kinetics of leucine in response to consuming a low-leucine mixed meal enriched with free leucine. **Overview:** This study was a single-blind placebo control trial, with a cross-over design, with three experimental conditions; Bolus-fed free leucine (BOLUS), Pulse-fed free leucine (PULSE) and Placebo - Maltodextrin (CON); with a low leucine meal. Five healthy active males, (age 25.6 years ± 2.4 years), visited the laboratory on three separate occasions, in which they were randomly assigned to one of three conditions. Blood samples were taken at 15, 30, 45 and 60 min, and every 30 min thereafter for the subsequent 3 hours. Blood samples were later analysed for plasma AA concentrations using high performance liquid chromatography.

1.7.4 Chapter 7: The effect of leucine supplementation on delayed onset muscle soreness and recovery of muscle function after intense exercise

Aim: The aim of this study was to determine whether recovery from resistance exercise in young resistance trained males can be enhanced by either intermittent or bolus feedings of leucine during recovery. **Overview:** This study was a double-blind placebo-controlled cross-over design, including three different experimental conditions; Bolus feeding (BOLUS) - 3g free leucine every 4 hours for 14 hours, pulse feeding (PULSE) - 1.5g free leucine fed every 2 hours for 14 hours, a control group (CON) - 3g maltodextrin fed every 2 hours for 14 hours. Thirteen healthy, active males (age 25.5 years ± 5.2 years) performed 10 sets of 10 repetitions of a leg press at 60% 1RM. Participants were given one of the three supplementation strategies to follow during the 14-hour post-exercise period in addition to a low-leucine meal plan. Plasma concentrations of creatine kinase and lactate dehydrogenase; ratings of perceived soreness and recovery; and performance measures using a counter-movement jump; were assessed immediately before, after, 24 hours after and 48 hours after the exercise bout.

1.7.5 Chapter 8: The effect of a nutrition intervention targeting leucine-rich meals on changes in calorie, macronutrient and micronutrient intake, and protein distribution in older adults

Aim: The aim of the present study was to determine if instruction on a nutrition intervention, prescribing the equivalent of 3g of leucine per meal, can augment protein intake and protein distribution in older adults. **Overview:** This study was a randomised control trial with three groups: Exercise and Nutrition group (EX+NUTR), Nutrition only group (NUTR ONLY), Exercise only group (EX ONLY). Fifty-six community-dwelling older males and females (m/f, 28/28; age, 69.3±4.0 years) were randomly assigned to one of three groups for 12 weeks. NUTR+EX and NUTR were instructed to follow a leucine-rich dietary strategy, in which meals equating to 3g of leucine were translated to user-friendly portion sizes. EX were asked not to change their diet for the duration of the study. All participants completed a 3-day portion estimate food diary before (week 0), during (week 6) and after (week 12) the 12-week intervention, which were later analysed (using Nutritics Nutrition Analysis Software) to determine energy, macronutrient and micronutrient intake. Protein distribution was assessed, in which the number of meals reaching ≥20g and ≥30g protein per day; and ≥0.4g/kg body mass protein per day was calculated.

1.7.6 Chapter 9: The effect of concurrent exercise training and/or a high protein diet intervention targeting leucine-rich meals on body composition and physical function in older adults

Aim: The aim of this study was to determine if the nutrition intervention prescribed in chapter 8 augmented exercise training-mediated effects on LBM and physical function in older adults. **Overview:** This study was performed in combination with the analysis in chapter 8. Participants in NUTR+EX and EX performed exercise training, which consisted of 24 min of combined resistance and aerobic exercise per session, three times per week for 12 weeks. Body composition and physical function were assessed before (week 0), during (week 6) and after (week 12) the 12-week intervention.

Chapter 2

2.0 Review of Literature

2.1 Introduction

The aim of this chapter is to (1) Summarise the current understanding of the role of dietary essential amino acids (EAAs), in particular leucine, in muscle protein synthesis (MPS); (2) Discuss the potential ways in which this understanding can be used to maximise the anabolic response to a meal, namely protein quality, protein quantity, protein distribution and leucine-enrichment; (3) Discuss the potential of these strategies to delay the age-related decline in muscle mass and contribute to the preservation of muscle mass and muscle function in older adults; (4) Identify the potential of these strategies to ameliorate the deleterious effects of exercise induced muscle damage (EIMD) and thereby enhance recovery from an intense training bout in athletic populations.

2.2 Muscle Protein Turnover

2.2.1 Muscle Protein Synthesis and Muscle Protein Breakdown

Muscle protein remains in a state of constant remodelling through the dynamic processes of the formation of new proteins and the degradation of existing proteins. This state, termed muscle protein balance, is regulated by MPS and muscle protein breakdown (MPB), that both fluctuate over the course of a typical day (McGlory and Phillips, 2014; Norton and Layman, 2006). When the magnitude of MPS outweighs that of MPB, a positive protein balance is achieved, which is conducive to muscle accretion over time. In contrast, when MPB outweighs that of MPS, a negative protein balance ensues, which over time can result in muscle loss (Damas *et al.*, 2015; Koopman *et al.*, 2007). These processes are sensitive to external stimuli, in particular, the intake of dietary protein (Atherton and Smith, 2012). During periods of fasting (between meals or after an overnight fast) MPB is elevated above MPS. When a protein meal is eaten, MPS is elevated above MPB, as per **figure 2.1**.

The importance of dietary protein in the regulation of skeletal muscle mass is based on the principle that the amino acids (AAs) within the matrix of dietary protein are used to synthesise new muscle protein, which are incorporation into the skeletal muscle cell (Kreider and Campbell, 2009). The way in which dietary protein ultimately signals the synthesis of new proteins will be discussed in detail in **section 2.4**. Much of the research to date has focused predominantly on MPS as opposed to MPB, as a proxy for measuring muscle protein turnover, mainly because MPS is more sensitive to the external stimuli such as nutrient and exercise, in some instances, 4-5 fold more responsive than MPB to the same stimulus (Damas *et al.*, 2015; McGlory and

Phillips, 2014). Furthermore, MPB is technically challenging to measure compared to MPS. MPB can be determined by assessing the dilution of amino acid tracer across the limb, in conjunction with blood flow. However, blood flow can be difficult to measure and the calculation of MPB involves the use of complex mathematical modelling (McGlory and Phillips, 2014; Atherton and Smith, 2012). Quantifying MPS is therefore widely used as a means of determining the efficacy of nutrition and training strategies in skeletal muscle remodelling and the potential for muscle accretion over time.

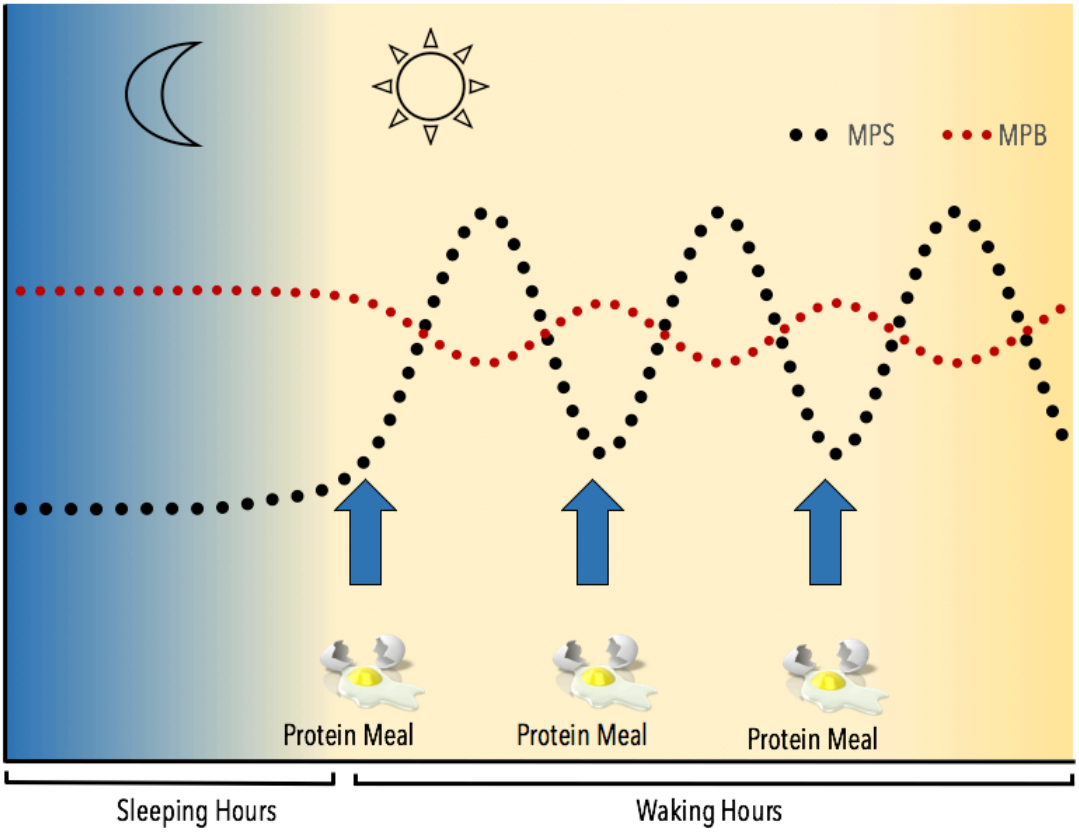


Figure 2.1: Graphic representation of the daily fluctuations of muscle protein synthesis (black dotted line) and muscle protein breakdown (red dotted line), which affect net protein balance. Fasting acts as a stimulus for muscle protein breakdown, while dietary protein acts as a stimulus for muscle protein synthesis.

2.2.2 Quantifying Muscle Protein Synthesis

The use of stable isotopes is now most widely used as a measure of MPS. Isotope tracer method involves the use of non-radioactive stable isotopes, which are identical to exogenous AAs, but can be differentiated based on their mass. Through muscle biopsy, the incorporation of isotopically labelled AAs, such as [1,2-$^{13}C_2$] leucine and [$^{13}C_6$] phenylalanine, into the skeletal muscle can be identified, therefore quantifying MPS (McGlory and Phillips, 2014; Atherton and Smith, 2012). Fractional synthetic rate (FSR) is most frequently used to quantify

the rate of synthesis of new proteins (% per h) in the muscle compartments. Using this technique, incorporation of the labelled tracer can be identified in the sub fractions of muscle, such as the contractile myofibrillar proteins (e.g. actin, myosin and troponin) and the non-contractile myofibrillar proteins (e.g. sarcoplasmic and mitochondria)(Witard *et al.,* 2016). However, this measurement is carried out in a controlled lab environment over several hours, and often requires several muscle biopsies, and is therefore not representative of a free-living environment. A new method of assessing MPS, involves the use of deuterated water (D_20) and has the ability to assess MPS over several weeks to months, outside of a lab setting (McGlory and Phillips, 2014; Atherton and Smith, 2012). This method involves the ingestion of D_20, and the measurement of the incorporation of deuterium into skeletal muscle. While this technique enables the collection of data in a free-living setting; nutrition, exercise and other lifestyle factors are therefore difficult to control.

2.3 Current Protein Recommendations

2.3.1 Current Protein Recommendations

Nitrogen balance studies have traditionally been used as method for quantifying protein requirements (Rand, Pellett and Young, 2003) and have informed the current population reference intake (PRI) for dietary protein, which now stands at 0.83 grams per kilogram body mass per day (g/kg/d) (European Food Safety Authority, 2012). The premise of using nitrogen balance studies for quantifying protein requirements is that protein is the only nitrogen-containing macromolecule in the body. Through monitoring nitrogen intake and nitrogen excretion, nitrogen balance, and therefore an individual's minimum protein requirement can be determined (Rand, Pellett and Young, 2003). A major limitation of this technique is accounting for the various routes of nitrogen intake and loss from the body (Bauer *et al.*, 2013). Furthermore, the studies which inform protein recommendations use predominantly young adults in good health and in energy balance, therefore not accounting for populations, particularly, athletes and older adults (Traylor, Gorissen and Phillips, 2018; Witard *et al.*, 2016). With the recent advances in techniques for assessing skeletal muscle metabolism, our understanding of protein requirements across populations has changed. In particular, the indicator amino acid oxidation (IAAO) method is emerging as an adjunct technique in assessing protein requirements. This is a technique in which a labelled tracer amino acid is infused and its rate of oxidation is observed and correlated to protein requirements (Humayun *et al.*, 2007). When protein intake is insufficient, the rate of incorporation of the tracer AA into the cell for MPS will be limited, and the tracer AA will remain in the free AA pool and be

oxidised. As protein intake increases and approaches sufficiency, the rate of oxidation of the tracer AA will decrease, eventually reaching a plateau when the sufficient protein is reached.

The IAAO method has determined that protein requirements in young adults are indeed higher than the current population reference intake (PRI), at 0.93g/kg (Humayun *et al.*, 2007). While the current recommendations inform protein requirements to prevent deficiencies across the entire population, minimum requirement is not the same as optimal requirement, and the current recommendations potentially fail to address what is optimal and enables an individual to thrive. This is particularly relevant for athletes, whose primary goals are optimal adaptation to training and ultimately, maximal athletic performance (Phillips and van Loon, 2011). Furthermore, there are additional factors which may call for even higher protein needs in athletes, such as, their chosen sport, energy intake, physique characteristics, performance goals, training volume, intensity, frequency, phase and goals (Egan, 2016; Thomas, Erdman and Burke, 2016; Tipton and Wolfe, 2004). As a consequence, there is an emerging recognition that 1.2g to 2g/kg/d of dietary protein represents a more appropriate recommendation for athletic populations (Egan, 2016; Kato *et al.*, 2016; Thomas, Erdman and Burke, 2016; Morton, McGlory and Phillips, 2015). Resistance trained athletes may require more protein than their endurance trained counterpart, and protein intake at the highest range may offer benefit in minimising skeletal muscle loss during periods of a caloric deficit (Phillips and van Loon, 2011).

A further limitation of the current recommendations is that it does not account for older adults who exhibit a blunted muscle protein synthetic response to ingestion of exogenous protein, when compared to their younger counterpart, a phenomenon termed 'anabolic resistance' (Wall *et al.,* 2015; Katsanos *et al.*, 2006; 2005; Cuthbertson, 2004; Volpi *et al.*, 2000). The IAAO method has estimated that protein requirements are 0.94-1.24g/kg in older males and 0.96-1.29g/kg in older women (Traylor, Gorissen and Phillips, 2018; Rafii *et al.*, 2016; 2015). As a result of these acute metabolic studies, it is well accepted that older adults have higher protein requirements than the current PRI, closer to 1.2g/kg/d (Bauer *et al.*, 2013), which will be discussed further in a later section.

In summary, protein requirements vary largely, and need to be individualised based on the nature of the training stimulus and goals in athletic populations. Meanwhile, older adults possess a higher protein requirement, predominantly due to the presentation of anabolic resistance. These unique requirements are

not reflected in current 'one size fits all' recommendations, which, based on ample evidence, necessitate revision.

2.4 The Role of Nutrition and Exercise in Muscle Protein Synthesis

2.4.1 Dietary Protein and Muscle Protein Synthesis

Dietary protein is a potent anabolic stimulus that results in an increase in MPS in the postprandial (post-meal) period (Witard *et al.*, 2014; Moore *et al.*, 2009). Essential amino acids (EAAs) in particular are responsible for eliciting this postprandial anabolic response, whilst non-essential amino acids (NEAAs) do not elicit this same stimulation in MPS (Smith *et al.*, 1998). Leucine is an essential amino acid that not only acts as a substrate for the synthesis of skeletal muscle, but is also involved in the initiation of a signalling cascade which ultimately results in an increase in MPS, above postabsorptive (period following absorption of nutrients, i.e. between meals) values (Haran, Rivas and Fielding, 2012; Pasiakos, 2012).

2.4.2 Leucine and mTOR Signalling

Increased availability of AAs in the plasma and intramuscular compartment triggers MPS. EAAs act as an anabolic stimulus, with leucine in particular playing a key role in initiating this anabolic response by the activation of proteins that mediate the process of MPS (Pasiakos, 2012; Atherton et al., 2010a). Following AA uptake via large neutral amino acid transporter (LAT1), leucine directly binds Sestrin-2 and CASTOR1, respectively, relieving their repression of GATOR2. Sestrin-2 is seen as the primary leucine sensor for the activation of mTORC1 (Wolfson and Sabitini, 2017). In turn, GATOR2 inhibits GATOR1, which maintains Rag GTPases in their respective inactive nucleotide bound configurations. GATOR1 and Rag GTPases are tethered to the lysosomal surface by the ragulator and KICSTOR complexes, respectively. Once activated, Rag proteins bind Raptor on mechanistic target of rapamycin complex 1 (mTORC1), thus facilitating its translocation to the lysosomal surface where it interacts with Rheb, an obligitary activator (Saxton and Sabitini, 2017). mTORC1 subsequently regulates mRNA translation via the phosphorylation of its downstream effectors; 1. The activation of p70 S6 kinase; 2. The activation of the repressor of mRNA translation, eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1). mTORC1 phosphorylates and activates S6K1, which enhances translation initiation. In addition, mTORC1 also phosphorylates and inactivates 4E-BP1, a protein that binds to eIF4E, thereby blocking the formation of eIF4E-eIF4G, a complex involved in upregulating translation initiation. However, when phosphorylation of 4E-BP1 occurs, eIF4E is released, binding to eIF4G and forming eIF4E-eIF4G. This

complex upregulates mRNA binding to the 40S ribosomal subunit, resulting in increased translation initiation, and therefore MPS (Laplante and Sabatini, 2012). Both of these signalling cascades ultimately result in the initiation of protein translation in the overall process of MPS, and thus, increased formation of new proteins. Leucine is unique in its ability to stimulate the signalling cascade associated with MPS. Leucine increases the phosphorylation of mTOR and 4E-BP1, 1.7-fold and 2.5-fold, respectively, with other AAs exhibiting no effect on change in phosphorylation of these signalling proteins (Atherton et al., 2010a). Leucine also exhibited a 6-fold greater increase in signalling through p70 S6 kinase, compared to 1.6-2-fold for other EAAs (Atherton et al., 2010a). Due to the anabolic potential of leucine, it remains a nutrient that has received much interest in recent years, both in athletic populations and in ameliorating age-related atrophy in older adults.

2.4.3 The Role of Insulin in MPS

Leucine stimulates MPS via an insulin-dependent and insulin-independent pathway (Haran, Rivas and Fielding, 2012), but insulin is unlikely to be the primary mediator of MPS after AA ingestion. Leucine is seen to stimulate MPS when insulin is kept constant (Atherton et al., 2010b). Hyperaminoacidemia (an increase in plasma amino acid concentration above baseline) shows a dose dependent relationship to MPS, without any increase in plasma insulin (Cuthbertson *et al.* 2005). Hyperinsulinemia promotes MPS only when there is an increase of muscle blood flow, AA delivery and availability (Fujita *et al.*, 2006). Insulin is therefore suggested to be permissive of MPS as oppose to modulatory (Haran, Rivas and Fielding, 2012). Insulin may instead be more effective in preventing MPB, and it has been shown that MPB is inhibited with an increase in plasma insulin, and in the absence of AA availability (Abdulla *et al.*, 2016). In conclusion, insulin is seen as permissive to MPS, as opposed to its concentration exhibiting a direct influence on MPS.

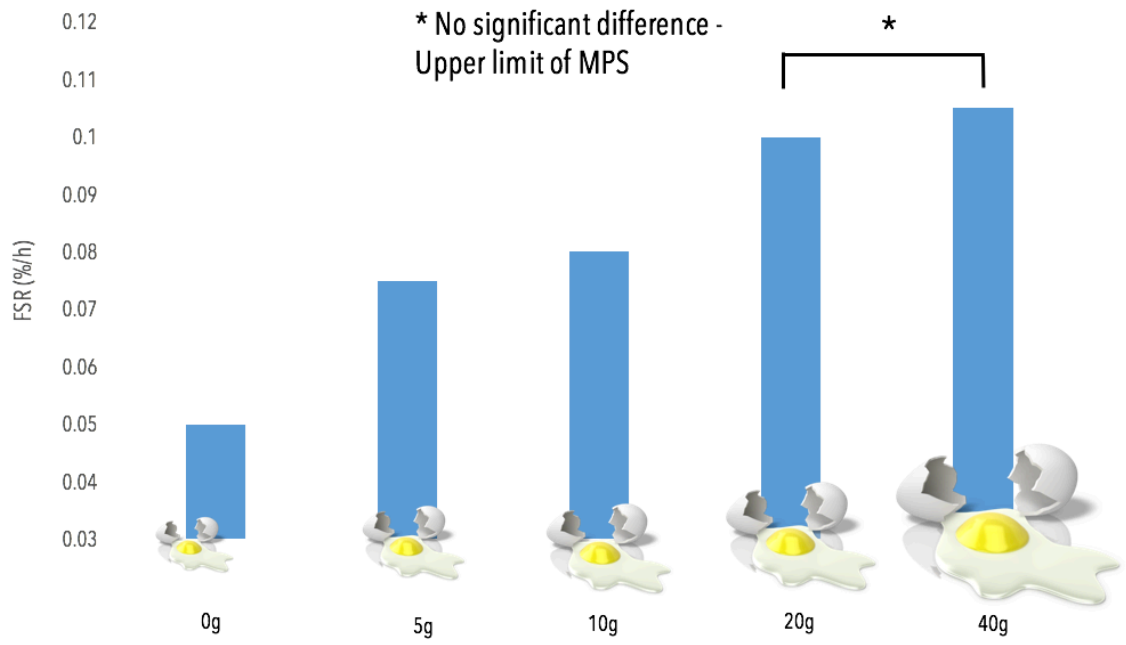


Figure 2.2: Mixed muscle fractional synthetics rate (FSR) after the ingestion of 0g, 5, 10g, 20g and 40g egg protein, in young healthy males, after exercise. Adapted from Moore *et al.*, (2009). MPS, muscle protein synthesis.

2.4.4 The Leucine Threshold Hypothesis and The Upper Limit of Muscle Protein Synthesis

It is well established that the ingestion of exogenous EAAs stimulates the mTOR pathway and acutely elevates MPS above postabsorptive values (Dreyer *et al.*, 2008; Fujita *et al.*, 2007). Hyperaminoacidemia has been shown to directly modulate MPS rates in a dose-dependent manner (Witard *et al.*, 2014; Moore *et al.*, 2009; Cuthbertson, 2004). When EAAs are ingested, thereby causing a sufficient increase in plasma aminoacidemia and leucinemia (plasma leucine concentration), MPS increases above postabsorptive values. The point at which mTOR signalling and MPS is significantly elevated above postabsorptive values is termed the 'leucine threshold' (Breen and Phillips, 2011). This threshold is not absolute, and has been shown to differ between young and old. While the leucine threshold is purported to be ~1g in young, older adults exhibiting a decreased sensitivity to increases plasma leucinemia, and therefore a higher leucine threshold of >2g, the quantity found in 20g high quality protein (Phillips, 2015). As a result of the key role played by leucine in the muscle protein synthetic response to a meal, the use of crystalline leucine as a supplement, and in particular, the enrichment of lower leucine foods, has received much interest in recent years (Murphy *et al.*, 2016; Trabal *et al.*, 2015; Casperson *et al.*, 2012; Verhoeven *et al.*, 2009). Adding leucine to a lower leucine meal may 'rescue' the inferior anabolic response of that lower leucine meals. Indeed, a 3g EAA beverage rich in leucine (1.2g), has been shown to cause a postprandial stimulation in MPS, similar to that of 20g whey protein

(Bukhari *et al.*, 2015). Similarly, 6g EAA, containing 2.4g leucine, stimulates MPS to the same extent as 40g whey protein in older adults (Wilkinson *et al.*, 2017). This is particularly relevant in the case of plant-based protein, which are inherently lower in leucine, and therefore result in an inferior muscle protein synthetic response when compared to the ingestion of isonitrogenous animal-based proteins (Burd *et al.*, 2012; Yang *et al.*, 2012a; 2012b; Tang *et al.*, 2009). Leucine-enrichment, as a nutrition strategy for increasing the anabolic potential of a meal, will be discussed further in **section 2.6.3.**

Witard *et al.*, (2014) sought to determine myofibrillar protein synthesis with graded dose of whey protein. 0g, 10g, 20g, and 40g, containing 0g, 1.1g, 2.2g, 4.4g leucine, respectively, was ingested 10 min after resistance exercise. 10g of whey protein did not cause an increase in MPS above values apparent after the ingestion 0g whey. The ingestion of 20g and 40g whey resulted in a 49% and 56% increase in MPS, respectively, compared to 0g whey. There was no statistically significant difference between myofibrillar FSR after the ingestion of 20g and 40g whey. Therefore, the equivalent of 20g of a high quality protein source is likely to be sufficient to maximise MPS. Similarly, Moore *et al.* (2009) investigated rates of mixed muscle FSR in response to egg protein, containing 0g, 0.42g, 0.84g, 1.68g and 3.36g of leucine, respectively. Plasma leucine concentration peaked at 101, 120, 146 and 167μmol/L with the ingestion of 0g, 5g, 10g, 20g and 40g at 45 min, after the ingestion of 5g, 10g, 20g and 40g protein, respectively. Mixed muscle FSR increased by ~37%, ~56%, ~93% and ~93% after the ingestion of 5g, 10g, 20g and 40g egg protein, above fasted values, as per **figure 2.2**. In accordance with finding by Witard *et al.* (2014), MPS reached a plateau after the ingestion of 20g protein, and there was a marked increase in leucine oxidation after the ingestion of 40g protein. This suggests that when dietary protein is ingested in excess of the rate needed for incorporation into skeletal muscle, irreversible oxidation occurs. This 20g dose of whey protein equates to approximately 10g EAAs, which is consistent with studies demonstrating that 10g EAAs maximally stimulates MPS (Glynn *et al.*, 2010; Moore *et al.*, 2009; Cuthbertson, 2004). Glynn *et al.* (2010) compared the ingestion of 10g EAA enriched with 1.7g leucine with 10g EAA enriched with an additional 2.8g leucine. There was no difference in area under the curve (AUC) of mixed muscle FSR or net protein balance between groups. In this study, 1.8g leucine in an AA mixture was sufficient to elicit maximal MPS, such that further increase in leucine content (3.5g in total) did not have an additive effect on MPS or net protein balance. In summary, MPS is stimulated in a dose dependent manner to graded increases in dietary protein. However, MPS has an upper limit of activation in response to a given quantity of leucine, meaning above a certain ingested dose or resultant plasma concentration of leucine, MPS is not further stimulated.

2.4.5 The Refractory Period and The Muscle Full Hypothesis

Not only does MPS possess an upper limit of activation, but muscle can also exhibit a 'refractory period' during this upper limit, in which MPS cannot be further stimulated despite plasma leucine concentration remaining elevated (Glynn *et al.,* 2010; Moore *et al.,* 2009; Cuthbertson, 2004). This concept was well demonstrated by Bohé *et al.* (2001). During a constant AA infusion over 6 h, MPS responded after 30-60 min and peaked with a 2.8-fold increase for 1.5 h. Plasma AA concentrations remained elevated during the 6 h infusion, but rates of MPS returned to baseline 4 h after the infusion had commenced. In short, MPS increases transiently, but after a time, muscle exhibits a 'refractory' response to sustained elevation in plasma leucine concentration. Thereafter, MPS returns to baseline and cannot be further stimulated (Atherton, *et al.*, 2010a; Bohé *et al.,* 2001). Atherton *et al.* (2010a) demonstrated myofibrillar FSR rate in response to ingestion of 48g whey protein in a single bolus. Similar to findings by Bohé *et al.* (2001), MPS increased 3-fold at 45-90 min but returned to baseline by 120 min despite plasma AA concentrations remaining elevated. Interestingly, mTORC1 signalling and S6K and IF4eF phosphorylation remained elevated despite the decline in MPS, mimicking the rise and decline of plasma AA concentrations, and returning to baseline after >180 min. This suggests that discordance exists between signalling and MPS, which has been reported elsewhere (Glynn *et al.*, 2010; Norton *et al.*, 2009) and a drop in intracellular signalling regulating MPS does not explain the refractory response of muscle to sustained hyperaminoacidemia (Atherton et al., 2010b). Despite elevated plasma and intramuscular leucine, MPS declined, which suggests that the muscle possesses a mechanism that monitors its capacity to synthesise new proteins, and terminates the process when a limit is reached. The phenomenon is termed the 'muscle full' hypothesis (Atherton et al., 2010b). This hypothesis is based on the 'bag full' theory first coined by Millward, (1995). Millward akined the endomysium which surrounds each muscle fibre to a bag. Due to the minimal elasticity of the endomysium, the 'bag' has a fixed volume, which limits the production of muscle proteins after a certain capacity has been reached. This refractory characteristic of muscle in response to a sustained concentration of plasma EAA, has led to the hypothesis that pulse fed AAs is more appropriate than a continuous supply of AAs, in maximising MPS. In this instance, pulse-fed refers to the ingestion of intermittent boluses of AAs, separated by a time period, while bolus-fed tends to be greater doses on AAs, separated by greater time periods. Bolus-fed is most often similar to a normal diet, in which meals are separated by several hours.

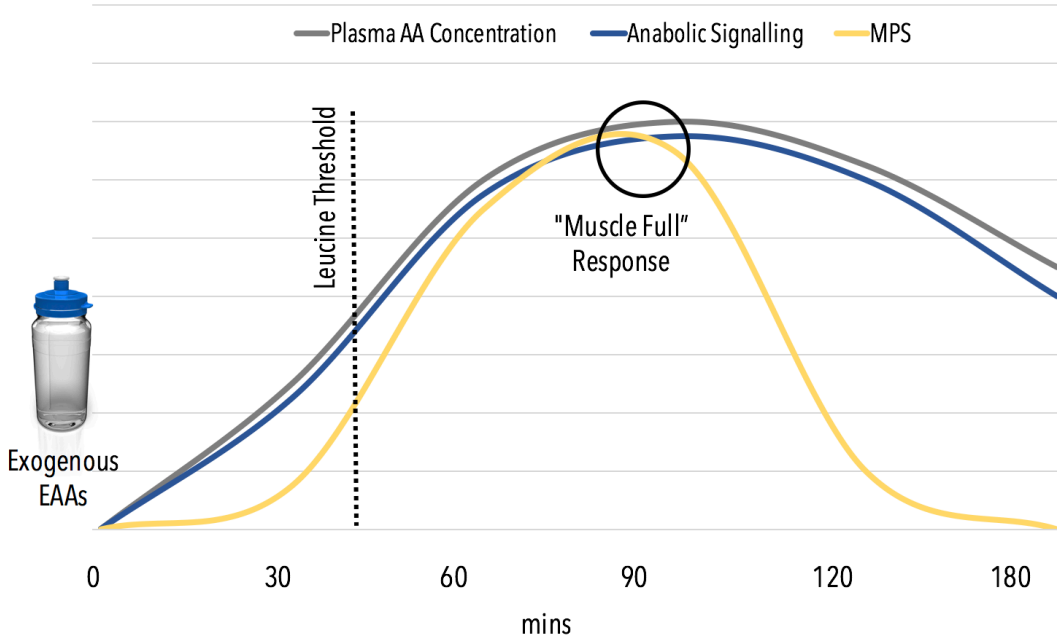


Figure 2.3: Graphic representation of the leucine threshold* and the discordance between sustained elevation of plasma amino acids* and muscle protein synthesis*, termed the muscle full response*. Despite plasma aminoacidemia and anabolic signalling* remaining elevated after the ingestion of exogenous amino acids, MPS returns to baseline after 90 min. MPS, muscle protein synthesis; *, arbitrary figures.

2.4.6 Protein Timing and Meal Distribution

The refractory characteristic of muscle to a sustained concentration of plasma EAA, has led to the hypothesis that feeding sufficient amounts of AAs in one bolus, is more appropriate than a continuous supply of AAs, in maximising MPS. West *et al.* (2011) compared a single 25g whey protein bolus (BOLUS), to 2.5g whey fed every 20 min (PULSE) on rates of MPS in the post-exercise period after reistsance exercise. 60 min after exercise, plasma AA concentration peaked in BOLUS at 290μmol/L, while PULSE was ~120 μmol/L at the same time-point. However, PULSE, due to the continous feeding nature of the condition, maintained a sustained aminoacidemia, which was higher than of BOLUS 180-220 min after exercise. While the AUC for plasma EAA concentration did not differ between conditions, myofibrillar FSR was elevated by 92% and 42% above basline in BOLUS and PULSE respectively, between 1-3 h post-exercise. At 3-5 h post-exercise, FSR was elevated by 193% and 121% above baseline, in BOLUS and PULSE respectively. This shows that a rapid and pronounced rise in amniacidemia in the post-exersise period stimulates MPS to a greater extent than a gradual and sustained rise in aminoacidemia. Indeed, consumption of 2-3 meals containing 20-30g of high quality protein

has been shown to cause greater rate of MPS over 24 h, when compared to one large protein meal, or several small protein meals (Areta *et al.*, 2013). Areta *et al.*, (2013) compared the effects of three different feeding strategies of 80g of whey protein on post-exercise MPS rates over 12 h– PULSE, 10g protein every 1.5 h; INTERMEDIATE, 20g protein every 3 h; BOLUS, 40g every 6 h. Myofibrillar FSR increased above baseline in all conditions. Cumulative myofibrillar FSR of INTERMEDIATE condition was 32% and 49% greater than BOLUS and PULSE condition. Therefore, 20g protein in the INTERMEDIATE condition was sufficient to stimulate a robust anabolic response, which is consistent with the dose that maximises MPS as reported elsewhere (Witard *et al.*, 2014; Moore *et al.*, 2009). Furthermore, the 3 h gap between 20g feeds seems to be sufficient in duration for recovery of the refractory response of muscle, enabling repeated MPS stimulation over 12 h. Taken further, this implies a more 'even', as opposed to 'skewed', distribution of daily protein intake is therefore considered optimal for maximising diurnal MPS rates. Strategic dosing and distribution of AAs with sufficient protein boluses, fed ≥3 h apart, is therefore likely to overcome this refractory response by modulating relative changes in plasma AA concentration. This is a fundamental consideration for the appropriate design of nutrition interventions to maximise muscle accretion, since it is clear evidence that total protein intake is not the only factor modulating to MPS. However, extrapolating acute rates of MPS to positive changes in muscle accretion over-time should be interpreted with caution. Long duration intervention studies, which assess lean body mass (LBM) and strength, are more representative of chronic adaptations to changes in protein distribution. The current literature available in this area, specifically using an older cohort, will be explored further in **section 2.8.4.**

2.4.7 The Role of Exercise in Muscle Protein Synthesis

The refractory response of MPS to sustained hyperaminoacidemia may be minimised by exercise. Similar to increased plasma concentrations of EAAs, exercise is a potent stimulator of MPS. Intramuscular mechanical strain created during the contractile forces elicited during exercise, ultimately results in the stimulation of intracellular anabolic signalling pathways that regulate MPS, a phenomenon known as mechanotransduction (Pasiakos, 2012). Akin to EAA, and leucine in particular, the upregulation of the master regulator of protein synthesis, mTORC1, is responsible for the increased rate of MPS seen in the post-exercise period. Of note, resistance exercise (8 sets of 8 repetitions at 80% 1RM) has been shown to increase positive net protein balance for 2 days after exercise cessation (Phillips *et al.,* 1997). This exercise bout also resulted a 112%, 65% and 34% increase in muscle FSR, 3 h, 24 h and 48 h after exercise, respectively. The anabolic effects of AA alone are greater than that of exercise alone, as per **figure 2.4** (Phillips, 2012b). However, the combination of

both exogenous EAA and exercise elicits a robust effect on MPS, greater than of AA or exercise alone. Exercise may enhance the sensitivity of skeletal muscle to the anabolic potential of AA, since mechanical loading has been reported to increase the muscle intracellular permeability to extracellular AAs (Pasiakos, 2012). Furthermore, blood flow, which increases with exercise, enhances the delivery of AAs to the muscle cell (Biolo *et al.*, 1997). Indeed, increased stimulation of intracellular signalling regulating MPS and greater rates of myofibrillar FSR are apparent when AA are ingested in the exercised state, compared to at rest, in both young (Witard *et al.*, 2014; Burd *et al.*, 2012; Churchward-Venne *et al.*, 2012; Moore *et al.*, 2011) and old (Yang *et al.*, 2012a). In young, myofibrillar FSR was 44% higher at 3-5 h post ingestion, in the resistance exercised leg, compared to the rested leg, after the ingestion of 25g whey (Churchward-Venne *et al.*, 2012). In older adults, Yang *et al.* (2012a) demonstrated that while 40g of whey conferred no additional increase in FSR compared to 20g of whey at rest, after resistance exercise, 40g of whey elicited a 32% greater increase in FSR compared to 20g whey. These results demonstrate that in an acute setting, exercise results in a greater increase in postprandial MPS, when compared to ingesting protein in a rested state, in both young and older adults. The potential effects of exogenous EAA in combination with resistance training on changes in LBM and strength over time in older adults will be discussed in **section 2.8.4.**

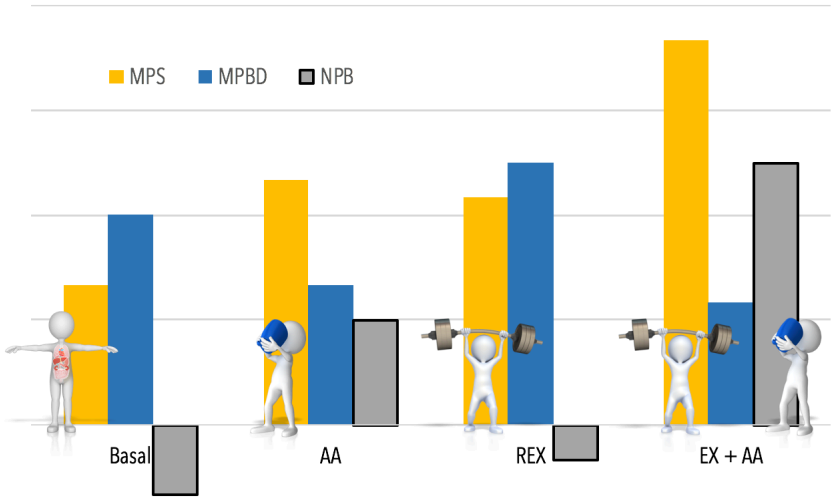


Figure 2.4: Muscle protein synthesis*, muscle protein breakdown* and the resulting net protein balance* at rest, after the ingestion of amino acids, after exercise, and after a combination of amino acids and exercise. Adapted from Phillips (2012b). MPS, muscle protein synthesis; MPB, muscle protein breakdown; NPB, net protein balance; AA, amino acids; REX, resistance exercise; *, arbitrary units.

2.5 Postprandial Aminoacidemia and Muscle Protein Synthesis

2.5.1 Digestion and Absorption Kinetics of Leucine

After ingestion of a protein-containing food source, whole proteins are denatured by acid in the stomach, and broken down into smaller peptides. These proteins and peptides then pass into the small intestine, where peptide bonds are further broken down by proteolytic enzymes, into single AAs. These AAs are transported into the mucosal cell by AA specific carriers (Gropper and Smith, 2012; Kreider and Campbell, 2009). Leucine, being a neutral AA, crosses the gut wall by active transport (Adibi and Gray, 1967). AAs with the greatest transport affinity compete more effectively for carrier membranes, consequently impeding the transport of low affinity AAs, particularly at higher concentrations (Webb, 1990). Indeed, leucine, isoleucine, valine and methionine show greater absorption rates than threonine when presented to the intestinal mucosa in equimolar mixtures (Adibi and Gray, 1967). AAs are subsequently absorbed in to the portal blood system, while some are metabolised within the mucosal cell. Absorbed AAs pass into the liver, where some are metabolised, and the remainder are released into circulation and delivered to peripheral tissues. The metabolic fates of the AAs include the formation of function protein such as enzymes, signalling proteins such as hormones, and structural proteins such as the skeletal muscle (Gropper and Smith, 2012; Kreider and Campbell, 2009).

A rise in plasma aminoacidemia, and in particular leucinemia, is a potent stimulator of MPS. Consequently, factors that influence the plasma kinetics of AAs are of interest in the present review. Both protein/AA dose, protein/AA source and co-ingestion of other macronutrients influence the postprandial plasma AA kinetics. Matsumoto *et al.* (2014) compared the plasma kinetics of leucine after the ingestion of graded doses (10, 20, 30, 45, 60, 75, 90mg/kg) of crystalline leucine diluted/partially diluted in water. Plasma concentrations of leucine peaked at 15-30 min for all conditions. Peak leucinemia was achieved at a dose of 45mg/kg, or ~3g leucine, at ~800 μmol/L, with no further increase in peak leucinemia apparent after the ingestion of 60, 75 and 90mg/kg (~4g, 5g and 6g leucine). This suggests that the absorption rate of leucine is maximised at ~3g leucine in crystalline form. This may be explained by the poor solubility of leucine, which did not completely dissolve in water at doses ≥60mg/kg. After the ingestion of 10-30mg/kg leucine, plasma leucine concentrations had returned to baseline at 240 min, while plasma leucine remained elevated at 240 min after the ingestion of 45-90mg/kg (~3-6g leucine). Peak leucinemia is apparent after the ingestion of ~3g leucine, however, the ingestion of ~4, 5 and 6g leucine results in a sustained elevation of plasma leucinemia for up 4

h after ingestion. The source of dietary protein has also been shown to influence postprandial plasma kinetics of AA. Burke *et al.* (2012) demonstrated that 20g protein from various food exhibit marked differences on plasma leucinemia, with liquid-form protein achieving peak aminoacidemia twice as fast, compared to the ingestion of protein in solid form. Indeed, free form AAs results in a faster and greater peak leucinemia when compared the AAs given in a mixed meal, with a decrease circulation of EAAs apparent in the meal condition (Rondanelli *et al.*, 2017). This may be explained by the presence of carbohydrates and fat in the mixed meal, which slows down gastric emptying, and therefore intestinal absorption of AAs (Have *et al.*, 2007). As mentioned previously, leucine-enrichment is a promising strategy for enhancing the anabolic response to a low leucine meal. However, the plasma kinetics of AAs after the ingestion of a leucine-enriched mixed meal remains unexplored, and warrants further investigation.

2.5.2 Enhanced Food Ingredient Delivery by Microencapsulation Technology

With our present understanding of the modulatory effects of plasma aminoacidemia on MPS, it is within scientific reason that optimising the plasma kinetics of leucine to favour MPS, shows promise for enhancing the anabolic potential of a meal. However, the ideal postprandial plasma kinetics of leucine required to maximise MPS remains unclear. West *et al.* (2011) demonstrated that rapid postprandial aminoacidemia enhances anabolic signalling and myofibrillar MPS to a greater extent than an identical amount of whey protein that has a slower digestion kinetics, concluding that a more pronounced peak in aminoacidemia is optimal for enhanced MPS in young after exercise. However, while not entirely conclusive, several studies have pointed to the notion that a prolonged low amplitude elevation in postprandial leucinemia may instead facilitate an extended MPS response after meal ingestion (Gorissen *et al.*, 2016; Mitchell *et al.*, 2015a; Areta *et al.*, 2013). Maintaining a low amplitude leucinemia after a single meal may offer a means of overcoming the refractory response to a sustained high elevation in leucinemia, by preventing the muscle full response. Interestingly, microencapsulation is an emerging technology that has shown potential for the optimal delivery of drugs and nutrients (Dias, Ferreira, & Barreiro, 2015; Singh, Hemant, Ram, & Shivakumar, 2010; Champagne & Fustier, 2007). This technology is based on the immobilization of a core ingredient in a miniature-sealed capsule that maintains structural integrity until degradation and release of the ingredient at an appropriate time or site in the body (Dias, Ferreira, & Barreiro, 2015). Furthermore, through appropriate design, the degradation and release of the ingredient can be manipulated to potentially create the desired plasma kinetics. A microencapsulation technology has been recently developed by Anabio Technologies, who part-funded the work undertaken in this PhD thesis, which can be used to encapsulate leucine and claims

enhanced bioavailability and a sustained-release design (Brodkorb and Doherty, 2015). This patent relates to a process for producing microcapsules, whereby the core ingredient (leucine) is encapsulated within a protein matrix (whey protein). This protein matrix, acting as carrier system for the core ingredient, is purported to have the optimal pH to minimise degradation of the core ingredient by gastric acids, enabling enhanced bioavailability and timely release and absorption in the intestine. Furthermore, the use of microencapsulated leucine may also enhance the palatability of leucine-containing protein supplements, as masking the bitter taste properties of free leucine proves difficult (Kato, Rhue, and Nishimura 1989). Microencapsulated leucine may also show promise in leucine-enrichment of plant-based proteins. The addition of microencapsulated leucine to a plant-based protein supplement may achieve the desired sustained low amplitude plasma leucinemia, with the potential to stimulate MPS to a greater magnitude compared to a plant-based protein alone.

2.5.3 Protein Quality and Postprandial Aminoacidemia

A high quality protein is defined as a protein that provides sufficient EAAs, that is easily digested and can readily be used for the synthesis of new proteins (Food and Nutrition Board, 2005). Animal-based protein, such as meat, dairy and eggs are inherently high in protein, EAAs and leucine, per 100g, as per **table 2.1.** Meanwhile, plant-based proteins, such as cereals and legumes, provide a lower proportion of protein, EAAs and leucine (USDA National Nutrient Database for Standard Reference, 2009). Postprandial hyperaminoacidemia is a potent stimulator of MPS. Consequently, foods that are higher in EAAs and leucine, and elicit greater and faster digestion and absorption kinetics, have been shown to elicit a superior muscle protein synthetic response. When 21-22g of whey protein hydrolysate, isolate soy protein and micellar casein were ingested, the resultant rise in aminoacidemia was more pronounced for whey, compared to soy and casein (Tang *et al.*, 2009). While matched for total EAA content, the whey, soy and casein beverage contained 2.3g, 1.8g and 1.8g leucine, respectively.

When compared to soy, the AUC for leucine after whey ingestion was ~73% greater. Despite whey and soy both being classed as 'fast' digesting proteins, whey resulted in a steeper and greater overall increase in plasma EAA, BCAA and leucine concentration compared to soy. The difference in digestion and absorption kinetics between whey and soy can be attributed to greater splanchnic uptake and subsequent lower uptake by the peripheral tissues (i.e. skeletal muscle) compared to milk protein. The ingestion of soy is associated with greater gut protein synthesis and an increase in ureagenesis, meaning a greater uptake of AAs by the portal

vein to the hepatic tissue, which ultimately leads to less AAs being available for release into the plasma and use by peripheral tissue (Bos *et al.*, 2005, 2003; Fouillet. *et al.,* 2002). The reason for a greater AA splanchnic tissue extraction following the ingestion of soy protein is not fully understood, but it is suggested to be attributed the lower EAA content of the protein. In support, Engelen *et al.* (2007) demonstrated that splanchnic extraction can be reduced with the addition of BCAA to a soy protein meal. However, as discussed, beverages were matched for EAA content (Tang *et al.*, 2009), therefore the reason for increased gut sequestration and uptake by hepatic tissue in soy protein, is not fully understood. Meanwhile, the more modest increase in aminoacidemia after the ingestion of micellar casein, classed as a 'slow' protein, is attributed to the slower rate of gastric emptying, due the coagulant nature of micellar casein (Hall *et al.*, 2003). In any case, Tang *et al.* (2009) observed mixed muscle MPS was ~18% and ~31% greater after the ingestion of whey, compared to soy, at rest and after exercise, respectively. The superiority of whole milk over soy protein in stimulating net protein balance and FSR is also reported elsewhere in young adults (Wilkinson *et al.*, 2007). However, in contrast to findings by Tang *et al.* (2009), authors reported a more modest and sustained elevation of plasma total amino acids (TAAs) in the milk condition compared to soy, albeit EAA and leucine plasma kinetics were not measured by Wilkinson *et al.* (2007). Nonetheless, Tang *et al.* (2009) concluded that differences in net balance and FSR were due to differences in aminoacidemia and attributed this to increase splanchnic uptake of AAs in the case of the soy condition. Similar to Tang *et al.* (2009), Wilkinson *et al.* (2007) attributed soy's anabolic inferiroirty to increased splanchnic uptake of EAAs. When compared to casein, the AUC for leucine after whey ingestion was ~200% greater. MPS was also ~93% and ~122% greater after the ingestion of whey, compared to casein, at rest and after exercise, respectively (Tang *et al.*, 2009). This is consistent with findings in older adults (Burd *et al.*, 2012). The divergent MPS response between whey and casein is attributed to the blunted aminoacidemia peak and smaller AUC following the ingestion of casein. These results underline the potential importance of achieving optimal aminoacidemia, when stimulating post meal anabolism. However, several studies have contested the modulatory effects of postprandial plasma leucinemia on the muscle protein synthetic response (Van Vliet *et al.*, 2017; Gorissen *et al.*, 2016; Burd *et al.,* 2015). Burd *et al.* (2015) compared the postrandial plasma leucine kinetics and resultant muscle protein synthetic response after the ingestion of a portion of beef protein and an isonitrogenous portion of milk protein. Authors concluded that the ingestion of beef protein resulted in a more accelerated and greater increase in postprandial leucinemia but had an inferior anabolic response when compared to milk in the early (0-2 hours) postprandial period. This suggests that an increase in plasma leucine kinetics is not the sole determinant of MPS in the postprandial period. Gorissen *et al.* (2016) compared the plasma leucie kinetics and the postprandial muscle protein synthetic response to 35g

whey protein, 35g hydolysed wheat protein and 35g casein. The ingestion of whey protein resulted in the greatest increase and area under the curve for plasma leucine in the four hour postprandial period, with wheat and casein ingestion exhibiting similar leucine plasma kinetics. However, while the ingestion of casein resulted in a 48±16% increase in FSR, the ingestion of whey and wheat protein did not result in a significant change in FSR above basal values (4±17% and 33±24%, respectively). This study refutes the hypothesis that the magnitude of postprandial plasma leucinemia dictates MPS and instead suggests that other factors, potentially the mileu of other AAs ingested, have an influence on postprandial MPS; a hypothesis which has been supported elsehwhere (Churchward-Venne *et al.,* 2012). Finally, Van Vliet *et al.*, (2017) demonstated that despite a similar response in postpradial plasma leucine kinetics, a portion of whole egg protein results in a greater increase in FSR compared to an isonitogenous portion of egg white protein. This suggests that there are other factors which modulate postprandial MPS, such as the co-ingestion of macronutrients and/or micronutrients, since whole eggs have a higher fat content, as well as vitamins and minerals, which may exhibit an anabolic effect. In summary, while studies have shown that postprandial plasma leucine kinetics has an infleunce on MPS, it is not the only factor than influences postprandial MPS, and its importance has been challenged recently. Other factors such as the accompanying AAs and the co-ingestion of macronutrients and micronutrients are important considerations when discussing the muscle protein synthetic response to the ingestion of dietary protein.

Changes in MPS in an acute setting may not necessary represent muscle accretion and changes in strength over time, and longer interventions are a more accurate representation of the potential for longitudinal muscle accretion and strength gains. Long term intervention studies suggest that animal protein exhibits no added benefit on changes in LBM when compared to plant-based proteins. Joy *et al.* (2013) investigated the effect of whey protein compared to rice protein isolate administered post workout on changes in LBM and strength. After 8 weeks resistance exercise training (RET), both the group consuming rice protein and whey protein experience an increase in LBM (+2.5kg and +3.2kg, respectively), strength and power and a decrease in fat mass. However, there was no difference between treatment groups. Babault *et al.* (2015) investigated the effect of 25g whey protein vs. 25g pea protein, administered twice daily, on changes in muscle thickness. After 12 weeks RET, rice protein was equally as effective as whey protein in increasing biceps brachii thickness. Brown *et al.* (2004) demonstrated that soy and whey protein bars, administered three times per day, results in similar increases in LBM after 9 weeks RET. In contrast, Hartman *et al.* (2007) compared the effect of 17.5g skimmed milk protein, isonitrogenic soy protein and an isoenergetic maltodextrin beverage, ingested

immediately post training and again 1 h post training, 5 days per week, on changes in LBM and strength, in novice weightlifters. After 12 weeks resistance exercise training (RET), fat and bone free mass (FBFM) increased to a similar extent in the control and soy protein group, 3.7% and 4.4% change from week 1, respectively. Participants consuming the skimmed milk showed the greatest increase in FBFM, a 6.2% change from week 1, and also a greater loss in fat mass, -5.5% from week 1, compared to control and soy group. While all treatment groups experienced an increase in strength from baseline, there were no differences between groups. Cross sectional area (CSA) of type I fibres was greater in the milk and soy group, compared to control, while the increase is CSA of type II fibres was greatest in milk group. Both groups showed a greater protein intake at week 6 and week 12 (1.6-1.8g/kg/d,) compared to week 1 (1.2-1.4g/kg/d), with no differences between groups. Despite the superiority of milk protein over soy protein in the study, there was no difference apparent in plasma kinetics of leucine, BCAA, EAAs or total AAs after the ingestion of a single bolus of each beverage. This would suggest that the differences in LBM and muscle fibre CSA over 12 weeks cannot be explained by differences in digestion and absorption kinetics between milk and soy.

In summary, the superiority of beef, whey and milk protein over plant-based protein in stimulating MPS in an acute setting is well reported and is attributed predominantly to the relative leucine content of respective proteins and the difference in postprandial digestion and absorption kinetics, with beef and whey protein resulting in a greater hyperleucinemia and hyperaminoacidemia when compared to soy protein. In longer intervention studies in combination with RET, the difference between animal-based and plant-based proteins, when daily protein intake is matched, in eliciting differential changes in LBM is less clear. However, the development of strategies to enhance the anabolic properties of plant-based proteins remains an important consideration, and has particular potential in ameliorating age-related decline of muscle mass.

2.6 Strategies to Enhance the Anabolic Potential of a Meal

2.6.1 Increasing Quantity of Plant-Based Protein Dose

Strategies to enhance the anabolic potential of a meal and plant-based proteins have received increased interest in recent years. These approaches include ingestion of higher amounts of plant-based proteins, combining two or more plant-based proteins to yield a more optimal EAA profile, and leucine-enrichment of meals. However, research supporting these strategies in increasing MPS in an acute setting and increase LBM and strength over a prolonged period is scarce. As discussed, the postprandial plasma aminoacidemia, in

particular, leucinemia, is an independent driver of the MPS response to a meal. Hence, it is intuitive that consuming a greater quantity of plant-based foods with the aim of achieving a similar plasma kinetics to that achieved with beef or whey ingestion, may 'rescue' the inferior anabolic properties of plant-based protein (van Vliet, Burd and van Loon, 2015). Furthermore, when plant-based proteins are more refined and processed, in which anti-nutritional factors that impede protein digestion and absorption are removed, it may be possible to render greater postprandial plasma aminoacidemia, similar to that of animal-based proteins (Food and Agriculture Organization of The United Nations, 2011). While research documenting the anabolic effect of plant-based whole foods is limited, there are several studies in older adults, which investigate graded quantities of plant-derived protein powders on MPS, at rest and following exercise (Gorissen *et al.*, 2016; Yang *et al.*, 2012a; 2012b). Firstly in terms of animal protein, 20g of whey was sufficient to elevate FSR above postabsorptive values in older adults at rest (~66% increase) and following exercise (~33% increase) in older males (Yang *et al.*, 2012a). While the ingestion of 20g or 40g of soy protein was unable to stimulate myofibrillar FSR above postabsorptive values at rest, 40g soy protein after exercise was sufficient to stimulate MPS above baseline, to a magnitude similar to that of 20g whey protein at rest (Yang *et al.*, 2012a; 2012b). This suggests that the inferiority of soy protein in stimulating postprandial anabolism can be 'rescued' by ingesting a sufficient amount of protein, and administered following exercise when the anabolic sensitivity of skeletal muscle is likely to be enhanced (Yang *et al.*, 2012b). Gorissen *et al.* (2016) compared the plasma AA kinetics and myofibrillar protein synthesis in older males after the ingestion of 35g and 60g wheat protein hydrolysate and 35g micellar casein, providing 2.5g, 4.4g and 3.2g leucine, respectively. Leucinemia peaked at ~290 μmol/L at ~75min for both 25g wheat and casein conditions, however, ingestion of casein resulted in a more prolonged hyperaminoacidemia compared to wheat protein. Postprandial myofibrillar FSR increased 48% above postabsorptive values after casein ingestion over 4 h, while there was no change in MPS after 35g wheat protein ingestion. Of interest, both casein and wheat ingestion results in a similar peak leucinemia, and at the same time, but resulted in a marked difference in rates of MPS. This would suggest that the accompanying milieu of other AAs from a given protein source, and/or the kinetics of plasma leucine, and not simply the magnitude and time to reach peak concentration of plasma leucine has an effect on postprandial rates of MPS. In support of the latter, the ingestion of 60g wheat protein had a similar peak leucinemia to 35g casein, but a more prolonged elevation in plasma leucine. Postprandial myofibrillar FSR increased ~77% above postabsorptive values between 2-4 h after the ingestion of 60g wheat protein. This suggests a plant-based protein can stimulate MPS to a similar extent to casein when a greater amount plant-based protein is ingested in older men. However, the ingesting of these large quantities of plant-based protein sources may

not be feasible in a real world setting, particularly in older adults, who experience a deterioration in appetite with advancing age (Morley, 2001), termed 'anorexia of ageing'. Often a higher calorie intake needs to be ingested to enable a plant-based protein to reach a plasma and/or intramuscular leucine threshold, which older adults may struggle with. **Table 2.1** gives an insight into the approximate calorie content required to attain 3g leucine from a food item. Although promising for the efficacy of plant-based proteins, these results signify a need for other adjunct strategies to maximise the effectiveness of post-meal anabolic effect of plant-based proteins.

Food	Leucine (g) Per 100g	3g Leucine (g food item)	3g Leucine (kcal)
Cereals and Legumes			
Cereals, corn grits, yellow, regular and quick, enriched, dry	1.1	278.8	1026.8
Chickpeas, canned, drained solids	0.5	593.9	826.3
Kidney beans, canned, drained solids	0.7	443.1	549.7
Oats, dry	1.0	291.4	1126.4
Peanuts	1.7	179.4	1019.0
Potatoes, flesh and skin, raw	0.1	3040.5	2351.4
Quinoa, uncooked	0.8	357.1	1315.1
Rice, white, long-grain, parboiled, unenriched, dry	0.7	457.2	1710.0
Seeds, hemp seed, hulled	2.2	138.7	767.3
Meat, Poultry and Fish			
Bacon, raw	1.7	180.1	199.2
Beef, loin, boneless, separable lean only, all grades, raw	2.1	145.4	201.8
Chicken, breast, skinless, boneless, meat only, raw	1.9	161.2	193.4
Fish, cod, Atlantic, raw	1.4	207.3	170.7
Fish, salmon, Atlantic, wild, raw	1.6	186.0	264.8
Eggs and Dairy			
Cottage cheese	1.1	268.8	264.1
Egg, whole, raw	1.1	276.2	397.8
Milk, whole, 3.25% milkfat	0.3	1002.7	612.3
Mozzarella cheese, whole milk	1.8	164.3	492.9
Soymilk, original and vanilla, unfortified	0.2	1612.8	869.5
Yogurt, Greek, plain, low-fat	0.5	567.1	414.0

Table 2.1: The quantity of leucine in 100g of each food item, the quantity (g) and calories (kcal) of each food item which would provide 3g of leucine. g, grams; kcal, calories, adapted from USDA National Nutrient Database for Standard Reference (2009).

2.6.2 Combining Amino Acid Profile

Animal-based proteins, such as meat, fish, dairy and eggs, are termed 'complete protein', due to the presence of all 9 EAAs (Food and Nutrition Board, 2005). Most plant-based proteins, such as cereal, grains and legumes, which are lacking in one or more EAAs, are termed 'incomplete proteins' (Trumbo *et al.*, 2002). While soy and quinoa contain 9 EAAs, and are thus termed complete proteins, their EAA content is relatively low in comparison to animal-based proteins (Young and Pellett, 1994). Since postprandial plasma EAA is a driver of MPS, it is rational that the combination of one or more plant-based proteins may yield a more complete AA profile, thereby increasing its anabolic potential. The premise of combining plant-based protein is that one protein source will be high in the most limiting AA of the other protein. An example of this is the combination of soy, which is inherently low in methionine and high in lysine, with a cereal protein, which are inherently high in cysteine and methionine, and low in lysine, resulting in a synergistic effect and a better overall EAA content. (Gorissen and Witard, 2018; van Vliet, Burd and van Loon, 2015). However, it remains to be investigated if these plant-based protein blends can elicit an MPS response similar to that of an animal protein.

2.6.3 Leucine-Enrichment

Table 2.1 offers insight into the quantity and energy (kcal) of a food item, both plant and animal derived, which would provide 3g leucine, the quantity of leucine previously shown to maximise MPS in older adults (Koopman *et al.*, 2006). However, this table does not infer optimal plasma aminoacidemia or maximal MPS rates, as studies on whole food source in this regard have not been done. Nonetheless, it highlights the problem of relying solely on plant-based foods to attain 3g leucine, since high quantities of food, and therefore high energy intake is often required to reach the purported leucine threshold to stimulate MPS. Leucine is a key driver in of MPS, indeed as discussed above, Wilkinson *et al.* (2013) demonstrated that the ingestion of 3.42g leucine can elicit a robust increase in postprandial MPS, that is similar to that results in by 48g whey protein (Atherton et al., 2010b). The enrichment of whey protein with leucine has yielded promising results in acute studies for changes in MPS (Devries *et al.*, 2018; Kramer *et al.*, 2017; Churchward-Venne *et al.*, 2014), and long-term intervention studies in changes of LBM (Bauer *et al.*, 2015). Therefore, the idea of fortifying plant-based foods and lower leucine meals appears to be a promising strategy in promoting postprandial anabolism. Several studies have investigated the potential of EAA and leucine-enrichment in the context of promoting both MPS in an acute setting, and the accretion of skeletal muscle over time.

Bukhari *et al.* (2015) demonstrated that the ingestion of 3g 40% leucine EAA beverage stimulated MPS to a similar extent as 20g whey protein (which is most often ~11% leucine), both at rest and post–exercise in older women. In a follow up study within the same cohort profile, Wilkinson *et al.* (2017) compared the effect of 1.5g EAA, 6g EAA and 40g whey protein, containing 0.6g, 2.4g and 4g leucine, respectively, on myofibrillar PS. While 1.5g EAA was sufficient to initiate a robust MPS response 0-2h post-ingestion, this increase in MPS was not maintained over the 4 h postprandial period. Meanwhile, 6g EAA and 40g whey protein maintained MPS throughout the postprandial period, with no difference between each condition. This suggests that a 6g of a leucine-rich EAA beverage providing 2.4g leucine results in an MPS response similar to that of 40g whey protein, which has previously been shown to elicit a maximal MPS response in an older cohort (Yang *et al.*, 2012a). In young adults, Churchward-Venne *et al.* (2012) compared the effect of three beverages; [25g whey protein (WHEY), containing 3g leucine; 6.25g whey with additional leucine (WHEY+LEU), containing 3g leucine; and 6.25g whey with additional EAAs without leucine (WHEY-LEU), containing 0.75g leucine], for their effect on MPS in exercised and non-exercised state. Results showed that all three beverages increase MPS, but there was no difference between groups for the non-exercised muscle. Similarly, no differences were apparent in the exercised muscle, in the first 3 h post-exercise. However, 25g whey appeared to show greater MPS rates in the 3-5 h period after exercise. The addition of both leucine, and a mixture of EAAs void of leucine, to a suboptimal dose of whey are as effective as 25g whey in stimulating MPS in the rested state and in the early post-exercise period. In this instance, 0.75g leucine, the amount for leucine provided by 6.25g whey, is sufficient to mount a maximal anabolic response, at rest, and 0-3 h after exercise, in young, since additional leucine in WHEY and WHEY+LEU did not have any further effect on MPS. Therefore, it is difficult to determine the effect of additional leucine, since the MPS response had already been maximised before leucine was added. However, despite 25g whey having the same leucine content as WHEY+LEU, 25g whey alone was superior than WHEY+LEU and WHEY-LEU in stimulating MPS in the later post-exercise stage (+3 h). This suggests that the more sustained and prolonged peak in leucinemia after WHEY ingestion stimulated MPS to a greater extent in the 3-5 h after exercise, which has been reported elsewhere (Gorissen *et al.*, 2016; Mitchell *et al.*, 2015a; Areta *et al.,* 2013). Another explanantion is that other AAs may have become rate limiting for MPS in the later hours of exercise, since WHEY, WHEY+LEU and WHEY-LEU beverages contained 12g, 5g, and 9g of EAA and 13g, 3g and 3g non-essential AAs, respectively. This suggests that plasma leucine concentration is not the only driver of MPS, and other AAs are required as a substrate to maintain the synthesis of proteins in skeletal muscle. The addition of EAAs excluding leucine was as effective as 25g of whey protein at stimulating MPS in the early post-exercise stage. In this instance, that additional leucine in WHEY+LEU is not responsible

for this equivalent MPS response, since 6.25g of whey in the absence of additional leucine has the same effect on MPS. Similar results have been reported elsewhere, in which the addition of leucine to a beverage had no further enhancement on MPS in young (Glynn *et al.*, 2010; Tipton *et al.*, 2009; Koopman *et al.*, 2008). Nonetheless, in a follow up study Churchward-Venne *et al.* (2014) determined that the addition of a higher dose of leucine to 6.25g whey, totalling 5g leucine, within a mixed meal, results in an increase in MPS that was equivalent magnitude and duration to that of 25g whey in the later post-exercise period. This suggests that when a sufficient quantity of leucine is used to enrich a suboptimal protein beverage, MPS in the later hours of exercise can be maximised. In the latter studies, the purported suboptimal protein dose may not necessarily be suboptimal, particularly in the rested state. The supplementary leucine and EAAs is being added to a beverage that possibly has sufficient leucine and EAA content to maximise rates to MPS in young at rest, therefore rendering no added benefits. A more appropriate assessment for the potential of leucine-enrichment to maximise the anabolic response of a meal, would be to enrich a truly suboptimal leucine/EAA beverage with leucine at rest. Engelen *et al.* (2007) demonstrated that enriching soy protein with BCAA to an amount equating that found in casein results in an increase in whole body protein balance compared to soy alone in patients with chronic obstructive pulmonary disease. To the best of our knowledge, no studies have observed the co-ingestion of leucine with a low leucine/EAA or plant-based protein on direct measurement of MPS.

In older adults, the co-ingestion of 5g of leucine supplement with three main meals enhances 3 day rates of MPS, and to a greater extent after exercise (Murphy *et al.*, 2016). Murphy *et al.* (2016) demonstrated that leucine-enrichment of a diet containing 0.8g/kg/d and 1.2g/kg/d was equally effective at increasing newly synthesised myofibrillar proteins, assessed using deuterated water technique in older men. Similarly, Casperson *et al.* (2012) concluded that enrichment of three main meals with 4g leucine, over 2 weeks, results in greater postabsorptive and postprandial mixed muscle FSR in older adults. These data suggest that leucine supplementation is a means of acutely improving MPS and chronically improving basal and fed MPS in older adults. However, these promising results are not reflected in studies assessing changes in muscle mass and function over time (Trabal *et al.*, 2015; Verhoeven *et al.*, 2009). A criticism of these long-term studies is that total protein, and not just leucine, plays a key role in the phenotypic changes to AA feeding over time. Furthermore, these studies may be too short in duration to detect these changes, particularly with the use of dual-energy x-ray absortometry (DXA) which may be insensitive to the detection of subtle changes in lean body mass (LBM)(Hind *et al.,* 2018). While leucine-enrichment yields positive results for changes in MPS in the short

term (3-14 days), the potential outcome of chronic application of leucine-enrichment on changes in LBM and strength remains relatively underexplored.

While its capability is promising, enriching a meal or ingestion crystalline leucine alone has its potential drawbacks. The ingestion of leucine has been shown to elicit a reduction in concentrations of other BCAA, both in the postprandial state and in the basal fasted state after long-term supplementation (Matsumoto *et al.*, 2014). Pitkänen *et al.* (2003) reported a 28% increase in plasma leucine, and a 14% and 25% reduction in valine and isoleucine, respectively, after the ingestion of total ~15g leucine ingested 50 min before exercise, and during exercise. During 6 months of leucine supplementation (2.5g leucine at three main meals), Leenders *et al.* (2011) reported a 13% increase in basal plasma leucine concentrations after 12 weeks, and a 23% and 16% reduction in basal plasma valine and isoleucine values, respectively, after 4 weeks, in males with type 2 diabetes. These changes in aminoacidemia stayed stable for the duration of the intervention, while no change in basal aminoacidemia occurred in the control group. Since these EAA are crucial substrates during MPS, their decline may introduce a limitation to MPS and thus, may be counterproductive. Of note, Leenders *et al.* (2011) reported no significant changes in LBM or muscle strength after 3 and 6 months of supplementation. Nonetheless, despite this marked drop in BCAA plasma concentration after chronic leucine supplementation, the values of valine and isoleucine remained within physiological norms, so it is possible that this would exhibit no change in MPS rates.

In summary, enrichment of lower EAA meals with leucine has shown promise in stimulating MPS in older adults, in an acute setting. However, positive findings for changes in LBM or strength when leucine is supplemented over time are not apparent, but research in the area is scarce. Despite the critical role of plasma AA, in particular leucine, on stimulating postprandial MPS, the effect of leucine ingestion, both alone, and with a mixed meal, on plasma AA kinetics remains underexplored.

2.7 Muscle Mass and Healthy Ageing

2.7.1 Age-Related Decline in Muscle Mass & Function

Muscle mass is well maintained throughout the fifth decade of life, with a modest 10% decrease in muscle mass between the mid-20s and 50 years. However, between 50 years and 80 years, muscle mass deteriorates more rapidly, and a further 30% loss in muscle mass is apparent, meaning 40% muscle mass is typically lost

between the 24th and 80th year of life (Lexell, Taylor and Sjostrom 1988). Sarcopenia, defined as the age-related decline in muscle mass and function, has been associated with functional impairment, physical disability, increased frailty and a decrease in quality of life (Fielding *et al.* 2011; Doherty, 2003). Age-related decline of muscle mass and function has been shown to be directly and independently associated with all-cause mortality (Ruiz *et al.*, 2008), making it an issue that has significant societal consequences for the development of healthcare planning (Fielding *et al.*, 2011; Janssen, Heymsfield and Ross, 2002).

Irrespective of whether an individual has defined sarcopenia or not, the pathophysiology of age-related declines in muscle mass and function is considered to possess a multifaceted aetiology. Non-modifiable contributors include the ageing process, which results in a reduction in sex hormones and mitochondrial dysfunction (Beasley, Shikany and Thomson, 2013). Modifiable contributors include increase in fat mass, a decrease in physical activity, inadequate daily calorie and protein intake and a blunted a response to anabolic stimuli (Burd, Gorissen and Van Loon, 2013; Malafarina *et al.*, 2013; Thompson, 2007; Latham *et al.*, 2004). Systemic inflammation as a result of an increase in fat mass, is associated with the aetiology of sarcopenia, more specifically referred to as sarcopenic obesity (Bano *et al.*, 2017). Furthermore, reduced physical activity (PA) has been shown to contribute to the development of sarcopenia, whilst PA shows a protective role against sarcopenia development, even reducing the likelihood of progressing to sarcopenia later in life (Steffl *et al.*, 2017). Meanwhile, inadequate energy and protein intake (Morley, 2001) combined with the manifestation of blunted anabolic response to the ingestion of protein meals (Katsanos *et al.,* 2006; Katsanos *et al.,* 2005; Cuthbertson, 2004), remains one of the most significant and potentially modifiable contributors to the age-related decline in muscle mass.

2.7.2 Factors Contributing to Anabolic Resistance in Older Adults

Age-related decline in muscle mass is attributed to an imbalance between the rate of MPS and MPB, resulting in a negative net protein balance, and a decline in muscle mass over time (Burd, Gorissen and Van Loon, 2013). There remains debate on the potential deterioration of basal MPS and increase in basal MPB with advancing age, with some research suggesting there is a difference between basal MPS values between young and old (Trappe *et al.,* 2004; Welle, Thornton, Jozefowicz, Statt 1993) and others suggesting that there is no difference in MPS between young and old in the basal state (Wall *et al.,* 2015; Katsanos *et al.,* 2006; Katsanos *et al.,* 2005; Cuthbertson, 2004). However, research supports the thesis that there is indeed impairment in the

MPS response to anabolic stimuli in older adults, which offers an explanation for age-related decline in muscle. **Figure 2.5** represents the discordance apparent between MPS stimulation in young and elderly, in response to an anabolic stimulus, for example, exogenous protein or EAAs, which is well supported in the literature (Wall *et al.,* 2015; Katsanos *et al.*, 2006; Katsanos *et al.,* 2005; Cuthbertson, 2004; Volpi *et al.*, 2000). Indeed, older adults show a 3-fold smaller capability to elevate MPS above postabsorptive values when compared to younger adults, after the ingestion of 20g of casein (Wall *et al.*, 2015).

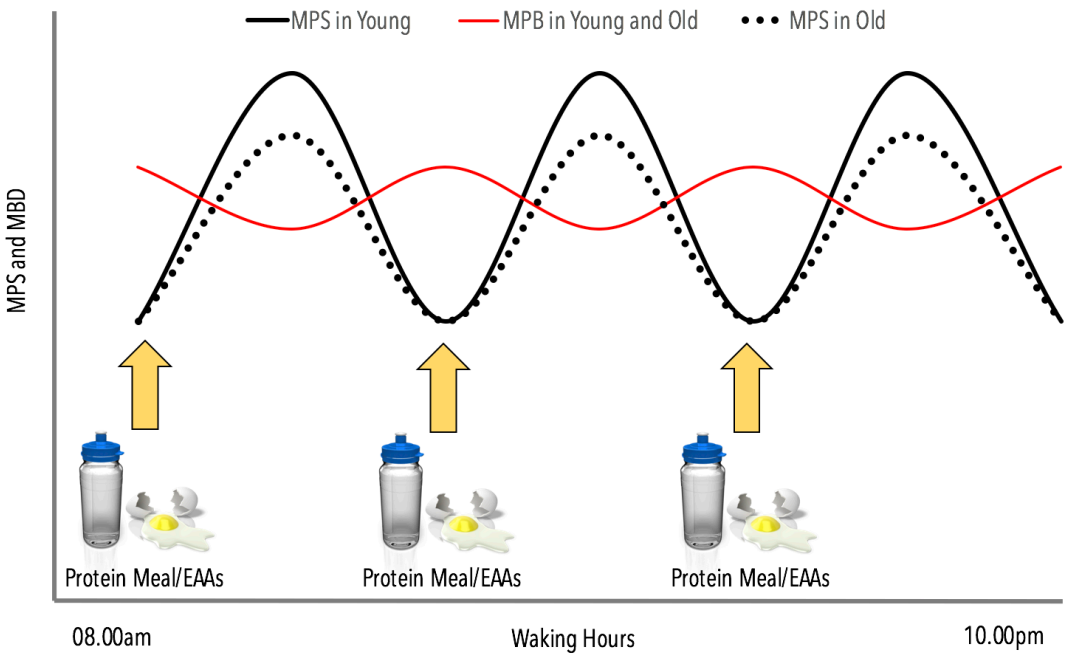


Figure 2.5: Muscle protein synthesis and muscle protein breakdown in response to a protein meal or EAAs in young vs. old. Older adults exhibited a blunted anabolic response (dotted line) to the same protein dose, when compared to young (solid line). Adapted from Breen and Phillips (2011).

As mentioned previously, this blunted response to anabolic stimuli in older adults is termed anabolic resistance. Potential contributing factors to anabolic resistance after the ingestion of protein-containing meal include; compromised digestion and absorption kinetics, increased splanchnic AA sequestration, inflammation, decreased satellite cell content and microvascularity (i.e. capillary density and therefore blood flow to the muscle) (Morton *et al.*, 2018).

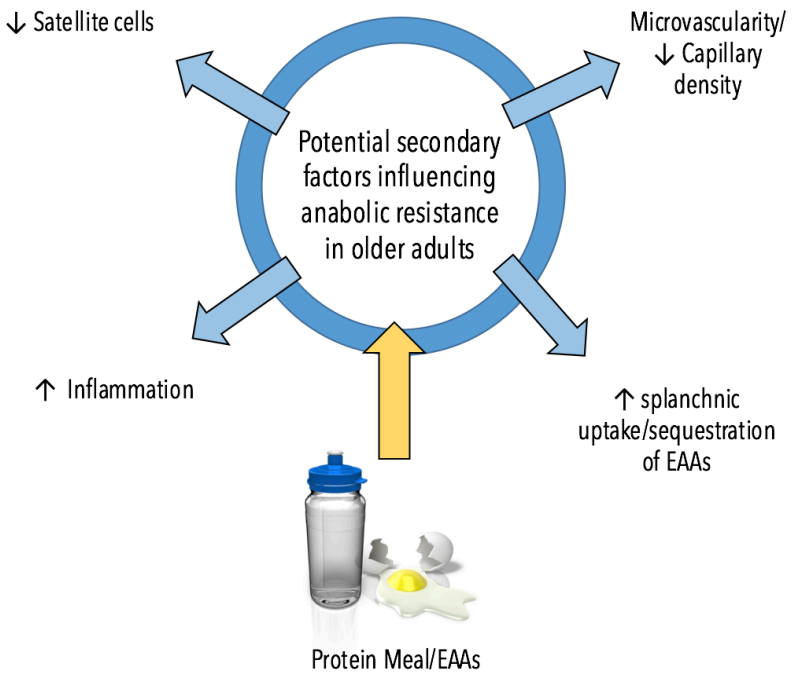


Figure 2.6: Graphic representation of the potential factors which contribute to anabolic resistance in older adults, in response to a protein meal or EAAs. Adapted from Burd, Gorissen and van Loon, (2013).

Considering the critical role of plasma aminoacidemia in the regulation of rates of MPS (Dreyer *et al.*, 2008; Fujita *et al.*, 2007; Bohé *et al.*, 2001), compromised digestion and absorption kinetics of plasma EAAs after the ingestion of a protein meal might explain anabolic resistance seen in older adults. Indeed, after the ingestion of 8g EAA mixture, Rondanelli *et al.* (2017) reported a greater increase in aminoacidemia, in terms of difference between each time-point and baseline, in old compared to young, but the rate of this increase was slower in old compared to young, represented by a marked shift to the right in the dose response curve for EAAs and BCAA. Peak aminoacidemia (1822 μmol/L) was achieved at 30 min in young and disappearance from plasma occurred at 150 min. Meanwhile, peak aminoacidemia (4298μmol/L) was reached 90 min after ingestion in elderly and disappearance from the plasma occurred at 270 min. This slower rate of appearance in elderly could be related to increased first pass splanchnic extraction of dietary AAs; indeed phenylalanine extraction by the gut and liver is reported to be higher in old compared to young, 29% in young and 47% in old (Volpi *et al.*, 1999). Similarly, splanchnic extraction of leucine was 23% in young and 50% in old after the ingestion of mixed liquid meal (Boirie, Gachon and Beaufrère 1997). Furthermore, the slower clearance of AAs is considered related to reduced uptake by other extra-intestinal organs and tissues, namely skeletal muscle. In contrast, Koopman *et al.* (2009) reported the ingestion of a 35g casein, results in a 71%, 51%, 38%, 37%

and 57%, greater increase in plasma phenylalanine, tyrosine, leucine, valine and isoleucine concentrations, respectively, in older adults, compared to young adults. Similar findings have been reported elsewhere after the ingestion of 20g protein (Pennings *et al.*, 2011a). Therefore, there are discrepancies in the literature. Nonetheless, the differences in aminoacidemia between young and old may reflect the difficulty of skeletal muscle to uptake and use AAs (i.e a reduced rate of peripheral disappearance rather than increased rate of appearance), therefore compromising the anabolic response.

Although difficult to distinguish from co-morbidities such as cardiovascular disease, obesity, arthritis and insulin resistance, chronic low-grade inflammation is apparent in older adults, a term known as 'inflammaging' (Franceschi *et al.*, 2007; Roubenoff, 2003). Ageing is associated with increased circulation of interleukin 6 (IL-6), tumour necrosis factor (TNF-a) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) (Colbert *et al.*, 2004; Visser *et al.*, 2002). This inflammation is associated with a marked decrease in MPS sensitivity to the presence of hyperaminaocimia from exogenous AAs. Indeed, Cuthberston *et al.* (2004) demonstrated that NF-kB, an atrophy mediating signalling protein, associated with inflammation, and activated by TNF-a, was 4-fold higher in elderly. In addition, the concentration and degree of activation of AA sensing/signalling anabolic pathways were 30-50% less in elderly after the ingestion of exogenous EAA, when compared to young adults (Cuthberston *et al.,* 2004). Moreover, anti-inflammatory agents such as fish oils/omega 3 fatty acids, non-steroidal anti-inflammatory drugs, Cox-2 inhibitors and anti-cytokine therapy are gaining increased interest in the preservation of muscle mass with advancing age (Dalle, Rossmeislova and Koppo, 2017; Jensen, 2008).

Skeletal muscle satellite cells, stems cells which are critical in the regulation of muscle fibre repair and growth, experience a decline with advancing age (Snijders *et al.*, 2015). In addition, decreased muscle fibre vascularity with advancing age is seen as a factor that contributes to impaired regulation of satellite cells in older adults (Snijders and Parise, 2017). Indeed, Timmerman *et al.* (2010) demonstrated that increased muscle perfusion, using exogenous vasodilators, resulted in an increase in postabsorptive or postprandial MPS. In summary, the underlying mechanisms of anabolic resistance are not fully understood, and are likely a combination of several aforementioned contributors.

2.8 Ameliorating The Age-related Decline in Muscle Mass - The Role of Protein and Exercise

2.8.1 Overcoming Anabolic Resistance in Older Adults with Protein/EAA Feeding

Despite factors previously outlined, this blunted anabolic response associated with aging can be overcome by ingesting a greater quantity of exogenous EAAs. This is well supported when comparing acute MPS rates in elderly, to that of young adults. Katsanos *et al.* (2006) compared the ingestion of 6.7g EAAs containing either 1.7g or 2.8g leucine, in both young and elderly. In young participants, FSR showed an increase after ingestion of 1.7g leucine, but no further enhancement occurred at 2.8g leucine. However, in elderly participants, there was no significant increase in FSR above baseline after ingestion of 1.7g leucine, while 2.8g leucine did cause an increase in FSR that was not different from young values. This demonstrates that MPS is not different between young and old when a substantial amount of leucine is consumed and underscores the importance of providing adequate EAA and leucine content in a meal when aiming to recover the difference in postprandial anabolism between young and old. Moreover, the ingestion of 35g whey protein is sufficient to increase postprandial MPS above postabsorptive values, with 10g and 20g causing no substantial increase above postabsorptive values in older adults (Pennings *et al.*, 2012). Meanwhile in young, 20g of quality protein renders maximal MPS stimulation, with 40g having no further benefit (Moore *et al.* 2009). This decreased sensitivity of elderly to the anabolic stimulus of hyperaminoacidemia may be overcome by increased leucine ingestion. This suggests that elderly may have a higher 'leucine threshold' that young, meaning a greater plasma leucine concentration is required to elevate MPS rates above postabsorptive values (**Figure 2.7**). Indeed, Paddon-Jones *et al*. (2003) demonstrate that the ingestion of 15g EAA, containing 2.8g of leucine, produced similar increases in mixed-muscle FSR in elderly (0.103%/h) and young individuals (0.088%/h).

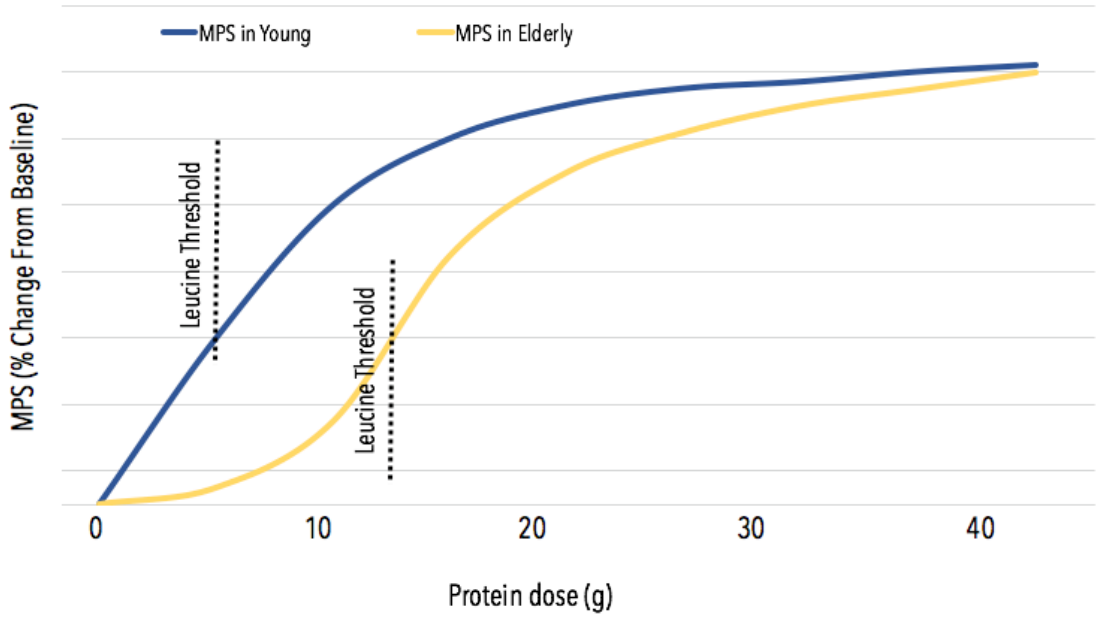


Figure 2.7: Graphic representation of the rightward shift in the 'leucine threshold' with advancing age. Adapted from Breen and Phillips (2011).

Furthermore, physical activity in combination with casein and whey ingestion has been shown to cause an additive anabolic response, when compared to a meal alone in the post-exercise recovery period (Pennings *et al.*, 2011b; Yang *et al.*, 2012a). Pennings *et al.* (2011b) achieved a 30% increase in mixed muscle FSR during, and 26% increase after, 30 min of moderate intensity cycling and resistance type exercises, when compared to non-exercise values. Similarly, Yang *et al.* (2012a), demonstrated that exercise results in an enhancement in myofibrillar FSR at 10g, 20g, 40g whey protein ingestion, when compared to non-exercised values. Of interest, while 40g of whey conferred no additional increase in FSR compared to 20g of whey in the absence of exercise, after resistance exercise, 40g of whey elicited a 32% greater increase in FSR compared to 20g whey. This is in contrast with data from young, in which 20g of protein is sufficient to maximise the MPS response whether prior exercise has been performed or not (Witard *et al.*, 2014; Moore *et al.*, 2009). Optimising the meal-induced anabolic response in older adults should be a main focus in the preservation of skeletal muscle mass with advancing age. Exercise should therefore form an integral part of this effort, due to the combined synergistic effect of exercise and protein feeding on MPS.

2.8.2 Habitual Protein Intake in Older Adults

With the present understanding of the potential importance of protein in healthy ageing, and the diurnal MPS and MPB fluctuations in response to meal ingestion, there has a been an increased interest in habitual intake

and pattern of protein distribution in older adults in recent years. The general pattern of protein intake in older adults is 'skewed', in which the majority of protein is eaten at one meal (usually lunch or dinner) with other meals representing a suboptimal per meal intake of protein (Cardon-Thomas *et al.*, 2017; Tieland *et al.*, 2015; Almoosawi *et al.*, 2013; Bollwein *et al.*, 2013; Ruiz Valenzuela *et al.*, 2013). Habitual dietary intake in the Dutch community determined daily protein intake averaged of 71g, 71g and 58g/day in community dwelling, frail and institutionalized older adults, accounting for 16%, 16% and 17% of their energy intake, respectively (Tieland *et al.*, 2015). Breakfast, lunch and dinner accounted for over 80% of daily protein intake, at 10-12g, 15-23g and 24-31g, respectively, with dinner accounting for 38%-44% of total daily protein intake. Meanwhile, other countries show a trend towards a higher protein lunchtime meal, compared to breakfast and dinner (Bollwein *et al.*, 2013; Ruiz Valenzuela *et al.*, 2013).

In addition to total and per meal protein intake, the habitual sources of protein intake in older adults has also been explored. In the Dutch community, ≥60% of dietary protein consumed originated from animal sources, with meat and dairy as dominant sources. 40% of protein intake in community dwelling, 37% in frail and 29% in institutionalised elderly, respectively, is derived from plant-based sources, with bread being the primary source (Tieland *et al.*, 2015). Similarly, Cardon-Thomas *et al.*, (2017) reported that the contribution of meat, other animal sources and plants to total protein consumption was 42%, 21% and 37%, with animal protein accounting for 73% of total protein intake. At dinner, >70% of the protein intake originated from animal protein, whilst at breakfast and lunch, a large proportion of protein is derived from plant-based protein sources (Tieland *et al.*, 2015). Of note, contributors to inadequate intake of energy and protein include, the cost of these more nutrient dense foods, difficulty chewing fibrous foods, diminishing as we age, perceived food intolerances and fear of eating too much fat and cholesterol in foods (Bauer *et al.*, 2013; Malafarina *et al.*, 2013; Chernoff, 2004), which are also factors to consider. To date, neither the protein distribution pattern nor sources of protein have been examined in Irish adults.

2.8.3 Rationale for Advocating Protein Intake on a Per Meal Basis in Older Adults

Daily protein requirements are currently provided relative to body mass, with the PRI currently standing at 0.83g/kg/d. However, there is a growing acceptance that the recommendations of protein intake for elderly populations need reviewing, and increasing the PRI to 1.0-1.5g/kg/d represents a more appropriate strategy for ameliorating age-related decline in muscle mass in older adults (Bauer *et al.*, 2013). Furthermore, there is

an emerging recognition that per meal protein recommendation, termed 'meal thresholds' should be adopted in lieu of this broad daily requirement, since it offers an effective strategy in maximising MPS over the course of the day (Layman *et al.*, 2015; Areta *et al.*, 2013; Paddon-Jones, 2004). Research suggest that modulating daily protein distribution to an even protein intake at each meal, for example ≥30g high quality protein, or 0.4g/kg of protein, ingested at three main meals, has the potential to maximise MPS, thereby mitigating this decline in skeletal muscle mass over time (Moore *et al.*, 2015; Paddon-Jones and Leidy, 2014; Symons *et al.*, 2009).

Indeed, when habitual meal intake of older adults is compared to the 0.4g/kg threshold suggested for maximal MPS, the proportion of participants meeting this threshold for eating occasion (EO) 1, 2 and 3 is 3%, 42% and 68%, respectively (n=38)(Cardon-Thomas *et al.*, 2017). No participant consumed ≥0.4g/kg threshold for all 3 meals. 8% of participants did not meet the threshold for any meals, 71% met the threshold for one meal, and 21% met the threshold for two meals. These habitual dietary patterns are therefore representative of a suboptimal protein intake at given meals for maximising MPS, while meals that exceed the optimal protein intake will not cause an additive anabolic response (Areta *et al.,* 2013; Moore *et al.,* 2009). This has led to the hypothesis that spreading daily protein intake evenly throughout the day can result in a greater cumulative anabolic response compared to a skewed pattern of protein intake (Areta *et al.*, 2013; Moore *et al.*, 2012). Of note, a more evenly distributed pattern of protein intake has been associated with non-frail older adults. This relationship was investigated in community dwelling older adults (≥75 years of age) in the region of Nürnberg, with frailty being defined as three or more, and pre-frailty being defined as one or two of the following criteria: weight loss, exhaustion, low physical activity, low handgrip strength and slow walking speed. Median total protein intake was 77.5g, 1.07g/kg/d and 16% of total energy intake across participants. While there were no difference between groups (frail, pre-frail and non-frail group) for total protein intake, frail participants tended to have a more uneven protein distribution, with a lower intake at breakfast and higher at lunch (11.9% and 61.4% of total protein consumed at breakfast and lunch in frail, 17.4% and 55.3% in non-frail) (Bollwein *et al.*, 2013). Furthermore, using data from NHANES 1999-2001, Loenneke *et al.* (2016) determined that participants who consumed 1 or 2 main meals over the 30g protein per meal threshold is associated with greater LBM and strength. Furthermore, there was a positive dose-response relationship between protein meal thresholds (15g/meal, 20g/meal, 25g/meal) and leg lean mass and strength, with this association plateauing at 30g/meal when two meals met this threshold and plateauing at 45g/meal when one meal met this threshold. These cross-sectional and epidemiological data provide evidence that a more evenly

distribution protein intake and/or a greater number of meals reaching the purported threshold to elicit MPS is associated with decreased frailty and greater LBM and strength in older adults. However, intervention trials are required to determine if changing distribution in turn has a positive effect of change in muscle mass and function.

In addition to optimising the per meal protein dose and meal frequency in older adults, the source of protein intake also bears significant importance, due to the role of hyperaminoacidemia and therefore high quality proteins in elevating postprandial MPS (Tang *et al.*, 2009). As discussed previously, animal-based sources of protein have been shown to elevate postprandial MPS to a greater extent than plant-based sources in younger adults, with a similar response also apparent in older adults (Yang *et al.*, 2012b). Indeed, 20g and 40g of soy protein were unable to stimulate myofibrillar FSR above postabsorptive values in older adults at rest. Meanwhile 20g of whey was sufficient to elevate FSR above baseline values, with no added elevation when 40g of whey was consumed at rest (Yang *et al.*, 2012a; 2012b). This is related to faster digestibility rates and the greater EAA content of animal-based protein, namely, the amino acid leucine. In older adults, breakfast and lunch are most often lacking in adequate EAA content, since a large proportion of protein at these meal times is derived from plant-based protein sources (Tieland *et al.*, 2015). These sources contain of a lower EAA content (USDA National Nutrient Database for Standard Reference, 2009), in particular leucine and therefore representing a missed opportunity to maximise postprandial anabolism for that meal time. Emphasising adequate, high quality, leucine-rich protein at meal times, particularly at breakfast and lunch where animal protein intake is reported to be lowest, is a necessary consideration when applying the per meal protein targets in practice in older populations.

2.8.4 Nutrition and Training Interventions in Older Adults

Resistance exercise training (RET), in the absence of a change in habitual nutritional intake, has been shown to have a positive effect on muscle mass and function in older adults (Onambélé-Pearson, Breen and Stewart, 2010; Liu and Latham, 2009). Considering the anabolic properties of resistance training and protein/EAA ingestion, combining both strategies may lead to a greater improvement in muscle mass and function than resistance exercise intervention alone. Indeed, the ingestion of a protein supplement alongside resistance-type exercise in elderly, increases muscle mass and strength gains in older adults, during prolonged (≥6 weeks) resistance exercise interventions (Cermak *et al.*, 2012). However, a limitation of this meta-analysis is

the low minimum age criteria for exclusion of participants, with the cut off being as young as 50 years of age. While muscle mass and muscle function has been shown to decline with advancing age, there is a significant sharp rise in the incidence of sarcopenia after the age of 65 (Bautmans *et al.*, 2007), meaning studies using a higher age cut off point for inclusion criteria may be more representative of an 'elderly' cohort. Meanwhile elsewhere, using a more appropriate age cut-off criteria (≥70 years of age), it is reported that intervention trials using protein/EAA supplementation alongside RET, did not result in an additive effect on muscle size, body composition and functionality, in elderly populations (Thomas *et al.,* 2016). Authors concluded that overall improvement from pre-intervention levels were apparent for the majority of outcomes, indicating a positive effect of RET. However, across the 15 studies, these improvements were not significantly different in groups receiving protein/EAA supplements and partaking in RET, when compared with groups partaking in RET alone. Discrepancies in findings likely exist due to differences in supplement use (AA content, EAA content, leucine content), timing (administered once/twice daily), frequency (administered solely on training days/daily) duration of intervention and cut off age of participants, between studies. **Table 2.1** focuses on RCTs which use sufficient per meal protein (~3g leucine/8g EAAs/20-30g protein/~0.4g/kg), in adults >60 years, which have LBM and changes in muscle strength as outcomes, in interventions lasting ≥12 weeks. **Table 2.3** represents the same study characteristics, but also in combination with RET interventions. These studies are discussed in further detail in the next sections. In summary, studies using leucine-enrichment alone did not result in changes in LBM and muscle function over time (Trabal *et al.,* 2015; Verhoeven *et al.*, 2009). When EAA/protein supplementation were only administered once per day, or only on training days there were no changes in LBM or muscle function (Arnarson *et al.*, 2013; Verdijk *et al.*, 2009; Godard, Williamson and Trappe, 2002), while supplementing >1 per day everyday shows benefits (Dillon *et al.*, 2009; Solerte *et al.*, 2008). Whole food-based intervention, while scarce, may offer the most benefit in changes in LBM and muscle function (Daly *et al.*, 2014; Tieland *et al.*, 2012a). The use of whole foods in combined with RET represents an obvious gap in the literature.

Reference	Design and Participants	Protocol, Intervention, total protein intake	Outcome Measures Associated Changed in LBM	Significant Findings For Treatment Compared To Placebo/Control	Conclusions
Norton *et al.* (2016)	Parallel, single blinded, RCT **N**=60, healthy males and females; age, 61(5) years	24-week intervention **PRO,** milk protein-based enrichment to achieve >0.4g/kg protein at breakfast and lunch, daily **PLA,** isocaloric non-nitrogenous maltodextrin, 1.2g-1.6g/kg/d total daily protein, NSD	**LBM Outcomes:** DXA	↑LBM	**Positive effect:** The enrichment of breakfast and lunch with a whey protein supplement resulted in a positive change of LBM compared to a isocaloric non-nitrogenous maltodextrin control in older adults.
Dillon *et al.* (2009)	Parallel, double blinded, RCT **N**=14, healthy women; age, 68(2) years	3 month intervention **AA,** 7.5g EAAs (1.4g leucine) twice daily between meals **PLA,** lactose capsules	**LBM Outcomes:** DXA **Strength Outcomes:** Upper and lower body 1RM **Other**: Basal FSR, postprandial FSR	↑LBM ↑ Basal FSR	**Positive effect:** Prolonged EAA supplementation for 3 months enhances LBM and basal FSR in older women.
Verhoeven *et al.* (2009)	Parallel, double blinded, RCT **N**=30, healthy males; age, 71(4) years	3 month intervention **LEU,** 2.5g leucine three times daily with meals	**LBM Outcomes:** quadriceps CT, DXA **Strength Outcomes:** 1RM leg press, leg	↔	**No effect:** Prolonged leucine supplementation (7.5g/day) for 3 months does not augment LBM or strength.

		PLA, wheat flour 1g/kg/d total protein NSD	extension		
Solerte *et al.* (2008)	Parallel, RCT **N**=41, sarcopenic adults; age, 66-84 years	6-month and 18-month intervention **AA**, 8g EAA (2.5g leucine) twice daily PLA, isocaloric placebo twice daily ~75g total protein daily NSD	**LBM Outcomes:** DXA	↑ LBM at 6 and 18 months	**Positive effect:** Long term supplementation with AA beverage twice per day results in a significant increase in LBM in sarcopenic adults. Participants reached normal nonsarcopenic LBM values at 16 months.

Table 2.2: Summary of RCTs using protein/amino acids/leucine supplementations ≥12 weeks, in older adults (≥60 years). n, participant size; **PRO,** protein supplement group; **LEU,** leucine supplement group; **AA**, amino acid supplement group; **PLA**, placebo group; **EAA,** essential amino acids; **CHO**, carbohydrate; **RCT**, randomised control trial; **PRT,** progressive resistance training; **RET,** resistance exercise training; **PRE**, pre exercise; **POST**, post-exercise; **LBM,** lean body mass; **LTM**, lean tissue mass; **FM**, fat mass; **DXA,** dual x-ray absorptiometry; **CT,** computer-tomography; **RM,** repetition max; **FSR,** fractional synthetic rate; **NSD,** no significant difference for dietary protein intake between groups; **↑**, significant positive difference to PLA; **↓**, significant negative difference to PLA, **↔**, no difference to PLA.

Reference	Design and Participants	Protocol, Intervention, total protein intake	Outcome Measures Associated Changed in LBM	Significant Findings For Treatment Compared To Placebo/Control	Conclusions
Trabal *et al.* (2015)	Parallel, double blinded, RCT ***n*** =30, healthy older adults; age, >70 years	PRT three times per week, for 12 weeks **LEU,** 5g, twice daily, 60min after lunch and dinner 1.3g/kg/d protein **PLA**, maltodextrin placebo 1.2-1.4g/kg/d protein, NSD	**LBM Outcomes:** mid upper arm muscle area, calf circumference **Strength Outcomes:** Leg extension MVC, TUG, standing balance, 4m walk, chair stand	↑MVC, ↑TUG	**Minor positive effect:** The combination of exercise with a twice daily leucine supplement, results in moderate improvements in muscle strength and markers of muscle performance, in older adults, compared to a maltodextrin placebo.
Daly *et al.* (2014)	Parallel RCT ***n*** =100, healthy females; age, 60-90 years	PRT twice weekly, for 4 months **PRO,** 80g cooked red meat, twice daily, at lunch and dinner, 6 days per week (Additional 45g protein per day); 1.3g/kg/d protein **CON**, >1 serving 75g cooked pasta/rice; 1.15g/kg/d protein ($p<0.05$)	**LBM Outcomes:** quadriceps CT, DXA **Strength Outcomes:** 1RM leg extension, TUG, 30 sec STS, FSST	↑LBM, ↑leg LTM, ↓FM, ↑1RM leg extension	**Positive effect:** Protein enrichment with 45g protein from red meat, 6 times per week, enhances the effect of PRT on LBM and muscle strength in older adults.
Arnarson *et al.* (2013)	Parallel, double blinded, RCT ***n*** =161, healthy males and females; age, 65-91 years	RET three times weekly, 12 weeks **PRO,** whey protein (20g protein, 20g CHO) POST	**LBM Outcomes:** DXA **Strength Outcomes:** knee extensor MVC, TUG, 6min walk distance	↔	**No effect:** The ingestion of 20g whey protein in the post training period, did not lead to greater gains in LBM, muscle strength and muscle function,

		PLA, 40g CHO POST 0.9-1g/kg/d total protein, NSD			after 12 weeks RET in older adults
Chalé *et al.* (2013)	Parallel, double blinded, RCT ***n*** =80, mobility-limited adults; age, 70-85 years	PRT three times per week, for 6 months **PRO,** 20g whey and maltodextrin twice daily with breakfast and evening meal **PLA,** isocaloric maltodextrin	**LBM Outcomes:** Thigh CT, DXA **Strength Outcomes:** 1RM and PP leg press and leg extension, SPBB	↑ PP leg extension	**No effect:** Whey protein supplementation offers no further benefit to the effects of a 6 month PRT intervention in older adults
Tieland *et al.*(2012a)	Parallel, double blinded, RCT ***n*** =62, frail older adults; age, 78(1) years	2 sessions per week PRT, 24 weeks **PRO,** 15g milk protein with breakfast and lunch 1.3g/kg/d total protein **PLA,** flavoured placebo 1g/kg/d total protein	**LBM Outcomes:** DXA **Strength Outcomes:** 1RM leg press and leg extension, handgrip, SPPB	↑ LBM	**Positive effect:** Protein supplementation twice per day further augments changes in LBM associated with PRT, when compared to a placebo
Verdijk *et al.* (2009)	Parallel, randomised placebo control trial ***n*** =26, healthy males; age, 72(2) years	12 weeks RET x3 days per week **PRO,** 10g casein, PRE and POST **PLA,** flavoured water, PRE and	**LBM Outcomes:** Quadriceps CT scan, DXA scan, number of muscle fibres and mean fibre CSA via muscle biopsy, **Strength Outcomes:**	↔	**No effect:** Increases in LBM and strength are apparent after 12 weeks resistance training, however, the addition of pre training and post training protein has no additive effect.

		POST 1.1g/kg/d protein NSD	1RM leg press and leg extension		
Godard, Williamson and Trappe (2002)	Parallel RCT ***N*** =17, healthy males; age, >65 years	3 sessions per week PRT, 12 weeks **AA**, 12g EAA (2.2g leucine) and 72g once daily fructose and dextrose **CON,** no supplement Daily total protein, 16% total kcal NSD	**LBM Outcomes:** Mid-thigh CT **Strength Outcomes:** 1RM, MVC, IPT, work capacity test		**No effect:** Daily AA supplementation results in no further enhancement in muscle size and muscle strength and performance during 12 weeks PRT in older males

Table 2.3: Summary of RCTs using protein/amino acids/leucine supplementations, in combination with prolonged (≥12 weeks) RET in older adults (≥60 years). **n**, participant size; **PRO,** protein supplement group; **LEU,** leucine supplement group; **AA**, amino acid supplement group; **PLA**, placebo group; **EAA,** essential amino acids; **CHO**, carbohydrate; **RCT**, randomised control trial; **PRT,** progressive resistance training; **RET,** resistance exercise training; **PRE**, pre exercise; **POST**, post-exercise; **T2D**, type 2 diabetic; **LBM,** lean body mass; **LTM**, lean tissue mass; **FM**, fat mass; **DXA,** dual x-ray absorptiometry; **CT,** computer-tomography; **RM,** repetition max; **MVC,** maximal voluntary contraction; **IPT,** isokinetic peak torque; **PP**, peak power; **TUG,** timed up-and-go test; **FSST**, four square step test; **SPBB,** short physical performance battery; **FSR,** fractional synthetic rate; **NSD,** no significant difference for dietary protein intake between groups; **↑**, significant positive difference to PLA; **↓**, significant negative difference to PLA, **↔**, no difference to PLA.

2.8.5 Leucine-Enrichment in Maximising MPS and Muscle Accretion in Older Adults

Despite the potential superiority of higher leucine meals in stimulating MPS and the potential for muscle accretion over time, few studies have focused on leucine-enrichment of meals in older adults. Murphy *et al.* (2016) demonstrated that the co-ingestion of 5g of leucine supplement with three main meals enhances integrated 3 day rates of MPS in free-living older males using deuterated water to assess newly synthesised myofibrillar proteins, compared to a placebo. Authors demonstrated that this strategy was equally as effective in older males who consume 0.8g/kg/d (low protein, LP) vs. 1.2g/kg/d (high protein, HP) in increasing rates of MPS in rested and exercised conditions. Indeed, myofibrillar protein synthesis was higher in LP compared to placebo, in the unexercised leg (1.57%/d and 1.48%/d, respectively) and in the exercised leg (1.87%/d and 1.71%/d, respectively). This supports the caveat of solely relying on body mass relative recommendation for total protein intake in elderly, since protein intake was not different between treatment groups (LP leucine, 67g and LP placebo, 67g; HP leucine, 98g and HP placebo, 102g). This also reiterates the isolated importance of leucine in the MPS process in elderly, and underlines that leucine-rich meals, high quality protein, and the distribution of meals may have a significant application. Furthermore, myofibrillar protein synthesis values for leucine and placebo were higher in both exercise conditions when compared to rested condition, further emphasising the importance of combining exercise with the aforementioned dietary approaches. Casperson *et al.* (2012) investigated the effects of 2 weeks leucine supplementation (4g at each main meal =12g total), on basal and postprandial mixed muscle FSR and markers of nutrient signalling (mTOR, 4E-BP1 and p70S6K1) in older adults. Both postabsorptive FSR (pre, 0.063%/h; post 0.074%/hr) and postprandial FSR (pre, 0.075%/h; post, 0.1%/h) were greater on day 15, compared to day 0 values. Furthermore, these changes in FSR coincided with a 19% and 13% increase in phosphorylation of mTOR and 4E-BP1in the postprandial state, respectively, and a 23% increase in phosphorylation of p70S6K1 in the postabsorptive state. These data suggests that leucine is a means of chronically improving basal and fed MPS in older adults. While these results are promising in their potential to inform nutrition strategies for ameliorating age-related declines in muscle mass, acute changes in MPS may not necessary represent muscle accretion over prolonged periods of time, and longer interventions are a more accurate representation of the potential for longitudinal muscle accretion. As per table 2.2, Verhoeven *et al.* (2009) demonstrated that supplementation of 2.5g leucine at 3 main meals did not cause changes in LBM or muscle strength over a 3 month period. In the presence of exercise, supplementation of 5g leucine twice daily showed a modest improvement in strength outcomes (Change in leg flexion MVC and change in TUG)) however, authors did not assess changes in LBM over the 12-week period (Trabal *et al.,* 2015). While leucine supplementation shows promise in acute protein synthesis studies, the

potential outcome of combining a leucine supplementation or focusing on leucine enrichemnt from whole foods, with a structured RET program in older adults, remains unclear.

2.8.6 Protein/AA Supplementation in Maximising MPS and Muscle Accretion in Older Adults

While leucine provides the trigger for the stimulation of MPS above postabsoptive values, additional AA are required for the synthesis of skeletal muscle proteins. The supplementation of leucine alongside other AAs yields more positive findings and suggests a potenital synergistic effect when AAs are provided together. As per **table 2.2**, Dillon *et al.* (2009) demonstrated that supplementation of 7.5g EAA, twice daily, results in a 1.7kg increase in LBM, compared to a control group who gained 0.3kg, over 3 months. Similarly, in sarcopenic men, 8g EAA supplementation, twice daily, results in improvements in LBM, with participants achieving normal non-sarcopenic LBM values after 16 months (Solerte *et al.*, 2008).

When similar nutrition strategies are combined with resistance exercise, as per **table 2.3**, the prevailing finding is that protein supplementation results in no further enhancements in LBM and muscle strength (Arnarson *et al.*, 2013; Verdijk *et al.*, 2009; Godard, Williamson and Trappe, 2002). A noticeable trend in the latter studies are the use of one single bolus supplement (Godard, Williamson and Trappe, 2002), or the administration of the supplement only on training days (Arnarson *et al.*, 2013: Verdijk *et al.*, 2009). Therefore, a rational explanation for these results is that protein distribution was not optimised, and there were meals that fell below the leucine threshold purported to maximise MPS, thereby resulting in minimal muscle accretion over time. Since multiple adequate protein meals throughout the day can result in a greater cumulative anabolic response compared to a skewed pattern of protein intake (Paddon-Jones *et al.*, 2015; Mamerow *et al.*, 2014), using >1 per day supplementations may result in more beneficial outcomes for muscle hypertrophy and strength in older adults.

2.8.7 Optimal Per Meal Protein in Maximising MPS and Muscle Accretion in Older Adults

Protein distribution studies that inherently focus on per meal muscle protein anabolism yield conflicting results for changes in acute MPS and long term muscle accretion and strength gains. Kim *et al.* (2015) compared a skewed (SKEWED, 15%, 20% and 65% at EO 1, 2 and 3, respectively) and even (EVEN, 33%, 33% and 33% at EO 1, 2 and 3 respectively) daily protein intake for 0.8g/kg/d of protein and 1.5g/kg/d protein, which represents approximately twice the recommended daily allowance (RDA) of protein. Whole protein kinetics and MPS were assessed on day 4 after 3 days of diet habituation. Net protein balance was 61% greater

and MPS was 17% greater in the 1.5g/kg/d protein group compared to the 0.8g/kg/d protein group, however, protein intake pattern exhibited no effect on net balance or MPS. A limitation of the present study is that the skewed group consisted of just four participants, which means the study may have been underpowered to detect significant differences between groups, and the lack of change may therefore represent a type II error. However, in the aforementioned studies, these acute changes in MPS do not necessarily inform changes in LBM and strength over time. Bouillanne *et al.* (2013) reported that malnourished hospitalised older adults who followed a skewed protein diet (4.5g, 47.8g, 2.3g and 10.9g at EO 1, 2, 3 and 4 respectively), experienced a 0.91kg increase in LBM over a 6 week period, compared to a more evenly distributed protein diet (12.2g, 21g, 13.5g and 21.2g at EO 1, 2, 3 and 4 respectively), in which a loss of 0.41kg was reported. While these results are contradictory to what we have discussed previously, a criticism of the study is the insufficient per meal protein quantity in the EVEN group, meaning each meal potentially failed to reach the leucine threshold required to stimulate MPS above postabsorptive values. Meanwhile, in SKEWED, 47.8g of protein would be sufficient to stimulate a robust anabolic response (Pennings *et al.,* 2012). To further support this thesis, with a similar design Arnal *et al.* (1999) reported a 2-fold enhancement in net protein balance in older women when a skewed protein distribution was adopted (7%, 79% and14% for EO1, EO2 and E03) compared to a more evenly distributed intake (22%, 31%, 19% and 28% for EO1, EO2, EO3 and E04) over 14 days. However, daily protein intake was 1.05g/kg/d, which translates to no more than 13g-20g, 0.19g-0.33g/kg of protein for each meal in EVEN, which falls short of the purported intake for increasing MPS above postabsorptive values. Meanwhile, SKEWED consumed 56g, 0.83g/kg protein for one meal per day, which is more than sufficient to maximise MPS in older adults (Moore *et al.*, 2015; Bauer *et al.*, 2013). Adopting a more evenly distributed protein intake may only offer value when total protein intake is sufficient, since the per meal threshold for maximising MPS would be met. In support, Norton *et al.* (2016) reported a positive change in LBM when breakfast and lunch were enriched with whey protein, over 24 weeks in older adults, resulting in a daily protein intake 1.6g/kg/d. The pertinent focus of the additional protein was to enrich breakfast and lunch, in which a protein intake of 0.4g/kg and 0.47g/kg was achieved, which is line with previous 'meal threshold' recommendations for maximising MPS in elderly (Moore *et al.*, 2015). An increase of 0.6kg in LBM occurred in the protein group, compared to an isocaloric maltodextrin control group, who experienced a 0.16kg loss in LBM over the 24 week period. Of note, protein intake increased from 83g (1.2g/kg/d) to 106g (1.6g/kg/d) with the addition of the protein supplement. Therefore, it is not possible to determine if the positive effect on LBM was a result of an increase in overall daily protein or the more even protein distribution. This is an issue for many studies in that the treatment groups are often not isonitrogenous. Nonetheless, this study was well

designed and long enough in duration to represent muscle accretion in older adults. However, in summary, the effect of strategic feeding of sufficient total protein and EAA/leucine-enrichment of lower protein meals, in modulating MPS and changes in LBM over time in older adults, remains relatively underexplored.

2.8.8 Food-Based Interventions for Increasing Protein Intake and Maximising MPS and Muscle Accretion in Older Adults

The consumption of whole food meals, as opposed to the consumption of supplements via capsules and powders, is representative of a normal diet. To date, much of the research aimed at ameliorating age-related decline in muscle mass in older adults has focused on the use of protein powders, such as whey and casein protein powder, and AA mixtures, which signifies a gap in the literature for the use of a whole food-based nutrition intervention in community-dwelling older adults. Furthermore, there is evidence to suggest that protein its whole-form may have greater anabolic properties; such is the case in young, for whole egg vs. egg whites (Van Vliet *et al.*, 2017), and whole-milk vs. skimmed-milk (Elliot *et al.*, 2006). The few studies which have focused on increasing per meal protein intake with whole foods have indeed reported positive results. Daly *et al.* (2014) investigated the effect of 80g red meat consumed at lunch and dinner, 6 days a week, in older adults following a 4 month resistance training program. There was 0.5kg greater gain in whole body LBM, which was predominantly leg LBM (0.33kg), and an 18% greater increase in 1RM leg extension in the red meat group, when compared to a control group. Of note, dietary protein intake was 1.3g/kg/d for the group consuming red meat, while the total protein intake of the control group was 1.15g/kg/d. Similarly, 15g protein from whole milk, consumed directly after breakfast and lunch, had a greater effect on changes in LBM alongside 24 weeks PRT, when compared to a placebo (Tieland *et al.*, 2012a). The authors reported an increase of 1.3kg in LBM in the group consuming milk, while the placebo group experience a 0.3kg decline in LBM. Similarly, total protein intake increased from 1g/kg/d to 1.3g/kg/d in the milk-consuming group, with no change in the control group. These studies concluded that the increasing per meal protein intake using whole food protein source (15g-22.5g protein, twice daily) results in changes in LBM and strength when combined with PRT. While these whole food-based interventions yield promising results for muscle accretion and strength gains in older adults, a diet which solely focused on deriving addition protein from just one food/food group (i.e. milk and red meat) may not be representative of a long term, sustainable approach for healthy ageing, particularly with our understanding of the factors which influence food choice in elderly (the cost of these more nutrient dense foods, difficulty chewing fibrous foods, diminishing appetite as we age, perceived

food intolerances and fear of eating too much fat and cholesterol in foods (Bauer *et al.*, 2013; Malafarina *et al.*, 2013; Chernoff, 2004). To the best of our knowledge, the effect of a whole food-based intervention, containing a variety of food options and targeting optimal protein intake at three main meals, on muscle accretion in older adults, in combination with exercise training, has yet to be investigated.

2.9 Protein Feeding and Recovery from Exercise Induced Muscle Damage

2.9.1 Exercise Induced Muscle Damage and Delayed Onset Muscle Soreness

Acute, unaccustomed resistance exercise can produce micro-damage as a result of trauma to muscle fibres and connective tissue. This muscle damage is characterised by a mechanical disruption of the muscle membrane, the infiltration of inflammatory cells and an increase in the production of inflammatory cytokines (Proske and Morgan, 2001). Noteworthy, when compared to concentric and isometric training, eccentric training has been shown to create the most significant damage (Nosaka, Newton and Sacco, 2002). The pain and discomfort that often accompanies exercise-induced muscle damage (EIMD) is referred to as delayed onset muscle soreness (DOMS). DOMS is characterised by dull aching pain, tenderness and stiffness and strength loss, which can last up to 10 days after exercise cessation (Connolly, Sayers and Mchugh, 2003; Cheung, Hume and Maxwell, 2003; Clarkson, Nosaka and Braun, 1992). DOMS is experienced to a greater extent in muscles that are untrained (Connolly, Sayers and Mchugh, 2003). Furthermore, athletes who participate in regular resistance training often will experience DOMS to a lesser extent during subsequent exercise sessions of similar nature. The initial damaging exercise bout results in an adaptive response that acts as a protective mechanism against these subsequent bouts of exercise, which can last for several weeks, a phenomenon known as the 'repeated bout effect' (McHugh, 2003). Nonetheless, the combination of pain, stiffness and this decrement in muscle function after unaccustomed exercise can have a detrimental effect on athletic performance (Pearcey *et al.*, 2015). Thus, a nutritional strategy, aimed at ameliorating the deleterious effects of DOMS, may be advantageous by minimally compromising the quality of exercise sessions in the days following a muscle-damaging exercise bout or during intensified or overreaching training.

The mechanism of the development of DOMS is not fully understood and there is much debate about the role of muscle damage in the development of DOMS. Although unaccustomed exercise results in an increase in both DOMS and the concentrations in plasma of proteins associated with muscle damage, research suggests that they are not closely related, and muscle damage is not entirely causative of DOMS (Nosaka, Newton and

Sacco, 2002). Soreness typically peaks 24-48 h post-exercise, returning to pre-exercise values 96 h post-exercise (Connolly, Sayers and Mchugh, 2003). Closely mirroring the time course of these symptoms, creatine kinase (CK) concentrations, widely used as an index of muscle damage, increase 24 to 48 h post-exercise, peaking between 3-6 days and returning to baseline values in 7 to 14 days (Lieber and Friden, 2002). However, studies demonstrated poor correlation between DOMS and CK values (Nosaka, Newton and Sacco, 2002; Malm *et al.*, 2000). In support, certain diseases of skeletal muscle, such as Duchenne's muscular dystrophy, which results in disruptions of the myofibrillar and sarcotubular structures, does not cause muscle pain (Lieber and Friden, 2002). The current consensus is that one single mechanism is insufficient to explain the aetiology of DOMS, and instead a combination of lactic acid accumulation, muscle spasm, connective tissue damage, micro trauma, free radical production, nitric oxide, and inflammation best explain its pathophysiology (Kim and Lee, 2014; Lewis, Ruby and Bush-Joseph, 2012).

2.9.2 Role of Exogenous EAAs in Ameliorating Effects of EIMD

In an effort to reduce the negative symptoms associated with DOMS, several 'recovery strategies' have been explored which include; cold water immersion, ergogenic aids, antioxidant support, non-steroidal anti-inflammatories, and nutrition interventions which include caffeine, omega 3 fatty acids and protein supplements (Kim and Lee, 2014; Howatson *et al.*, 2012). Of note, the use of omega 3 fatty acids has shown promise in minimising the negative effects of EIMD. Tartibian, Maleki and Abbasi, (2009) demonstrated that 30 days supplementation of 1.8g/d omega-3 fatty acids resulted in a reduction in perceived soreness and thigh circumference compared to a placebo, 24-48 h following an intense exercise bout. Furthmore Philpott *et al.*, (2018) demonstrated that the addition of 1.1g omega-3 fatty acids to a whey protein, leucine and/or CHO beverage, results in a reduction in muscle soreness and plasma CK in the days following an intense exercise bout. Therefore, it appears that nutrition plays a role in ameliorating the deleterious effects of EIMD. Of particular interest to the present chapter and thesis is the use of AAs, namely branched chain amino acids (BCAA), which have shown mixed results in ameliorating symptoms of EIMD (Waldron *et al.*, 2017; Fouré *et al.*, 2016; Ra *et al.*, 2013; Howatson *et al.*, 2012; Jackman *et al.,* 2010). Research has focused on the ingestion of exogenous EAAs prior to and/or after resistance training, seeing this as a critical window to enhance recovery. The mechanism behind the beneficial effect of AAs on exercise recovery is not fully understood. It has erroneously been suggested that this enhancement in recovery is related to the resultant increase in MPS following AA ingestion. Indeed, while the stimulation of MPS following protein ingestion is critical to skeletal muscle remodelling over time, this process is long in duration. It is therefore unlikely to be responsible for an

enhanced recovery from exercise over a short window of several hours to several days. Studies that have investigated the effect of single bolus exogenous EAAs before and after exercise (from an AA mixture, whole milk and milk components, such as whey) in ameliorating symptoms of EIMD are summarised in **table 2.4.** Since the recovery period from EIMD typically lasts several days, with soreness, marker of muscle damage and performance only peaking +48-72 h after exercise, studies which have investigated these outcomes several days after exercise are included.

2.9.3 The Role of Single Bolus EAAs in Exercise Recovery

In summary, the present consensus is that a single bolus of exogenous EAAs, ingested prior to or after exercise, confers benefits to recovery from EIMD, when compared to a placebo. Indeed, supplementation with an AA mixture, milk or whey protein has been shown to ameliorate the deterioration of muscle function and increases in blood markers of muscle damage following a bout of muscle damaging exercise. It is within scientific reason that supplementing >1 per day or beyond the immediate post-exercise window would confer additive benefits for recovery. Studies that have investigated the effect of >1 boluses of exogenous AAs in the post-exercise period, in ameliorating the effects of EIMD in the days following exercise, are summarised in **table 2.5**.

Reference	Design and Participants	Protocol and Intervention	Outcome Measures Associated with EIMD	Significant Findings For Treatment Compared To Placebo/Control*	Conclusions
Shimomura *et al.* (2006)	Crossover design, double blinded randomised control trial ***n*** =34 untrained males, ***n*** =16 untrained females	20 squats x 7 sets **BCAA,** 5g (1:2.3:1.2), 1g green tea powder, 1.2g aspartame, 15min PRE **PLA,** Dextrin, 1g green tea powder, 1.2g aspartame	**DOMS:** Soreness **Muscle Function:** Self-reported muscle fatigue	↓Soreness in females (48, 72, 96, 120 h) ↓Muscle fatigue in males and females (96 h)	**Positive Effect:** BCAA supplementation may alleviate symptoms of muscle damage after a muscle damage bout of exercise, in untrained males and females.
Etheridge, Philp and Watt (2008)	Crossover design, double blinded randomised control trial ***n*** =9 trained males	30min downhill running at 75% age-predicted HR max **MILK,** 100g milk protein concentrate (40g EAAs) **PLA**, flavoured water Immediately POST	**DOMS:** Soreness **Muscle Function:** MVC **Blood Markers:** CK	↑MVC (48 h)	**Positive Effect:** The rate of force and power restoration following an exercise bout of muscle damage, can be accelerated by consumption of a milk protein mixture immediately post-exercise.
Cockburn *et al.* (2010)	Single blinded, parallel, randomised control trial ***n*** =32, trained males	10 repetitions x 6 sets, knee flexions **PRE,** chocolate milk, (33.4g PRO), consumed 3min PRE **POST,** chocolate milk, (33.4g PRO), consumed POST **24 POST,** chocolate milk, (33.4g PRO), consumed 24 h POST **CON,** water	**DOMS:** Soreness **Muscle Function:** PT, RSI **Blood Markers:** CK	↓Soreness, ↑PT, ↑RSI, POST and 24 POST, vs. PRE and CON ↓CK, PRE, POST and 22 POST, vs. CON	**Positive Effect:** Whether supplemented before, immediately post or 24 h post-exercise, a protein and carbohydrate drink exhibits a positive effect in ameliorating the decline in muscle function and increase in CK associated with EIMD.

* h denotes the hours post-exercise at which the difference between treatment group and control group for each variable was detected. Differences with h denotation is time-point not specified.

Shimomura *et al*, (2010)	Crossover design, double blinded randomised control trial ***n*** =12, untrained females	20 squats x 7 sets **BCAA,** 5.5g (1:2.3:1.2), 1g green tea powder, 1.2g aspartame, 15min PRE **PLA,** Dextrin, 1g green tea powder, 1.2g aspartame	**DOMS:** Soreness **Muscle Function:** MVC, **Blood Markers:** CK, Mb	↓Soreness (24, 48 h), ↑MVC (72 h), ↓Mb (24-72 h)	**Positive Effect:** BCAA supplementation may alleviate symptoms of muscle damage after a muscle damage bout of exercise in untrained females.
Cockburn *et al.* (2012)	Parallel, randomised control trial ***n*** =24, trained males	10 repetitions x 6 sets, knee flexions **1000ml milk,** 34g PRO **500ml milk,** 17g PRO **CON,** 1000ml water Immediately POST	**DOMS:** Soreness **Muscle Function:** PT **Blood Markers:** CK, Mb, IL-6	↑PT, 1000ml vs. CON ↓CK, 1000ml vs. CON ↔ PT, CK, between 1000ml and 500ml	**Positive Effect:** Decrements in isokinetic muscle performance and increases in CK can be ameliorating with the consumption of 500ml milk immediately post-exercise, with 1000ml showing no additive benefit.

Table 2.4: Summary table of studies which use a single bolus of exogenous amino acids before or after exercise induced muscle damage. n, participant size; **BCAA**, BCCA group; **PLA**, placebo group; **CHO**, carbohydrate; **EIMD**, exercise induced muscle damage; **PRE**, Pre EIMD; **POST**, Post EIMD; **RM,** repetition max; **MVC**, maximal voluntary contraction; **MIC**, maximal isometric contraction; **PIT**, peak isometric torque; **EMG,** electromyography; **PO**, power output; **C,** circumference; **VJ,** vertical jump**; CMJ**, counter movement jump; **CK**, creatine kinase, **LDH**, lactate dehydrogenase; **Mb**, myoglobin; **CRP,** c-reactive protein; **GEL,** granulocyte elastase; **IL,** interleukin; ↑significant positive difference to PLA; ↓significant negative difference to PLA, ↔no difference to PLA.

Reference	Design and Participants	Protocol and Intervention	Outcome Measures Associated with EIMD	Significant Findings For Treatment Compared To PLA*	Conclusions
Kraemer *et al.* (2006)	Double blinded, parallel, randomised control trial ***n*** =17, trained males	4 week total body, resistance training, designed to achieve overreaching **AA,** 0.1g/kg between meals **PLA,** cellulose Every day, 4 consecutive weeks	**Muscle Function:** 1RM back squat, 1RM bench press **Blood Markers:** CK **Others:** Total testosterone, human growth factors, sex hormone binding globulin, insulin-like growth factor, insulin, cortisol, haemoglobin, uric acid	↑1RM back squat, ↑1RM bench press	
Cockburn *et al.* (2008)	Single blinded, parallel, randomised control trial ***n*** =24, trained males	10 repetitions x 6 sets, knee flexions **CHO+PRO,** chocolate milk, (33.4g PRO) **MILK,** (34g PRO)	**DOMS:** Soreness **Muscle Function:** PT, **Blood Markers:** CK, Mb	↑PT in MILK vs. and CHO+PRO vs. CON (48 h)	**Positive Effect:** Decrements in isokinetic muscle performance and increases in CK, Mb can be ameliorating with the

* h denotes the hours post-exercise at which the difference between treatment group and control group for each variable was detected. Differences with h denotation is time-point not specified.

		CHO, sports drink **CON,** water Immediately POST and 2 h POST		↓CK in MILK and CHO+PRO vs. CHO (48 h) ↓Mb in CHO+PRO vs. CHO	consumption of milk based protein and carb drink, or milk, post-exercise.
Betts *et al.* (2009)	Crossover design, single blinded, randomised control trial ***n*** =17, trained males	90min intermittent shuttle runs **CHO+PRO,** ~21g PRO and ~62g CHO **CHO,** ~62g CHO POST, every 30 min x 8 boluses	**DOMS:** Soreness **Muscle Function:** PIT **Blood Markers:** CK, LDH, Mg, IL-1, IL-6, IL-10, CRP	↔	**No Effect:** The addition of protein to a carbohydrate drink, in the 4 h following exercise, exhibits no additive effect on ameliorating the negative effects of EIMD, when compared to carbohydrate alone.
Matsumoto *et al.* (2009)	Crossover design, double blinded, randomised control trial ***n*** =12, trained males and females	Uphill and downhill running, for 3 consecutive days **BCAA,** 8g (4g leucine) x2, between breakfast and lunch, between lunch and supper, 4g BCAA (2g leucine) x1 after supper, for 3 consecutive days **PLA,** dextrin	**DOMS:** Soreness, fatigue sensation **Blood Markers:** CK, LDH, GEL	↓Soreness (24 h), ↓fatigue sensation (24 h), ↓CK (24 h), ↓LDH (24 h), ↓GEL (24 h)	**Positive Effect:** BCCA supplementation during a three-day intensive training program, reduces soreness, fatigue and attenuates the increase in markers of muscle damage, compared to a placebo.
Hoffman *et al.* (2010)	Single blinded, parallel, randomised control trial	10 repetitions x 4 sets, squat, deadlift, lunges @80% 1RM, for 3 consecutive days	**DOMS:** Soreness	↑Repetitions (24, 48 h)	**Small Positive Effect:** Protein supplementation,

	n =15, trained males	**PRO,** proprietary protein blend (whey, casein, BCAA), 42g PRO **PLA,** maltodextrin 10min PRE and 15min POST, for 3 consecutive days	**Muscle Function:** Repetitions achieved in subsequent days, peak PO, mean PO **Blood Markers:** CK		consumed before and after exercise, performed for 3 consecutive days has a positive effect on muscle function in the days following exercise, but exhibits no effect on markers of muscle damage.
Jackman *et al.* (2010)	Single blinded, parallel, placebo control trial ***n*** =24, untrained males	10 repetitions x 12 sets eccentric leg extension at 120% MVC **BCAA,** (3.5g leucine, 2.1g isoleucine, 1.7g valine) x5 - 30min PRE, 1.5 h POST, before breakfast, between lunch and dinner, and bedtime. BCAA x4 consumed between meals for subsequent 2 days. **PLA,** Artificial sweetener and flavoured water	**DOMS:** Soreness **Muscle Function:** MIS **Blood Markers:** CK, Mb, IL-6	↓Soreness (48, 72 h)	**Small Positive Effect:** BCAA supplementation, consumed for 3 days post-exercise, attenuates muscle soreness, but has no affect no decreased muscle function and markers of muscle damage, following a bout of muscle damaging exercise.
Howatson *et al.* (2012)	Double blinded, parallel, randomised control trial ***n*** =12, trained males	1 EIMD session, Drop Jump x100 **BCAA**, 12 days, (7 day loading phase, 4 days POST) 10g x2 per day. Additional 20g 1 h PRE, and 20g POST.	**DOMS:** Soreness, Thigh C, Calf C **Muscle Function:** MVC, VJ	↓Soreness (24, 48 h), ↑MVC , ↓CK	**Positive Effect:** BCCA supplementation before and after muscle damaging exercise reduces markers of muscle damage and accelerates

		PLA, artificial sweetener	**Blood Markers:** CK		recovery.
Ra *et al.* (2013)	Double blinded, parallel, randomised control trial ***n***=18, untrained males	5 eccentric repetitions x 6 sets 90% MVC bicep curl x 3 consecutive days **BCAA,** 3.2g, x3 per day, 2 weeks PRE, 3 days of EIMD **PLA**, Starch, 2 weeks PRE, 3 days of EIMD	**DOMS:** Soreness, Upper Arm C **Blood Markers:** CK, LDH, aldolase, 8-hydroxydeoxyguanosine	↔	**No Effect:** BCAA supplementation alone three times a day, for 2 weeks prior and 3 days after a muscle damaging exercise has no added benefits on exercise induced DOMS or makers of muscle damage, compared to control.
Fouré *et al.* (2016)	Double blinded, parallel, randomised control trial ***n***=26,untrained males	Neuromuscular electrostimulation session **BCAA,** 100mg/kg (2:1:1) (mean 7g) 30min PRE, PRE and POST EIMD, and single dose PRE 4 subsequent daily testing sessions **PLA,** Not specified	**DOMS:** Soreness **Muscle Function:** MVC **Blood Markers:** CK	↔	**No Effect:** BCAA supplementation before and after muscle damaging exercise did not elicit any changes in DOMS or markers of muscle damage, when compared to a placebo.
Kephart *et al.* (2016)	Double blinded, parallel, placebo control trial ***n***=30, trained males	5 repetitions x 10 sets back squats at 80% 1RM, 3 consecutive days **BCAA,** leucine (3g), 1g isoleucine, 2g valine, and 2g CHO/day. POST x3 days	**DOMS:** Soreness **Muscle Function:** 1RM back squat, PIT, MIC, mean EMG, peak EMG	↓ Monocytes	**No Effect:** BCAA supplementation had no enhanced effect on markers of performance and muscle damage. BCAA did attenuate the increase

		PLA, 42g CHO	**Blood Markers:** Mg, WBC, Neutrophils, lymphocytes, monocytes		of monocytes compared to placebo.
Greer *et al.* (2007)	Crossover design, double blinded, randomised control trial ***n***=9, untrained males	90 minute 55% VO_{2peak} **BCAA,** 2.5g (1.22g leucine), 5 min PRE and 60 min later **PLA x2,** Isocaloric CHO beverage, and a non-caloric beverage with artificial sweetener	**DOMS**: Soreness **Muscle Function:** MVC **Blood Markers:** CK, LDH	↓Soreness (24 h) , ↓CK (4, 24, 48 h), ↓LDH (4 h)	**Positive Effect:** BCAA supplementation attenuates muscle damage after prolonged endurance exercise.
Waldron *et al.* (2017)	Double blinded, parallel, randomised control trial ***n***=16, untrained males	20 squats x 7 sets @70% 1RM **BCAA,** 0.087g/kg (2:1:1) (mean 8g) and dextrose, PRE and POST EIMD and testing at 24, 48, 72 hr. **PLA,** Dextrose	**DOMS:** Soreness **Muscle Function:** MIC, CMJ **Blood Markers:** CK	↓Soreness (24, 48 h), ↑MIC (24 h), ↑ CMJ (24 h), ↑CK (24, 48 h)	**Positive Effect:** Acute supplementation of BCAA before and after muscle damaging exercise improves performance markers and decreases perceived DOMS, 24 and 48 h post-exercise.

Table 2.5: Summary table of studies which use >1 bolus of exogenous amino acids after exercise induced muscle damage. n, participant size; **BCAA**, BCCA group; **PLA**, placebo group; **CHO**, carbohydrate; **EIMD**, exercise induced muscle damage; **PRE**, Pre EIMD; **POST**, Post EIMD; **RM,** repetition max; **MVC**, maximal voluntary contraction; **MIC**, maximal isometric contraction; **PIT**, peak isometric torque; **PT**, peak torque; **EMG,** electromyography; **PO**, power output; **C,** circumference; **VJ,** vertical jump**; CMJ**, counter movement jump; **CK**, creatine kinase, **LDH**, lactate dehydrogenase; **Mb**, myoglobin; **CRP,** c-reactive protein; **GEL,** granulocyte elastase; **IL,** interleukin; ↑ significant positive difference to PLA; ↓ significant negative difference to PLA, ↔ no difference to PLA.

2.9.4 The Role of >1 Boluses Per Day EAAs in Exercise Recovery

The present literature on the role of AAs, administered >1 time in the post-exercise period, in ameliorating the negative effects of EIMD, has yielded conflicting findings. This is likely due to the difference in participants recruited, the muscle damaging protocol employed, outcome measures, degree of dietary control, AA dose used, AA source used and supplement strategies used; with some studies using AA supplementation only before and after exercise, others having a loading phase in the days before exercise and some strategies continuing on for days after the exercise session.

Nonetheless, in summary, AA supplementation, namely BCAA, appears to be efficacious in ameliorating the outcome of EIMD, with a propensity to elicit greater benefits when the muscle damaging protocol is less severe. Indeed, BCAA supplementation yielded little to no benefit in outcomes of EIMD in studies which consisted of three consecutive days of muscle damaging exercises (Kephart *et al.*, 2016; Ra *et al.*, 2013; Hoffman *et al.*, 2010). However, these severe, consecutive day, repeated muscle damage protocols are not reflective of a real world periodised training plan in sports involving resistance training, therefore concluding that AA supplementation does indeed confer benefits for recovery from a single muscle damaging exercise session. Furthermore, there is a tendency for longer supplementation strategies to confer greater benefits in recovery (Kraemer *et al.*, 2006; Howatson *et al.*, 2012; Waldron *et al.*, 2017). With our current understanding of the superiority of multiple adequate high leucine/protein meals in maximising cumulative daily MPS (Layman *et al.*, 2015; Areta *et al.*, 2013; Moore *et al.*, 2012), it is possible that longer supplementation period is required to elicit the benefits of BCAA supplementation. Indeed, this thesis is well supported by RCTs using AA mixtures ingested for several days after EIMD. Nosaka, Sacco and Mawatari, (2006) demonstrated that extending AAs supplementation several days beyond post-exercise, confers greater benefits in exercise recovery, compared to a AAs ingested simply pre and post-exercise. Two boluses of 4.5g AAs (9 essential and 2 non-essential AAs) ingested 30 min before and immediately after exercise, was compared to a protocol in which the same boluses were ingested pre and post-exercise, with an addition 8 boluses ingested over the 4 days following exercise. CK, myoglobin (Mg) and muscle soreness using visual analogue scale (VAS) were significantly lower in the group which supplemented for an extra 4 days, concluding that AAs attenuate DOMS and markers of muscle damage when applied in the subsequent days recovery from a muscle damaging exercise bout. In support, Kraemer *et al.* (2006) investigated the effect of daily, evenly distributed AA ingestion on markers of recovery in a group of trained males, during 4 weeks of strategic overreaching training. 0.1g/kg

AA was ingested separate to meal (1 h before meals and 2 h after meals), four times per day. Based on mean body mass of 89.1kg, each dose of AA was approximately 4.5g of BCAA comprised of 2.2g leucine. Participants were weighed every 7 days and kept food diaries, in an aim of keep dietary intake isoenergetic for all participants, and was represented by a typical American diet, of 55% carbohydrates, 30% fat and 15% protein. Muscle strength and marker of muscle damage were assessed at every 7 days. 1 repetition max (RM) squat and bench declined in week 2 for placebo group, with no change apparent in AA group. In week 3, 1RM returned to baseline in placebo group, while the AA group experienced a significant increase in 1RM at this time-point. While both groups experienced an elevation in CK in week 1, this increase was significantly lower in the AA group. While these results support the use of AA supplementation after EIMD, a significant limitation in the present study is poor dietary control. While the aim was to keep calorie intake at maintenance levels for participants, quantity and source of protein was not controlled, which means it is difficult to attribute these benefits solely to the AA supplement. Matsumoto *et al.* (2009) demonstrated that when nutrition was controlled tightly, in which a BCAA and placebo group consumed the same meals in a cross-over design, results are still positive for the BCAA group. Following an intense 3 day training program, in which BCAA supplements were provide between meal, 2-4g leucine x 3 times per day for 3 days, muscle soreness and fatigue sensation were lower, when compared to a placebo group consuming dextrin only. BCAA supplementation also attenuated the increase in plasma CK, lactate dehydrogenase (LDH) and granulocyte elastase (GEL) following the training program, when compared to placebo group. The mechanism behind this enhancement in recovery following EIMD is not fully understood. It is unclear whether the benefits of AAs are related to a decrease in MPB, an increase in muscle protein anabolism, a combination of both mechanisms, or a mechanism yet to be identified. Nonetheless, these are promising results that potentially support the use of supplementary BCAA and AAs multiple times per day, for a prolonged period (i.e. beyond the immediate post-exercise period) in recovery from EIMD.

2.9.5 The Role of Leucine and Leucine-Enriched Beverage in Exercise Recovery

Of the three BCAA, leucine is the most evident contributor to postprandial anabolism and anti-catabolism. However, few studies have investigated the effect of leucine, in isolation, on recovery from resistance training. Positive results have been achieved in animal models, in which leucine-enriched AAs elicit improved rates of MPS and ameliorate muscle soreness after eccentric exercise in rats (Kato *et al.*, 2015). However, the role of solely leucine, in ameliorating the effects of EIMD in humans, remains underexplored. Thomson, Ali and Rowlands (2011) investigated the effects of a leucine-enriched protein and carbohydrate beverage on recovery

from subsequent cycling performances and markers of muscle damage. Trained cyclists performed 2-2.5 h of interval cycling on three consecutive evenings, and in a cross-over design consumed one of two beverages (one containing ~22g leucine, or an isocaloric control) alongside a carbohydrate and protein meal within the first 90 min post-exercise. Each morning following the three cycle trials, participants consumed the alternate beverage, thereby isolating the post training nutrition effect. Dietary intake was controlled for the duration of the trial, with protein intake fixed at 1.6g/kg/d. 39 h following the last exercise bout, the PRO+CHO+LEU group showed a 2.5% improvement in mean sprint power in a repeated sprint performance trial, 13% reduction in levels of fatigue, CK was 19% lower, with no difference in perceived tiredness and soreness between treatment groups. This suggests that a leucine-enriched beverage and meal in the 90 min following 3 consecutive days of cycle performance, enhances recovery and subsequent high intensity endurance performance. In contrast, when leucine is supplemented without the presence of other AAs, the results are contradictory; suggesting that supplementation of leucine alongside other AAs or a mixed meal results in a synergistic affect and promising results for recovery, as discussed previously in the context of MPS. Indeed, Stock *et al.* (2010) demonstrated that adding leucine to a carbohydrate beverage before and after exercise has no additive benefit on recovery from resistance exercise. Participants consumed a 0.35g/kg carbohydrate beverage, alongside 22.5mg/kg leucine, 30 min before and immediately after a muscle-damaging bout of exercise. Of note, the average leucine content of each beverage was 1.9g, at a mean body mass of 82.7kg, equating to a total of 3.7g leucine consumed within 60 min post-exercise. CK concentrations increased after exercise (24 h), and peaked at 48 h, returning to baseline at 72 h in both groups, but there were no differences between the treatment groups, nor were differences apparent in the lactate dehydrogenase (LDH) time course between groups. When asked to quantify their degree of DOMS using a VAS, participants self-reported DOMS peaked at 48 h in both groups, but again there was no difference between treatment groups. In addition, Kirby *et al.* (2012) investigated the effect of leucine supplemented 30 min before, immediately post-exercise and the morning of each subsequent recovery days following the exercise bout, compared to a placebo. 250mg/kg of leucine, represented an average intake of ~19g per bolus, resulted in no attenuation in CK, Mb or perceived soreness, assessed using VAS, with the supplement group reporting greater soreness after exercise. However, leucine supplementation attenuated the drop in peak force output during an isometric contraction, when compared the placebo group.

In summary, studies to date, which have investigated the effect of leucine alone supplementation on accelerating recovery from EIMD, have deemed leucine to elicit little to no benefit. A plausible explanation for

the insignificant differences in recovery markers between treatment groups in the latter studies, is that leucine was only consumed before and after exercise, or once per day in the subsequent days following the muscle damaging bout of exercise. A recovery protocol aimed at providing >2 leucine boluses, one or more days after training, may facilitate recovery from EIMD. To the best of our knowledge, no studies have investigated the effect of leucine supplementation on exercise recovery parameters in this way, or the role of enriching a low-leucine meal plan, given that this would also provide a range of other AAs to support recovery processes.

2.10 Conclusions

From the present review of current literature surrounding the role of leucine in the enrichment of meals, its role in the treatment and/or preventative for the age-related decline in muscle mass in older adults, and in recovery from EIMD, there are some obvious knowledge gaps which warrant further investigation.

With our present understanding of the role of the plasma leucinemia in postprandial MPS, leucine-enrichment is emerging as a promising strategy to increase the anabolic properties of a meal. While several studies suggest that leucine-enrichment offers an advantage in stimulating postprandial MPS compared to a meal alone, few studies to date have investigated the plasma kinetics of leucine ingested alone, and co-ingested with a mixed meal. Microencapsulation technology represents a means to alter the plasma kinetics of leucine, and through taste-masking, have broader application in enriching food matrices with leucine, but to date microencapsulated leucine has not been studied.

The well-established age-related decline in muscle mass with advancing age has a number of contributing factors, including a general decline in physical activity with age and a decrease in appetite which often results in a reduction in overall energy and protein intake. Modifications in exercise and diet would therefore appear a promising strategy in the treatment and/or prevention of loss of muscle mass and function with age. However, there still remains ambiguity of the efficacy of a training and nutrition intervention which positively influences changes in muscle mass and function in older adults. Furthermore, the manifestation of 'anabolic resistance' in which older adults have a dampened response to the anabolic characteristics of exercise and dietary protein, further exacerbates the issue. Older adults have been shown to possess a higher 'leucine threshold' when compared to young adults, in that a higher leucine or protein dose is required to elicit a robust postprandial anabolic response. Despite leucine being a key to potentially overcoming the anabolic resistance exhibited by

older muscle, few studies have focused on leucine as a nutrient in combatting the age-related decline in muscle mass. Furthermore, much of the research surrounding the modification of dietary protein intake in older adults, with an aim to positively influence changes in LBM, has focused on the use of powdered proteins and oral solutions. Indeed, few studies have focused on the use of whole foods, despite some research that suggests that food in its natural state has greater anabolic characteristics when compared to that which is more refined. Therefore, the use of a whole food-based intervention targeting leucine-rich foods as a strategy for increasing protein intake, and therefore positive influencing exercise-mediated changes on muscle mass and function, remains to be investigated.

Unaccustomed exercise can produce micro-damage to skeletal muscle and connective tissue which results in a decrease in muscle function and soreness in the days following exercise. These deleterious effects can result in a decrease in performance, compromising the quality of subsequent training sessions. Protein and AAs in the post-exercise period appears promising in ameliorating the deleterious effects of intense exercise. However, the role of leucine in particular, remains underexplored. Furthermore, the role of different temporal feeding strategies (i.e. bolus vs. pulse fed) of leucine in the hours following intense exercise, has not yet been investigated. The following chapters aim to address these present knowledge gaps and contribute to the current literature.

Chapter 3

3.0 Materials and Methods

3.1 Microencapsulation Preparation

The microencapsulation of leucine using a hydrolysed milk protein matrix material was created as per Hone *et al.* (2017) and described by Brodkorb and Doherty (2015). The method comprises the steps of providing a suspension of hydrolysed whey protein and an active component in a carboxylic ester, treating the suspension to generate droplets of the suspension and immediately curing the droplets by immersion in a basic curing solution. The ester in the suspension reacts with the basic curing solution to release a salt that polymerises the hydrolysed whey protein encapsulating the active component (Brodkorb and Doherty, 2015). Whey protein isolate (WPI) was dissolved in sterile water (9% w/v) for 16 hr at 4°C under slight agitation (180 rpm); the solution was adjusted to pH 7 with 100 mM HCl and filtered through Durapore® 0.45- μm HVLP (Millipore Ireland BV, Cork, Ireland). The appropriate formulation for a curing medium was investigated using calcium chloride, acetate, and citrate buffers systems. Uniform size whey protein micro-particulates were prepared for the encapsulation and extrusion of leucine using an extrusion ratio of 95:5 of leucine:WPI. Process temperatures were maintained at 35°C to optimize encapsulation efficiency, as per Hone *et al.* (2017).

3.2 Cannulation and Blood Handling

The participant was laid on a bed with their arm in a hyperextended position. A tourniquet was applied approximately 4 inches above the antecubital fossa. The puncture site was cleanses using a sterile pre-injection wipe. A single-use butterfly needle (or cannula in the event of multiple draws) was inserted in the antecubital vein, with the needle bevel upwards. After the puncture, the tourniquet was removed. For cannulation, the sample line was kept patent with saline. Blood samples (~4ml) were drawn into vacutainers containing lithium heparin (BD Vacutainers, Heparin Tubes). Once the sufficient blood has been collected, the needle was removed and disposed, and a swap was immediately placed with pressure on the participant's antecubital fossa until bleeding had ceased. The vacutainer was inverted eight to ten times and placed on ice. The blood was centrifuged at 4000 g for 10 min at 4°C and the upper layer of plasma was transferred into three separate 1.5ml tubes and stored at -80°C until further analysis.

3.3 High Performance Liquid Chromatography

The concentration of amino acids in plasma was measured using High Performance Liquid Chromatography (HPLC), according to the manufacturer's instructions (Henderson and Brooks, 2010) with minor adjustments as per Power-Grant *et al.* (2016). The principle of HPLC is a separation technique that involves the injection of the liquid sample into a column packed with porous particles, in which individual components of the sample are transported along the column by a mobile phase. The components of the sample are separated from one

another within the column. The separated components are collected at the exit of this column and identified by spectrophotometer measured via fluorescence detection. Fluorescence detection is more sensitive than utra violet detection and allows measurement of amino acids at an extremely low concentration.

3.3.2 Preparation of Reagents

2L of mobile phase A [10mM Na_2HPO_4, 10mM $Na_2B_4O_7$, pH 8.2, 5mM NaN_3] was prepared, using 2.8 g of Na_2HPO_4 (Sigma), 7.6 g of $Na_2B_4O_7$ (Sigma), 32 mg of sodium azide (Sigma). Solutes were dissolve in 800 ml of HPLC grade water. 2.4 ml of concentrated HCl. 1L of solution was made in a volumetric flask. 1L HPLC grade water was added to a pH of 8.2 using concentrated HCl.2 L of mobile phase B [Acetonitrile (Lennox, Romil SPS): methanol (Lennox, Romil SPS): water (45:45:10, v: v: v)] was prepared as follows 900ml acetonitrile, 900ml methanol, 200ml HPLC grade water. Injection diluent was made using 100ml mobile phase A and 0.4ml concentration of phosphoric acid (H_3PO_4, Sigma). This was stored at 4°C. 500 ml 0.1 N HCl was prepared using 4.2mL of concentrated HCl (VWR, 36 %) and make up to 500ml with HPLC grade water. 50:50 0.1M HCl and water was used for preparing amino acid stock solutions and internal standard stock solutions. Store at 4°C. 0.4M perchloric acid was also prepared.

3.3.3 Perchloric acid extraction and sample preparation

Once ready for analysis, plasma samples were defrosted at -20°C and then at room temperature. Samples were mixed using a 2 sec (speed 7) vortex. Samples were centrifuged at 4°C, 5000 rpm for 3 minutes. 50 µL of plasma was added to 15µl 1mM norvaline. 85µL of ice cold, 0.4M perchloric acid was added. All samples were gently vortexed for 5 sec (speed 7) and stored on ice. Samples were centrifuged at 4˚C, 8 min 10,000 rpm. 50 µl of supernatant was added to each well of the autosampler plate.

3.3.4 Reference Standards and Calibration

1mM Norvaline was used a standard added to each plasma sample. 0.0293 g of norvaline (Sigma) was prepared in 25ml of 50:50 water 0.1M HCl. Stored at 4 ˚C. 1ml of 10 mM norvaline added to 9ml of 50:50 HCl:water. A Sigma stock consisting of 18mM glutamine, 15 mM taurine and 4mM tryptophan was prepared using 65.7 mg of glutamine (Sigma), 46.9mg of taurine (Sigma), and 20.4mg of tryptophan (Sigma) weighed into a 50 ml tube. 10 ml of 50:50 water 0.1M HCl was added and vortexed to dissolve the powders. This was added to a 25 ml volumetric flask and topped up to the mark. This was stored at 4 ˚C. A working stock solution was also prepared as a reference standard using a combination of norveline, Sigma stock and 1nM Aligent amino acid stock. 3 ml of working stock solution was prepared as by adding 750 µL of agilent stock, 150 µL of

Sigma stock, 75 µL of the norvaline standard and 2025 µL of water to a plastic tube. Vortex mix and store at 4˚C.

A five-point calibration curve was constructed for each AA in concentration range as per **table 3.1**.

Calibration Level	Agilent standard (µM)	Glutamine (µM)	Taurine (µM)	Tryptophan (µM)	Norvaline (µM)
1	250	900	750	200	250
2	125.0	450	375	100	125
3	62.5	225	187.5	50	62.5
4	30	108	90	24	30
5	15.0	54	45	12	15

Table 3.1: Five-point calibration curve construction.

Calibration standards level 1 to 5 were prepared per **table 3.2.**

Calibration level	Volume Working stock (µL)	Volume Water (µL)
1	200	0
2	100	100
3	50	150
4	24	176
5	12	188

Table 3.2: Calibration standards.

3.3.5 Online Derivatisation

Chromatography condition were set as per **table 3.3** using a Chemstation interface. The elution programme used to separate amino acids consisted of a linear gradient 0–0.2min 2% B; 0.2–7.7min 2–43% B; 7.7– 7.8 min 43–100% B; 7.8–8.3 min 100% B; 8.3–9 min 100–2% B. The pump was operated at a flow rate of 2.0ml/min and 1.0ml of each derivatised sample was injected. O-phthalaldehyde derivatives were detected by fluorescence at an excitation of 230 and an emission of 450nm.

Column	Agilent ZORBAX Rapid resolution high throughput column 4.6*100 mm, C18 1.8 µM (Agilent Technology, Germany)
Guard column	Security Guard C18 (ODS; Phenomenex, UK)
Flow	1.5 ml/min
Injection volume	1 µl

Operating pressure	~350 bar
Temperature column	40°C

Table 3.3: Chromatography conditions.

3.4 Creatine Kinase (CK) and Lactate Dehydrogenase (LDH) Analysis

Analysis of CK and LDH concentration was performed using a semi-automated analyser (RX Daytona and RX Imola; Randox). Before calibration the system; the wash solutions, ultra-pure water and waste containers were assessed to ensure appropriate levels. The relevant reagents were loaded into the reagent carousel. CK NAC reagent (Randox) was used to assess CK, and Lactate Dehydrogenase NAD (Randox) was used to assess LDH. Calibration and control reagents (Randox) were specific to the CK and LDH analysis, as per the manufacturers guidelines. Once the calibration and control were satisfactory, plasma samples were defrosted to room temperature using mixer. The epindorphs were directly loaded into the carousel of the analyser. The sample sequence table was designed, and the analyser was run.

Chapter 4

4.0 Habitual Protein Intake, Protein Distribution Patterns and Protein Source Across the Lifespan in Irish Adults between 2008 and 2010

Background: Habitual dietary protein intake and distribution pattern across a population is an important consideration when designing nutrition strategies to combat the age-related decline of muscle mass and function. The National Adult Nutrition Survey (NANS) investigated habitual food and beverage consumption, lifestyle and health indicators in 1500 adults aged 18-90 years in Ireland between 2008 and 2010.

Objective: The aim of the current study was to complete a secondary analysis of the data collected in NANS to determine overall protein intake patterns and food sources by age and gender.

Data Analysis: The final sample size for the analysis was n=1051 (males, n=523; females, n=528), all of whom undertook a four-day semi-weighted food diary to include three weekdays and one weekend day. Total, body mass relative intake and percentage contribution to total energy of each macronutrient were determined. Protein distribution scores (PDS) were calculated to determine the number of eating occasions per day containing over the 20g, 30g, 0.24g/kg, 0.3g/kg and 0.4g/kg body mass of protein, averaged over the 4 days. 2,048 pre-existing food codes were aggregated into 16 food groups. The percentage contribution of these food groups to total protein intake (g/d) was determined. These foods codes were further aggregated into two broad animal- and plant-based food groups. Food source contribution to total and per meal protein intake was determined. A two-way mixed ANOVA (gender x age) was performed. Post-hoc analysis multiple comparisons with Bonferroni's adjustment was used to determine differences between age groups.

Results: Total protein intake, and protein intake relative to body mass was greatest in those aged 18-35 y (96±3g per day, 1.32±0.40g/kg/d), with lower protein intakes with increasing age, and the lowest intakes apparent in adults aged ≥65 y (82±22g, 1.15±0.34g/kg/d, P<0.001 for all). This difference in protein intake between age groups was more pronounced in males compared to females, with females between ages 35-50 y and 51-64 y showing no difference in protein intake. The average number of meals per day reaching the purported per meal protein threshold to maximise MPS was highest in adults aged 18-35 y, and lower with increasing age (P<0.001). Protein distribution follows a skewed pattern across each age group, in which dinner represents the highest per meal protein intake, followed by lunch and breakfast (44±17g, 30±15g and 15±10g, respectively) Plant-based protein is the predominant protein source at breakfast (57.5±37.1%). Overall protein is derived predominantly from animal sources (63.1±10.8%, animal protein; 36.9±10.8%, plant protein in total population), with meat and dairy having the largest contribution to total protein intake in both sexes across all age groups.

Conclusion: Protein intake and the number of meals reaching the purported threshold for maximising postprandial anabolism is greatest in young, and is lower with increasing age. Breakfast was the lowest total protein and animal protein-containing main meal across all age categories, and may represent an opportunity for improving overall protein intake, and protein distribution, thereby combatting the age-related decline in muscle mass and function.

4.1 Introduction

Dietary protein acts as an anabolic stimulus by resulting in the stimulation of MPS (Glynn *et al.,* 2010; Moore *et al.,* 2009; Paddon-Jones 2004). If each eating occasion is considered as an opportunity to increase MPS, the cumulative magnitude and duration of postprandial MPS after the ingestion of each protein-containing meal dictates the time spent in a positive net protein balance over the course of a day (Layman *et al.*, 2015; Areta *et al.*, 2013; Paddon-Jones, 2004). For that reason, there is an emerging recognition that 'per meal' protein recommendations offer an effective strategy for increasing positive net protein balance, and favouring muscle accretion over time (Areta *et al.*, 2013; Paddon-Jones, 2004). 20-30g protein per meal, or 0.24g/kg and 0.4g/kg body mass per meal, in young and old respectively, has been shown to maximise MPS after a single meal (Moore *et al.*, 2015, 2009; Witard *et al.*, 2014; Symons *et al.*, 2009). Furthermore, modulating protein distribution over the day to create an even protein intake at each meal, for example ≥20g high quality protein ingested at three main meals (Areta *et al.*, 2013) maximises rates of MPS over 12 hours (Paddon-Jones and Leidy, 2014; Moore *et al.*, 2015; Symons *et al.*, 2009).

Daily protein intake often follows a 'skewed' pattern of distribution, in which protein intake is highest at dinner, and lower at other meals and snacks (Cardon-Thomas *et al.*, 2017; Tieland *et al.*, 2015; Almoosawi *et al.*, 2013; Bollwein *et al.*, 2013; Ruiz Valenzuela *et al.*, 2013). This skewed pattern of distribution potentially exceeds the optimal protein dose at dinner, which does not cause an added anabolic response above ~20g high quality protein (Witard *et al.,* 2014; Moore *et al.,* 2009). Furthermore, the resultant lower protein intakes at breakfast, lunch and snack times are representative of a sub-optimal protein intake for maximising MPS (Witard et al., 2014; Areta, 2013; Moore *et al.*, 2009). This has led to the hypothesis that spreading daily protein intake evenly throughout the day can result in a greater cumulative anabolic response compared to this skewed pattern of protein intake (Layman *et al.*, 2015). Furthermore, an uneven protein distribution has been associated with an increase in incidence of frailty (Bollwein *et al.*, 2013), while the daily consumption of 1 or 2 main meals over the 30g protein per meal threshold is associated with greater lean mass and strength in older adults (Loenneke *et al.*, 2016). These data support the thesis that a more evenly-distributed protein intake is more favourable for augmenting LBM and strength, and preventing frailty in older adults. The source of dietary protein is also of relevance, since animal proteins, which have a higher essential amino acid (EAA) content (USDA National Nutrient Database for Standard Reference, 2009), exhibit greater anabolic properties, causing a superior postprandial anabolic response when compared to plant-based proteins (Gorissen *et al.*, 2016; Yang *et al.*, 2012a; 2012b; Tang *et al.*, 2009; Wilkinson *et al.*, 2007).

Habitual dietary protein intake and patterns in Irish adults remains underexplored. The National Adult Nutrition Survey (NANS) investigated habitual food and beverage consumption, lifestyle and health indicators in 1500 adults aged 18-90 years, in the Republic of Ireland, between 2008 and 2010. The series of interrelated databases, which has been compiled from the data collected in this survey, provide the most complete and up-to-date collection of food consumption data available for adults in Ireland, therefore offering valuable information about the protein intake, distribution and source across gender and ages. The aim of this current study is to complete secondary analysis of the data collected in this survey to determine overall protein intake patterns and food sources by age and gender.

4.2 Methods

4.2.1 Study Population

This study is based on secondary analysis of National Adult Survey (NANS), a cross-sectional food consumption survey in Irish adults. The surveys were carried out by the Irish Universities Nutrition Alliance (IUNA) in a sample of 1500 free-living adults aged 18-90 years (males, n=740; females, n=760), in the Republic of Ireland between 2008 and 2010. Respondents were randomly selected from a database of names and address from Data Ireland (An Post). Exclusion criteria include pregnancy, lactation and inability to complete the survey due to disability. The final survey response rate was 59.6%. The final sample was representative of the Irish population with respect to gender, age, location, social class and geographical location, when compared to the Irish censuses (IUNA, 2011). Informed consent from respondents was obtained before the survey commenced. Ethical approval was granted by University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals [ECM 3(p) 4 September 2008].

4.2.2 Primary Anthropometric Measures and Dietary Assessment

Anthropometric measurements were carried out by the researcher in the respondent's home. Body mass, height and body composition were measured. Body mass (kg), muscle mass (kg), body fat (g) and percentage body fat were assessed using a Tanita SC-331S body composition analyzer (Tanita, Tokyo, Japan). Height was assessed using a Leicester portable height measure to the nearest 0.1cm. Body Mass Index (BMI) was calculated as weight (kg) divided by height (m^2). A four-day semi-weighed food diary, at brand level where possible, was used to collect food, beverage and supplement intake. Participants were asked to report three weekdays and one weekend day. The researchers made three visits to the respondent's homes during the four days: A visit to demonstrate how to use a food weighing scales and log the food diary; a second visit to review

the diary 24-36 hours into the recording process; and a final visit 1-2 days after the recording period to review the last recording days and collect the diary. Food and beverage consumption was quantified using a food weighing scales (46%), a photographic food atlas (16%), a food portion size guide (11%), household measurements such as teaspoons, tablespoons, etc. (11%), manufacturers weights (10%), IUNA weight guide (4%) and an estimate made by the researcher (2%). Food and beverage intake was assessed using WISP version 3.0 (Tinuviel Software, Anglesey, UK). This analysis was based on data from the McCance and Widdowson's, The Composition of Foods, Sixth and Fifth Editions, as well as nine supplementary volumes. Modifications to the food composition database was also performed to include commonly consumed Irish foods. The anthropometric and dietary assessment carried out is described in further detail elsewhere (O'Donovan *et al.*, 2018; Hopkins *et al.*, 2015; Cashman *et al.*, 2013). The final food database comprised of 133,068 rows of data, with each row representing each food or beverage item at every eating occasion throughout the four days of recording.

4.2.3 Secondary Data Analysis

This secondary analysis was carried out using SPSS (IBM SPSS Statistics Version 24). Respondents who reported an energy intake BMR <1.1 (McGowan *et al.*, 2001) were determined as under-reporters (n=449) and were excluded from the present analysis. The final sample size was n=1051 (males, n=523; females, n=528). New variables were computed to determine body mass relative macronutrient intake on a gram per kg basis. Protein Distribution Scores (PDS) were calculated for the following: PDS^{20}, PDS^{30}, $PDS^{0.24g/kg}$, $PDS^{0.3g/kg}$, $PDS^{0.4g/kg}$. PDS^{20} and PDS^{30} represents the number of eating occasions per day containing over 20g and 30g of protein, averaged across the 4 days. $PDS^{0.24g/kg}$, $PDS^{0.3g/kg}$, $PDS^{0.4g/kg}$ represents the number of eating occasions per day containing over the 20g, 30g, 0.24g/kg, 0.3g/kg and 0.4g/kg body mass of protein, averaged over the 4 days. PDS is a scoring system adapted from MacKenzie *et al.*, (2015) with these values being representative of the recommended per meal protein target to maximise MPS in young (Witard *et al.*, 2014; Moore *et al.*, 2009) and old (Moore *et al.*, 2015; Symons *et al.*, 2009). 2,048 pre-existing food codes were aggregated into 16 food groups based on foods of similar type and protein content. The percentage contribution of these food groups to total protein intake was determined. These foods codes were further aggregated into two broad groupings described as either animal- or plant-based foods based on observation of the principal contributing protein source. The total and percentage contribution of animal- and plant-based foods to total protein intake, as well as per meal protein intake, was determined.

In general, the distribution of the data approximated normality, or was transformed as appropriate to approximate normality. Four age groups were created (18-35 y, 36-50 y, 51-64 y and ≥65 y). A two-way ANOVA (gender x age) was performed. When interaction or main effects were indicated, post-hoc analysis using multiple comparisons with Bonferroni's adjustment was used to assess the differences between age groups. Statistical significance was accepted at $P<0.05$.

4.3 Results

4.3.1 Anthropometric Measures

Table 4.1 represents anthropometric measures for the total population (aged 18->65 y), each age group 18-35 y, 36-50 y, 51-64 y and ≥65 y, and males and females. There was a gender x age interaction for body fat, body fat percentage and waist to hip ratio (WTHR) ($P<0.01$ for all), in which there were greater differences between ages 18-35 y and 36-50 y in males, compared to females, who had smaller differences. There were main effects for gender for all anthropometric measures ($P<0.01$ for all). Males tended to have higher measures in height, body mass, BMI, WTHR and muscle mass, compared to females, while females tended to have higher values than males for body fat percentage and fat mass.

There were main effects for age for differences in all anthropometric measures ($P<0.001$ for all). Body mass, BMI, WTHR, body fat percentage, fat mass tended to be greater with increasing age. However, there was a lower body mass in adults aged ≥65 y, compared to 51-64 y. Height and muscle mass tended to be lower with increasing age. However, for height, fat mass and muscle mass, there was no significant difference between adults aged 35-50 y and 51-64 y.

4.3.2 Energy and Macronutrient Intake

Table 4.2 represents average total daily energy, protein, carbohydrates and fat; and percentage of total energy intake for each macronutrient. **Table 4.3** represents energy and macronutrient intake relative to body mass, and expressed in gram/kilogram body mass per day. There was a gender x age interaction for total energy, protein (g/d) and relative protein intake (g/kg)($P<0.01$ for all). Energy intake was greater in males between ages 35-50 y compared to 51-64 y, while energy intake in females in these age categories were similar. Total and relative protein intake in males was greatest in young, and was lower with increasing age, while protein intake was similar between age groups for females. There were main effects of gender for total and relative calorie and macronutrient intakes ($P<0.001$ for all). There was a tendency for males to have higher intakes compared to females for all total and relative energy and macronutrient intakes.

There were main effects for age for differences in all total and body mass relative calorie and macronutrient intakes ($P<0.001$ for all). Total energy, protein, carbohydrate and fat intake was greatest in young, and had a tendency to be lower with increasing age. However, there was no significant difference between intakes for total energy and macronutrient intakes in adults aged 35-50 y and 51-64 y. Relative protein, carbohydrate and fat intake, were significant greater in adults aged 18-35 y compared to 36-50 y, 51-64 y and ≥65 y. Total protein intake in adults aged ≥65 y was 81.6±22.3g/d, which was significantly lower than that of adults aged 18-35 y (96.1±32.4g/d, $P<0.001$). Relative protein intake in adults aged ≥65 y was 1.15±0.34g/k/d, which was significantly lower than intakes in adults aged 18-35 y (1.32±0.40g/kg/d, $P<0.001$).

4.3.3 Protein Distribution Scores

Table 4.4 represents PDS, which were calculated for the following: PDS^{20}, PDS^{30}, $PDS^{0.24g/kg}$, $PDS^{0.3g/kg}$, $PDS^{0.4g/kg.}$ These values represent the number of eating occasions per day containing over the 20g, 30g, 0.24g/kg, 0.3g/kg and 0.4g/kg body mass of protein, averaged over the 4 days. There was a gender x age interaction for PDS^{20}, PDS^{30} and $PDS^{0.3g/kg}$ ($P<0.05$), in which there were greater differences between age groups for males, compared to females. There were main effects for gender for PDS^{20}, PDS^{30}, $PDS^{0.24g/kg}$ $PDS^{0.3g/kg}$ and $PDS^{0.4g/kg}$ ($P<0.05$ for all) in which males had higher scores than females for all PDS scores.

There were main effects for age in PDS^{20}, PDS^{30}, and $PDS^{0.4g/kg}$ and $PDS^{0.3g/kg}$ ($P<0.01$ for all). This revealed that the number of meals reaching these thresholds was lower with increasing age. However, for PDS^{30}, $PDS^{0.3g/kg}$ and $PDS^{0.4g/kg}$ there was no difference between ages 51-64 y and ≥65 y.

4.3.4 Contribution of Food Source to Total Calorie and Protein Intake

Table 4.5 represents the contribution of animal- and plant-based proteins to overall protein and energy intake. There was a gender x age interaction for animal protein only ($P<0.001$) in which there were greater difference between age groups for males, compared to females. There were main effects for gender for all variables in **table 4.5** ($P<0.05$ for all), in which females tended to have a higher percentage of protein and energy from plant-based foods compared to males, while males had a higher percentage of protein from animal-based foods compared to females. There were main effects for age for all variables in **table 4.5** ($P<0.05$ for all), in which total animal- and plant-based protein intake showed lower intakes with increasing age. Percentage protein intake from animal-based protein was significantly greater in adults aged ≥65 y

compared to all groups, while percentage protein intake from plant-based protein was significantly lower in adults aged ≥65 y, compared to all groups. **Figure 4.1** represents the average per meal protein intake, across each group, and the relative contribution of animal- and plant-based protein to total per meal protein intake. **Figure 4.2** represents the percentage contribution of 16 food groups to total protein intake across each age group. In each age group, meat, dairy and breads were the predominant protein sources, accounting for 40±15%, 15±9% and 12±6% in the total population, respectively.

		All Ages		18-35 y		36-50 y		51-64 y		≥65 y		*P* Value		
		mean±SD	*n*	mean±SD	*n*	mean±SD	*n*	mean±SD	*n*	mean±SD	*n*	Gender	Age	GxA
Height (m)	All	1.69±0.10	*966*	1.73±0.10^{bcd}	*357*	1.68±0.09^{ad}	*279*	1.68±0.10^{ad}	*186*	1.65±0.09^{bc}	*144*	<0.001	<0.001	0.224
	Male	1.76±0.07	*477*	1.79±0.07	*195*	1.75±0.07	*128*	1.75±0.08	*89*	1.72±0.07	*65*			
	Female	1.62±0.07	*489*	1.65±0.05	*162*	1.62±0.07	*151*	1.61±0.06	*97*	1.59±0.06	*79*			
Body Mass	All	75.1±14.6	*964*	73.7±14.1^{bc}	*356*	76.0±14.6^{a}	*279*	78.1±16.4^{ad}	*185*	72.9±12.2^{c}	*144*	<0.001	<0.001	0.27
(kg)	Male	82.6±12.8	*475*	80.2±12.8	*195*	85.1±11.8	*128*	86.5±14.3	*88*	79.6±10.6	*64*			
	Female	67.8±12.2	*489*	65.9±11.4	*161*	68.2±12.1	*151*	70.5±14.4	*97*	67.6±10.7	*80*			
BMI (kg/m^2)	All	26.2±4.3	*905*	24.5±3.6^{bcd}	*341*	26.8±4.2^{ac}	*270*	27.9±5.1a^{b}	*170*	26.9±3.6^{a}	*124*	0.002	<0.001	0.273
	Male	26.6±3.9	*441*	24.8±3.4	*186*	27.7±3.6	*123*	28.4±4.2	*78*	27.1±3.3	*54*			
	Female	25.8±4.6	*464*	24.2±3.8	*155*	26.0±4.5	*147*	27.4±5.7	*92*	26.8±3.9	*70*			
Waist to Hip	All	0.87±0.08	*856*	0.84±0.07^{bcd}	*321*	0.88±0.08^{ac}	*258*	0.91±0.08^{ab}	*163*	0.90±0.08^{a}	*114*	<0.001	<0.001	<0.001
Ratio	Male	0.91±0.08	*408*	0.86±0.07	*168*	0.93±0.07	*116*	0.96±0.07	*75*	0.95±0.07	*49*			
	Female	0.84±0.08	*448*	0.81±0.07	*153*	0.85±0.08	*142*	0.86±0.07	*88*	0.87±0.07	*65*			
Body Fat (%)	All	27.9±8.9	*870*	23.3±8.8^{bcd}	*339*	29.9±7.8^{ac}	*269*	31.7±7.8^{ab}	*163*	31.8±6.8^{a}	*99*	<0.001	<0.001	0.002
	Male	22.4±7.3	*431*	17.6±5.8	*185*	25.0±6.4	*123*	26.8±6.4	*76*	27.3±4.7	*47*			
	Female	33.2±6.9	*439*	30.0±6.8	*154*	34.1±6.4	*146*	36.0±6.3	*87*	35.9±5.7	*52*			
Fat Mass (kg)	All	21.3±9.4	*864*	17.2±7.9^{bcd}	*336*	23.5±9.9^{a}	*268*	25.1±10.0^{a}	*161*	23.3±6.3^{a}	*99*	<0.001	<0.001	0.003
	Male	19.5±10.0	*427*	14.6±6.8	*183*	22.8±11.2	*122*	24.6±11.1	*75*	22.5±5.8	*47*			
	Female	23.1±8.4	*437*	20.3±7.9	*153*	24.1±8.6	*146*	25.6±8.8	*86*	24.2±6.7	*52*			
Muscle Mass	All	51.1±10.7	*864*	53.2±11.1^{bcd}	*336*	50.3±10.3^{ad}	*268*	50.1±10.8^{ad}	*161*	47.4±9.5^{abc}	*99*	<0.001	<0.001	0.163
(kg)	Male	60.3±7.2	*427*	61.9±7.2	*183*	60.0±6.6	*122*	59.8±7.4	*75*	56.0±6.0	*47*			
	Female	42.1±3.7	*437*	42.9±3.2	*153*	42.3±3.8	*146*	41.7±3.9	*86*	39.7±3.5	*52*			

Table 4.1: Anthropometric measures for all participants, across genders and age groups. Values are expressed as mean ± standard deviation. A two-way ANOVA was performed (Gender*Age). When $P<0.05$, post-hoc analysis with Bonferroni's adjustment was used to determine where differences existed between age groups. Difference existing between age groups is indicated by: **a** $P<0.05$ vs. 18-35 years; by **b** $P<0.05$ vs. 36-50 years; **c** $P<0.05$ vs. 51-64 years; **d** $P<0.05$ vs. ≥65 years plus.

		All Ages	18-35 y	36-50 y	51-64 y	≥65 y	*P* Value		
		mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	Gender	Age	GxA
Energy intake (kcal/day)	All	2234±623	2457±663[bcd]	2190±565[ad]	2157±523[ad]	1899±555[abc]	<0.001	<0.001	0.006
	Males	2586±594	2816±556	2552±533	2475±510	2162±625			
	Females	1886±423	2020±501	1876±371	1862±327	1673±359			
Protein (g/day)	All	90.5±27.7	96.1±32.4[bcd]	89.6±26.2[ad]	88.4±21.4[ad]	81.6±22.3[abc]	<0.001	<0.001	<0.001
	Males	105.2±27.4	113.1±30.5	105.0±24.6	99.9±19.9	90.4±24.7			
	Females	75.9±18.8	75.4±20.2	76.3±19.3	77.8±16.9	74.1±16.7			
Protein intake	All	16.4±3.4	15.7±3.6[bcd]	16.6±3.4[ad]	16.7±3.0[a]	17.6±3.2[ab]	0.953	<0.001	0.08
(% Energy Intake)	Males	16.5±3.5	16.2±4.0	16.7±3.1	16.4±2.7	17.2±3.5			
	Females	16.4±3.3	15.1±3.0	16.5±3.6	16.9±3.2	17.9±2.9			
Carbohydrate (g/day)	All	252.9±76.6	272.5±80.5[bcd]	248.1±73.0[ad]	249.1±71.2[ad]	221.1±67.5[abc]	<0.001	<0.001	0.079
	Males	287.9±79.4	308.3±76.5	283.7±78.7	282.6±75.3	246.6±76.5			
	Females	218.2±54.9	228.9±61.6	217.3±50.6	218.1±50.5	199.2±49.3			
Carbohydrate	All	45.6±7.3	44.7±7.3[d]	45.5±7.4	46±7.0	46.9±7.2a	0.001	0.01	0.963
(% Energy Intake)	Males	44.7±7.9	44.0±7.8	44.5±8.3	46±7.3	46.0±8.0			
	Females	46.4±6.5	45.6±6.5	46.4±6.5	47±6.7	47.7±6.4			
Fat (g)	All	84.9±28.5	91.7±28.3[bcd]	84.0±27.7[ad]	82.2±26.2[ad]	74.3±29.3[abc]	<0.001	<0.001	0.739
	Males	96.6±30.3	102.4±27.9	96.8±30.1	93.4±28.7	84.5±35.1			
	Females	73.3±20.9	78.7±22.9	72.8±19.5	71.9±18.6	65.5±19.5			
Fat	All	34.2±6.3	33.8±6.1	34.4±6.0	34±6.4	34.9±6.9	0.04	0.367	0.291
(% Energy Intake)	Males	33.5±6.4	32.7±6.2	33.8±5.8	34±6.8	34.7±7.3			
	Females	34.9±6.1	35.0±5.7	34.9±6.1	35±6.1	35.1±6.6			

Table 4.2: Average energy intake (kcal) and macronutrient intake (g and % of total energy intake) for all participants, across genders and age groups. Values are expressed as mean ± standard deviation. N values, total populations, *n* =1051 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=377; 308; 204; 162]; males, *n*=523 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=207; 143; 98; 75]; females, *n*=528 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=170; 165; 106; 87]. A two-way ANOVA was performed (Gender*Age). When $P<0.05$, post-hoc analysis with Bonferroni's adjustment was used to determine where differences existed between age groups. Difference existing between age groups is indicated by: [a] $P<0.05$ vs. 18-35 years; by [b] $P<0.05$ vs. 36-50 years; [c] $P<0.05$ vs. 51-64 years; [d] $P<0.05$ vs. ≥65 years plus.

		All Ages	18-35 y	36-50 y	51-64 y	≥65 y	*P* Value		
		mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	Gender	Age	GxA
Energy intake (kcal/kg/day)	All	30.7±7.6	34.0±8.1[bcd]	29.8±6.3[ad]	28.8±6.2[a]	27.0±7.6[ab]	<0.001	<0.001	0.571
	Males	32.6±7.6	35.8±7.7	31.4±6.1	30.0±6.2	28.6±7.7			
	Females	28.9±7.3	31.8±8.0	28.3±6.1	27.7±6.1	25.8±7.3			
Protein (g/kg/day)	All	1.23±0.35	1.32±0.40[bcd]	1.21±0.31[a]	1.18±0.30[a]	1.15±0.34[a]	<0.001	<0.001	0.01
	Males	1.31±0.36	1.43±0.42	1.28±0.27	1.20±0.30	1.16±0.32			
	Females	1.16±0.33	1.18±0.33	1.15±0.33	1.15±0.31	1.13±0.35			
Carbohydrate (g/kg/day)	All	3.49±0.98	3.78±1.01[bcd]	3.39±0.92[a]	3.35±0.92[a]	3.16±0.94[a]	<0.001	<0.001	0.86
	Males	3.65±1.03	3.93±1.03	3.53±1.01	3.46±0.93	3.29±1.00			
	Females	3.34±0.91	3.59±0.95	3.27±0.81	3.25±0.91	3.06±0.89			
Fat (g/kg/day)	All	1.17±0.37	1.28±0.37[bcd]	1.14±0.32[a]	1.09±0.34[a]	1.06±0.43[a]	0.001	<0.001	0.944
	Males	1.22±0.39	1.31±0.38	1.19±0.34	1.13±0.36	1.12±0.47			
	Females	1.13±0.35	1.24±0.37	1.10±0.31	1.06±0.31	1.01±0.39			

Table 4.3: Overview of energy and macronutrient intake relative to body mass (grams per kilogram body mass) for all participants, across genders and age groups. Values are expressed as mean ± standard deviation. *n* =964 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=356; 279;185;144]; males, *n*=475 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=195; 128; 88; 64]; females, *n*=489 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=161; 151; 97; 80]. A two-way ANOVA was performed (Gender*Age). When $P<0.05$, post-hoc analysis with Bonferroni's adjustment was used to determine where differences existed between age groups. Difference existing between age groups is indicated by: [a] $P<0.05$ vs. 18-35 years; by [b] $P<0.05$ vs. 36-50 years; [c] $P<0.05$ vs. 51-64 years; [d] $P<0.05$ vs. ≥65 years plus.

		All Ages	**18-35 y**	**36-50 y**	**51-64 y**	**≥65 y**		***P* Value**	
		mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	Gender	Age	GxA
PDS (20g)*	All	1.64±0.57	1.73±0.60[cd]	1.64±0.57[d]	1.61±0.51[ad]	1.45±0.51[abc]	<0.001	<0.001	0.003
	Males	1.88±0.56	2.01±0.55	1.91±0.54	1.79±0.51	1.62±0.56			
	Females	1.39±0.47	1.40±0.48	1.41±0.49	1.45±0.46	1.30±0.41			
PDS (30g)*	All	1.08±0.51	1.21±0.57[bcd]	1.05±0.51[a]	0.98±0.43[a]	0.96±0.42[a]	<0.001	<0.001	0.002
	Males	1.34±0.49	1.48±0.52	1.35±0.47	1.21±0.38	1.13±0.45			
	Females	0.82±0.39	0.87±0.42	0.79±0.38	0.78±0.37	0.81±0.33			
PDS	All	1.81±0.56	1.86±0.55	1.81±0.54	1.76±0.56	1.73±0.60	0.02	0.105	0.057
(0.24g/kg)**	Males	1.89±0.54	1.97±0.55	1.91±0.50	1.75±0.50	1.77±0.59			
	Females	1.73±0.57	1.74±0.53	1.72±0.56	1.78±0.61	1.70±0.61			
PDS	All	1.47±0.52	1.55±0.53[cd]	1.45±0.50	1.42±0.49[a]	1.39±0.53[a]	<0.001	0.005	0.015
(0.3g/kg)**	Males	1.56±0.51	1.67±0.52	1.55±0.46	1.42±0.44	1.41±0.53			
	Females	1.39±0.51	1.41±0.49	1.37±0.51	1.41±0.53	1.37±0.52			
PDS	All	1.07±0.47	1.19±0.51[bcd]	1.02±0.44[a]	0.98±0.41[a]	0.98±0.43[a]	<0.001	<0.001	0.493
(0.4g/kg)**	Males	1.17±0.46	1.28±0.52	1.15±0.40	1.05±0.39	1.05±0.41			
	Females	0.97±0.45	1.07±0.47	0.91±0.45	0.92±0.42	0.93±0.43			

Table 4.4: Protein Distribution Scores (PDS) for all participants, across genders and age groups. Values are expressed as mean ± standard deviation. PDS^{20} and PDS^{30} represent the number of eating occasions per day containing over 20g and 30g of protein, averaged across the 4 days. $PDS^{0.24g/kg}$, $PDS^{0.3g/kg}$, $PDS^{0.4g/kg}$ represent the number of eating occasions per day containing over the 0.24g/kg, 0.3g/kg and 0.4g/kg body mass of protein, averaged over the 4 days. ***n* values, total populations, *n* =1051 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=377; 308; 204; 162]; males, *n*=523 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=207; 143; 98; 75]; females, *n*=528 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=170; 165; 106; 87]. ****n* values, total populations, *n* =964 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=356; 279;185;144]; males, *n*=475 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=195; 128; 88; 64]; females, *n*=489 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=161; 151; 97; 80]. A two-way ANOVA was performed (Gender*Age). When $P<0.05$, post-hoc analysis with Bonferroni's adjustment was used to determine where differences existed between age groups. Difference existing between age groups is indicated by: [a] $P<0.05$ vs. 18-35 years; by [b] $P<0.05$ vs. 36-50 years; [c] $P<0.05$ vs. 51-64 years; [d] $P<0.05$ vs. ≥65 years plus.

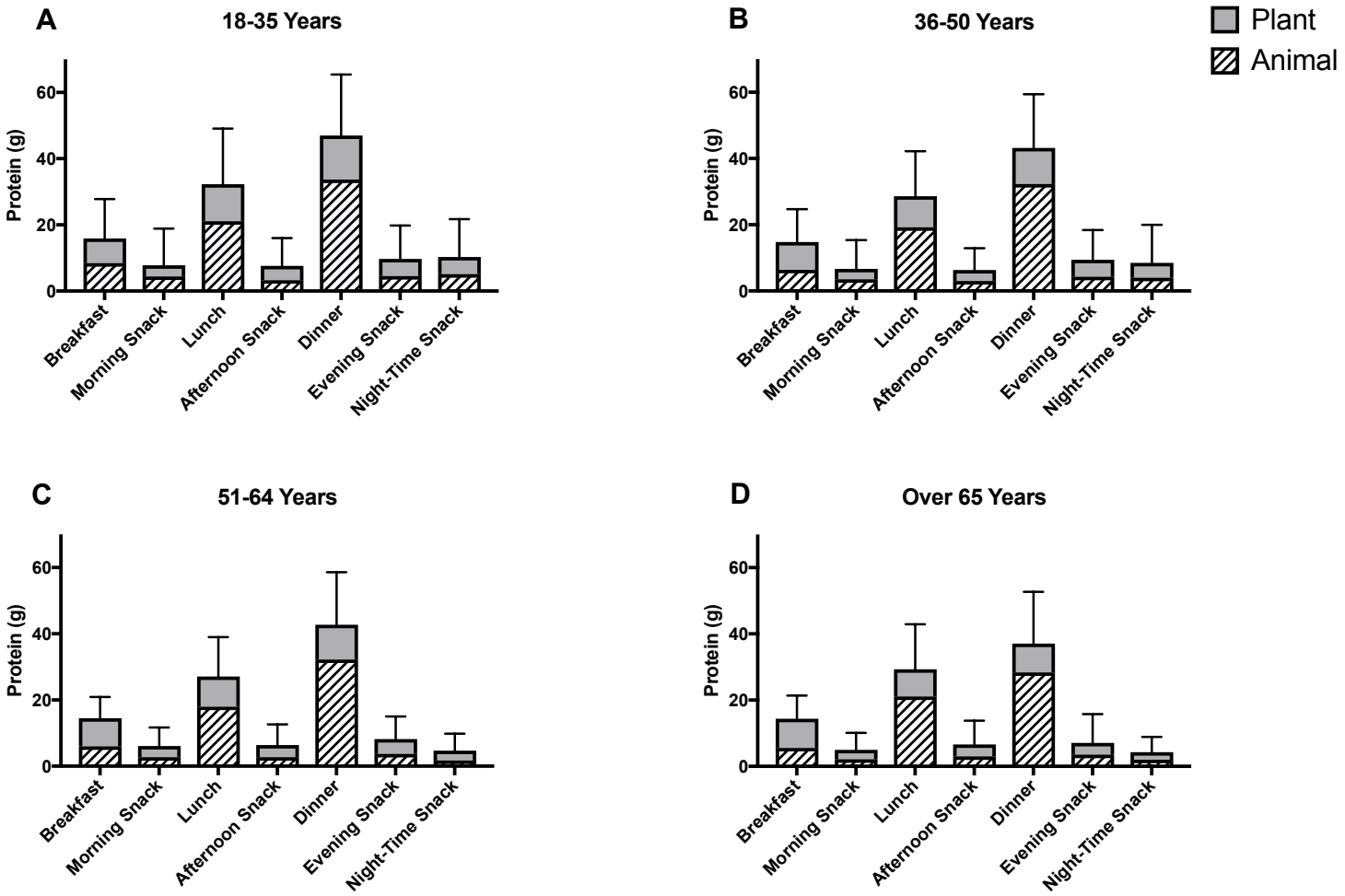


Figure 4.1: Total protein intake at each eating occasion in males and females aged 18-35 years (A), 36-50 years (B), 51-64 years (C) and over 65 years (D). Values are expressed as mean ± standard deviation.

		All Ages	18-35 y	36-50 y	51-64 y	65 y	*P* Value		
		mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	Gender	Age	GxA
Percentage Protein Contribution	**Animal Protein (%)**								
	All	63±11	63±11[d]	63±11[d]	63±11[d]	66±9	0.013	0.012	0.303
	Males	64±10	64±11	65±10	63±10	65±8			
	Females	62±11	61±12	61±12	62±11	66±9			
	Plant Protein (%)								
	All	37±11	37±11[d]	37±11d	37±11[d]	34±9	0.013	0.012	0.303
	Males	36±10	36±11	35±10	37±10	35±8			
	Females	38±11	39±12	39±12	38±11	34±9			
Total Daily Protein Intake	**Animal Protein (g)**								
	All	57.9±23	61.2±27.1c[d]	57.2±22.2	56.0±18.7[a]	53.9±17.3[a]	<0.001	0.021	<0.001
	Males	68.3±24.5	73.2±28.1	68.8±23.2	64.0±18.9	59.5±19.3			
	Females	47.6±15.5	46.5±16.5	47.1±15.4	48.7±15.3	49.1±13.9			
	Plant Protein (g)								
	All	32.6±12.4	34.9±13.7[bd]	32.4±12.9[ad]	32.4±10.2[d]	27.7±9.2[abc]	<0.001	<0.001	0.042
	Males	36.8±11.9	39.8±12.4	36.2±11.5	36.0±10.9	31.0±10.0			
	Females	28.4±11.4	28.9±12.8	29.2±13.1	29.1±8.2	25.0±7.3			
Percentage Total Energy Contribution	**Animal-Based Foods (%)**								
	All	36±10	33±9[bcd]	36±10[ad]	36±10[ad]	41±10[abc]	0.008	<0.001	0.313
	Males	37±10	35±10	37±10	37±10	41±10			
	Females	35±10	32±8	34±10	35±10	42±10			
	Plant-Based Foods (%)								
	All	64±10	67±9[bcd]	64±10[ad]	64±10[ad]	59±10[abc]	0.008	<0.001	0.313
	Males	63±10	65±10	63±10	63±10	59±10			
	Females	65±10	68±8	66±10	65±10	58±10			

Table 4.5: Percentage (%) and total (g) contribution of animal and plant protein to total protein intake, and percentage contribution (%) of animal and plant foods to total energy intake, for all participants, across genders and age groups. Values are expressed as mean ± standard deviation. *n* =1051 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=377; 308; 204; 162]; males, *n*=523 [18-35 y; 36-50 y; 51-64 y; ≥65 y, *n*=207; 143; 98; 75]; females, *n*=528 [18-35 y; 36-50 y; 51-64 y; ≥65 y, n=170; 165; 106; 87]. A two-way ANOVA was performed (Gender*Age). When *P*<0.05, post-hoc analysis with Bonferroni's adjustment was used to determine where differences existed between age groups. Difference existing between age groups is indicated by: [a] *P*<0.05 vs. 18-35 years; by [b] *P*<0.05 vs. 36-50 years; [c] *P*<0.05 vs. 51-64 years; [d] *P*<0.05 vs. ≥65 years plus.

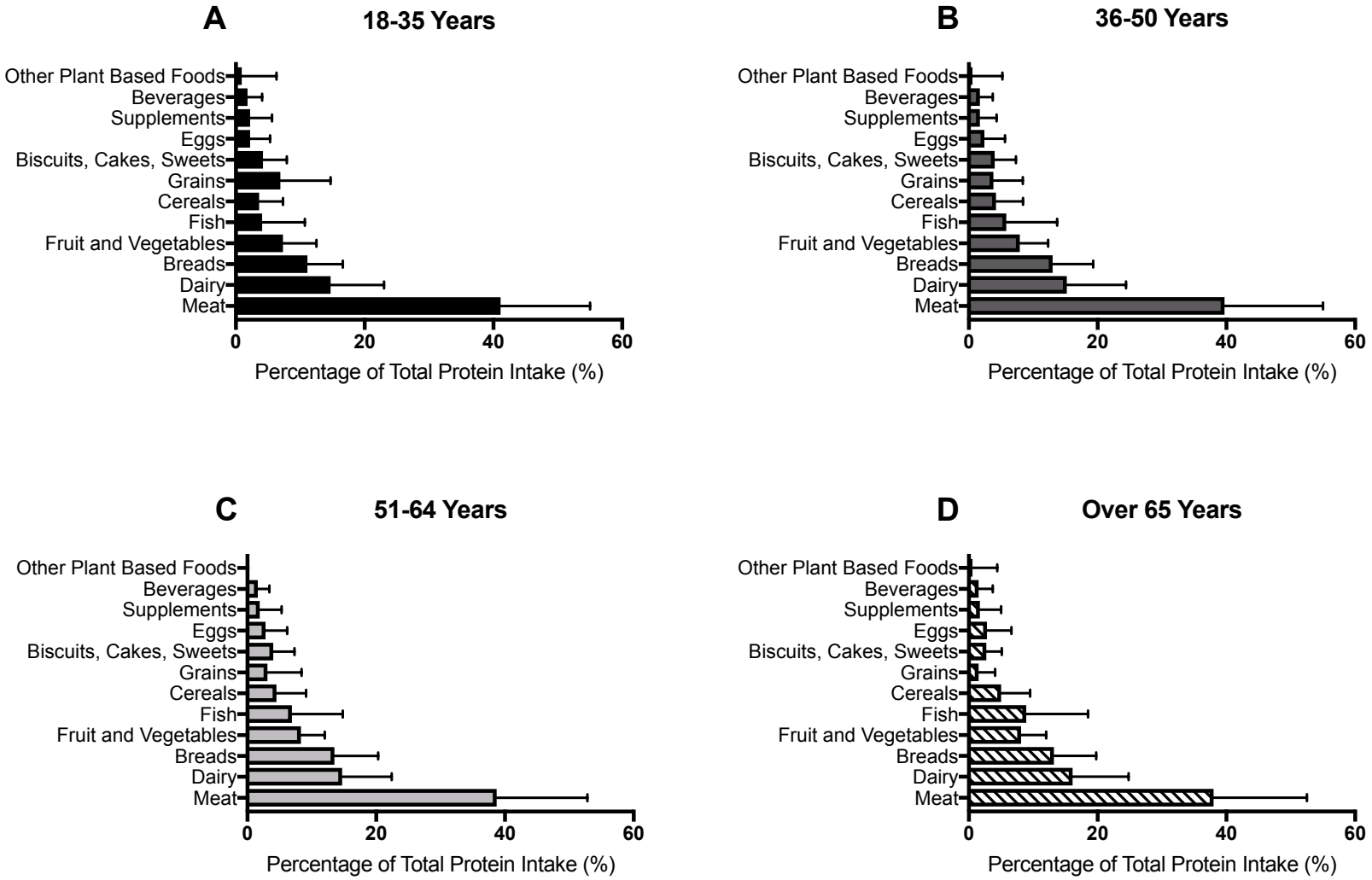


Figure 4.2: Percentage contribution of food groups to total protein intake in males and females aged 18-35 years (A), 36-50 years (B), 51-64 years (C) and over ≥65 years (D).
Values are expressed as mean ± standard deviation.

4.4 Discussion

The age-related decline in muscle mass and function is a fundamental threat to maintaining independence and a good quality of life throughout the lifespan (Fielding *et al.*, 2011; Janssen, Heymsfield and Ross, 2002). Dietary intake, and in particular protein intake, has been identified as a preventative and potential treatment in combatting the age-related decline in muscle mass and function (Norton *et al.*, 2016; Daly *et al.*, 2014; Liu and Latham, 2010; Onambélé-Pearson, Breen and Stewart, 2010). The aim of the present study was to identify age and gender related patterns in protein intake, distribution, and sources in Irish adults, thereby identifying areas where nutrition may improve the outcome of those at risk of declining muscle mass with age. This analysis identified that total protein intake, and protein relative to body mass is generally lower with increasing age, with males typically consuming more protein than females, and greater difference apparent between age groups for males. The average number of meals per day reaching the purported per meal protein threshold to maximise MPS is typically lower with increasing age, with males typically achieving a higher PDS score compared to females. Protein distribution follows a skewed pattern across each age group, in which dinner represents the highest per meal protein intake, followed by lunch and breakfast.

This analysis identified that total and relative protein intake are lower in older adults, compared to younger adults, in which adults aged 18-35 y typically consume 96±3g/1.32±0.40g/kg per day, while adults aged ≥65 y consume significantly less daily protein (82±22g/1.15±0.34g/kg). Males typically consume more protein than females (105±27g/1.31±0.36g/kg vs. 76±19g/1.16±0.33g/kg, respectively), as per **table 4.2.** However, there was no significant difference between intakes for total protein intake in adults aged 35-50 y and 51-64 y, suggesting that adults in these age groups tend to eat the same amount of protein. This same trend occurred for total energy, carbohydrates and macronutrients, suggesting that adults between the ages of 35 and 64 y show no obvious difference in overall energy and macronutrient intake. At the age of ≥65 y, there is a noticeably lower total energy and macronutrient intake compared to adults aged 35-51 y.

Of note, there was gender x age interaction effect for total and body mass relative protein intake, in which males tend to have consistent differences in protein intake with each increment in age category, while there was no difference in protein intake between females aged 35-50 y and 51-64 y. This absence of a difference may be related to an increase in dairy intake, which is often promoted by healthcare practitioners as females' approach peri-menopausal age category, as a preventative for osteoporosis (Munger, Cerhan and Chiu, 1999). However, dairy intake shows an arguably negligible difference across ages in males (~13% from age 18-35 y to 51-64 y), while females aged 51-64 y have ~5% greater dairy intake compared to females aged 18-35 y.

The National Health and Nutrition Examination Survey (NHANES) 2003-3004 from the USA reported a greater difference in protein intake between young and old, in which total protein intake in young adults aged 19-30 years was 91±22g/day/1.3g±0.4g/kg (*n*=874), and 66±17g/day/1.0±0.3g/kg in older adults aged >71 years (*n*=818) (Fulgoni, 2008). A survey in Dutch community-dwelling older adults aged 77±5 y (*n*=739), reported habitual dietary intake was 71g/day (Tieland *et al.*, 2015), which is similar to our findings. Similar to the present findings, in an Italian national food consumption survey, Sette *et al.*, (2011) reported that males aged 18-65 y consumed 93±25 protein per day, and females consumed 76±20 protein per day. From interpreting the present analysis, if Irish adults, in particularly males who show greater differences in protein intake across ages, could maintain the protein intake at ages 35-50 y and 51-64 y, into the latter years of life, they may have a decreased risk of age-related decline in muscle mass, thereby ageing with a better quality of life (Bauer *et al.*, 2013).

There was a significantly higher percentage contribution of protein to total energy intake with increasing age, since energy intake was lower with an increase in age, both of which have been reported in Spanish food consumption survey (Ruiz *et al.*, 2015). The reasons for this reduction in energy intake in older adults is considered related to the decrease in appetite with age (Morley, 2001). Additional contributors to this decrease in energy intake in older adults may also be the financial cost of these more nutrient-dense foods, difficulty chewing fibrous foods, perceived food intolerances and fear of eating too much fat and cholesterol in foods (Bauer *et al.*, 2013; Malafarina *et al.*, 2013; Chernoff, 2004). Of note, Tieland *et al.*, (2015) reported that energy intake is positively correlated to protein intake in older adults, and therefore daily energy intake is an important factor in determining habitual protein intake.

The way in which protein is distributed over the course of a typical day dictates the cumulative rates of MPS (Layman *et al.*, 2015; Paddon-Jones *et al.*, 2015; Mamerow *et al.*, 2014; Areta *et al.*, 2013). Furthermore, there is an increased recognition of importance of per meal protein recommendations, with 20-30g of high quality protein, or 0.24-0.4g/kg protein per meal, seen as the amount needed to maximise MPS in young (Witard *et al.*, 2014; Moore *et al.*, 2009) and old (Moore *et al.*, 2015; Symons *et al.*, 2009). The general pattern of protein intake across the total population was 'skewed', in which the majority of protein was eaten at dinner (15±10g, 30±15 and 44±17g at breakfast, lunch and dinner, for total population, respectively), as per **figure 4.1**. This skewed pattern of protein intake has been reported elsewhere (Cardon-Thomas *et al.*, 2017; Tieland *et al.*, 2015; Almoosawi *et al.*, 2013; Bollwein *et al.*, 2013; Ruiz Valenzuela *et al.*, 2013). In particular, in adults aged

≥65 y, breakfast, lunch and dinner accounted for 15%, 29% and 37% of average daily protein intake, which is similar to finding by Tieland *et al.*, (2015) in a similar aged cohort, in which these meals accounted for 15%–21%, 26%–32% and 38%–44% of total protein intake respectively. While similar in protein distribution, per meal protein intake in the present study is more optimal, as both lunch and dinner potential meet the 20-30g meal threshold (15±7g, 29±14g and 37±16g at breakfast, lunch and dinner, respectively), due to a higher overall protein intake compared to findings by Tieland *et al.* (2015). However, inevitably due to the large variability in protein intake for lunch and dinner, there are people who still fell below these protein thresholds for those meal times. Regardless, breakfast in the present cohort was representative of a suboptimal per meal protein intake, which is a worthy consideration when designing strategies to minimise the decline in muscle mass with age. Indeed, a nutrition intervention targeting supplemental protein intake at breakfast and lunch (0.17g/kg protein/~12g protein per meal) was successful in increasing in LBM over 24 weeks in Irish adults aged 50-70 y (Norton *et al.*, 2016).

With 20-30g of protein representing the total protein meal threshold for maximising MPS in young and old (Witard *et al.*, 2014; Moore *et al.*, 2009; Symons *et al.*, 2009), we sought to determine the age and gender patterns for the number of meals per day reaching this meal threshold, averaged over 4 days. PDS^{20} and PDS^{30} (the average number of meals per day reaching the 20g or 30g per day) was generally lower with increasing age, which was most notable between 51-64 y and ≥65 y for PDS^{20} (1.61±0.5 vs. 1.45±0.51, respectively) and 18-35 y and 35-50 y for PDS^{30} (1.21±0.6 vs. 1.05±0.5). Adults aged 18-35 y consumed 1.73±0.60 meals containing 20g, while adults aged ≥65 y consumed a smaller amount of meals providing ≥20g protein (1.45±0.51). Mirroring the trend for daily protein intake, there were greater differences in PDS^{20}, PDS^{30} and $PDS^{0.3g/kg}$ between age groups for males compared to females, who showed smaller differences between age groups.

Per meal protein intake was also determined relative to body mass, in which the number of meals reaching 0.24g, 0.3g and 0.4g/kg per meal protein was determined. This body mass relative protein intake has been reported as the per meal protein requirement to elicit a maximal/near maximal stimulation of MPS (Moore *et al.*, 2015). There was no difference in the number of meals reaching 0.24g/kg protein with age. At the 0.3g/kg per meal threshold, older adults consumed less meals at this threshold, compared to young, in which adults aged 18-35 y consumed 1.55±0.53 meals per day containing ≥0.3g/kg protein, while in adults aged ≥65 y a significantly smaller amount of meals ≥0.3g/kg protein (1.39±0.53). The same age and gender trend

occurred for meals reaching 0.4g/kg protein, in which older adults aged ≥65 y, on average, did not consume any meals reaching this threshold (0.98±0.43). In contrast to the present findings, Cardon-Thomas *et al.*, (2017) reported that older adults meeting the 0.4g/kg protein per meal threshold for eating occasion (EO) 1, 2 and 3 was 3%, 42% and 68%. Cardon-Thomas *et al.*, (2017), in community dwelling adults aged >70 y in United Kingdom, reported that 8% of participants did not meet the threshold for any meals, 71% met the threshold for one meal, 21% met the threshold for two meals and no participant consumed ≥0.4g/kg threshold for all 3 meals. These are different protein distribution trends to that found in the present analysis, however, Cardon-Thomas *et al.*, (2017) assessed a cohort of *n*=38, while the present analysis of ≥65 y was n=144, and was a nationally representative survey, therefore the dietary intakes reported by Cardon-Thomas *et al.*, (2017) is less likely to be representative of intakes for the entire older population. The habitual protein distribution patterns found in the present study are representative of a suboptimal protein intake at given meals for maximising MPS, while meals that exceed the optimal protein intake may not cause an additive anabolic response (Moore *et al.* 2009; Areta. 2013). This has led to the hypothesis that spreading daily protein intake evenly throughout the day can result in a greater cumulative anabolic response compared to a skewed pattern of protein intake (Layman *et al.*, 2015; Bauer *et al.*, 2013).

The source of protein (animal vs. plant) was a focus in the present study, since animal-based proteins have been reported to elicit a greater postprandial increase in MPS Gorissen *et al.*, 2016; Yang *et al.*, 2012a; 2012b; Tang *et al.*, 2009; Wilkinson *et al.*, 2007). In the present analysis, protein is derived predominantly from animal sources (63.1±10.8%, animal protein; 36.9±10.8%, plant protein in total population) (See **table 4.5**), with meat and dairy having the largest percentage contribution to total protein intake across both sexes and all age groups (39.8±14.5% and 15.0±8.5%, respectively, as per **figure 4.1**). This is similar to finding by Tieland *et al.,* (2015) in a Dutch population, in which 60% of dietary protein consumed originated from animal sources, with meat and dairy as dominant sources. Similarly, Sahni *et al.* (2015) reported that ~70% protein intake from animal protein and ~30% from plant protein, in adults aged 29-86 y in the USA.

When assessed on a per meal basis, dinner has the greatest animal protein contribution (72±16% animal protein), while lunch is lower (63±19% animal protein) and breakfast is predominantly plant protein (43±22% animal protein), as per **figure 4.2**. This is similar to finding by Tieland *et al.* (2015), in which >70% of the protein intake at dinner originated from animal protein, whilst lunch and breakfast was made up of ~63% and <50% animal protein, respectively. Similarly, in Dutch athletes aged 18-65 years, Gillen *et al.*,

(2017) reported the contribution of animal-based protein was greatest at dinner (70%), while animal-based protein contributed to 50% of protein intake at breakfast and lunch. Since a large proportion of protein at these meal times is derived from plant-based protein sources, these meal times, particularly breakfast, are likely to contain a lower EAA content (USDA National Nutrient Database for Standard Reference, 2009). These meals may therefore signify a missed opportunity to maximise postprandial anabolism for that meal time (Mamerow *et al.*, 2014; Areta *et al.*, 2013). Emphasising adequate, high quality protein at meal times, particularly at breakfast and lunch where animal protein intake is reported to be lowest, is a necessary consideration when applying the per meal protein targets in practice, particularly in older populations who are most at risk of age-related decline in muscle mass (Gorissen and Witard, 2018; van Vliet, Burd and van Loon, 2015). However, these recommendations should be interpreted with caution and not taken out of a context that may compromise the overall health of an individual's diet. Guidance towards better choices for protein at breakfast is imperative, particularly foods that are low in saturated fat, and/or high in polyunsaturated and monounsaturated fats should be emphasised. The consumption of highly processed meats, which are associated with an increased risk of heart disease, stroke, diabetes mellitus and cancers (Chan *et al.*, 2011; Micha, Wallace and Mozaffarian, 2010) should continue to be discouraged. Before recommendations based on this analysis could be incorporated into a public health framework, intervention trials investigating the effects of increasing animal-based protein and/or protein at breakfast and/or lunch on changes in LBM and health markers is required. These interventions would need to be long in duration and place a large emphasis on changes in health markers over this time course.

4.5 Conclusion

Protein intake and the number of meals reaching the purported threshold for maximising postprandial anabolism is highest in young, lower with increasing age, and lowest in adults aged ≥65 y. Breakfast is the lowest total protein-containing main meal across all age categories. Furthermore, it is the lowest animal protein-containing main meal. Since both protein dose and protein source strongly dictate the postprandial anabolic response to a meal, breakfast may represent an opportunity for improving overall protein intake, and protein distribution. This is an important consideration for strategies that would target age-associated declines in skeletal muscle mass and function.

Chapter 5

5.0 The plasma leucine kinetics after an oral load using free leucine and microencapsulated leucine in young, healthy males

Background: Amino acids (AAs) act as an anabolic stimulus, with leucine in particular playing a key role in initiating this anabolic response. Postprandial plasma leucine kinetics strongly influence rates of postprandial muscle protein synthesis (MPS). Microencapsulation is an emerging technology that has shown potential for the optimal delivery of drugs and nutrients, and may be used for the microencapsulation of leucine to optimise postprandial leucine plasma kinetics.

Objective: The aim of the present study is to investigate the plasma kinetics of leucine in response to the ingestion of leucine in its free form or in a novel microencapsulated form, in comparison to that of a maltodextrin control.

Design: Ten healthy active males [age (26.4±4.1y)] visited the laboratory on five separate occasions, and were randomly assigned to one of the following five conditions, in a single-blind cross-over design; A bolus of 3g free leucine (BOLUS), 3g microencapsulated leucine (ME), 3g maltodextrin (CONTROL), 1.5g free leucine consumed at 0 min and 1.5g consumed at 120 min (PULSE), 1.5g microencapsulated leucine + 1.5g free leucine (ME+LEU) combined at 0 min. Blood samples were taken every 15 min in the first hour, and every half hour for the subsequent three hours.

Data Analysis: Plasma AA concentrations were measured using high performance liquid chromatography. The difference from baseline over time and the difference between conditions, for plasma leucine, branched-chain amino acids (BCAAs), total AAs, essential amino acids (EAAs) and non-essential amino acids (NEAAs), was analysed using a two-way (time x condition) repeated measures analysis of variance (ANOVA). Post-hoc analysis was performed using a repeated measures ANOVA and pairwise comparisons with Bonferroni's adjustment.

Results: BOLUS, plasma leucinemia peaked at 30 min at 542±83μM, which was significantly different from baseline (P<0.001), with an AUC of 32±7μM.240min^{-1}, which significantly different from CONTROL (P<0.001). During ME, leucinemia peaked at 30 min, 212±38μM, which was significantly different from baseline (P=0.02), with an AUC of 6±3 μM.240min^{-1}. During PULSE, plasma leucinemia peaked from baseline at 30 min (325±72μM, P=0.001), trended towards baseline levels at 120 min (169±23.2μM) and peaked again at 273±81μM 30 min after the second leucine ingestion, which was significantly different from baseline (P=0.023). PULSE AUC was 24±7μM.240min^{-1}, which was not significantly different from BOLUS AUC 32±7μM.240min^{-1}, P=0.116).

Conclusion: The present form of microencapsulated leucine shows reduced bioavailability when compared to free leucine in BOLUS and PULSE conditions. The peak postprandial leucinemia achieved with ingestion of 3g free leucine is significantly greater than that of whole protein, such as egg and whey, providing the same dose of leucine, reported in the literature. Since plasma leucine concentration is a key regulator of activating MPS, dietary supplementation with free form leucine, or the enrichment of lower leucine meals, may show promise as a strategy for optimising the plasma kinetics required to maximise MPS.

5.1 Introduction

Skeletal muscle accretion and function is dictated by the dynamic balance between MPS and muscle protein breakdown (MPB). After feeding, when MPS exceeds MPB, an overall positive net protein balance is achieved, which enables the maintenance and growth of skeletal muscle (Atherton and Smith, 2012). Nutrition interventions to maximise this stimulation of MPS throughout the day may have a significant positive effect on recovery and adaptation in athletic training, and also slowing the rate of age-related decline of skeletal muscle mass in elderly populations. Muscle mass and function, is not only seen as a vital component of athletic performance, but its maintenance is fundamental to healthy ageing and correlates with longevity and enhanced quality of life (Kreider and Campbell, 2009; Paddon-Jones and Rasmussen, 2009).

Amino acids (AAs) act as an anabolic stimulus, with leucine in particular playing a key role in initiating this anabolic response. Protein ingestion results in the activation of signalling cascades that stimulate MPS, and the resultant increases in rates of MPS occurs in a dose-dependent manner to the quantity of protein ingested (Witard *et al.*, 2014; Moore *et al.*, 2009). However, MPS exhibits an upper limit of activation in response to the magnitude and duration of plasma leucine elevation, and after this threshold, MPS is not further stimulated (Witard *et al.*, 2014; Glynn *et al.,* 2010; Moore *et al.,* 2009; Cuthbertson, 2004). The magnitude of MPS shows a dose-dependent response to the ingestion of 0g, 5g, 10g, 20g and 40g of protein, with fractional synthetic rate (FSR) reaching an upper limit in response to 20g protein. At this upper limit, whole body leucine oxidation increases markedly, suggesting that the leucine content of 20g protein is sufficient to stimulate MPS maximally, and leucine provided in excess of this is simply oxidized (Witard *et al.*, 2014; Moore *et al.*, 2009). Furthermore, muscle exhibits a refractory response to sustained elevation of plasma aminoacidemia (Atherton *et al*. 2010a; Bohé *et al.*, 2001). This refractory response was first described when despite AAs remaining elevated during a 6 hour infusion and a sustained elevation in plasma leucine concentration, rates of MPS returned to baseline 2 hours after the beginning of the infusion (Bohé *et al.*, 2001). Similarly, a large bolus meal resulted in an increased magnitude of MPS at 45-90 min, but returned to baseline shortly thereafter, despite plasma AA concentrations remaining elevated (Atherton *et al.* 2010a). This refractory response has been termed the 'muscle full' phenomenon (Mitchell *et al.*, 2015b).

Several studies have pointed to the notion that a prolonged low amplitude elevation in postprandial leucinemia may facilitate an extended MPS response after meal ingestion (Gorissen *et al.*, 2016; Mitchell *et al.*,

2015; Areta *et al.,* 2013). Gorissen *et al.*, (2016) demonstrated that both 35g casein and 35g wheat protein ingestion resulted in a similar peak leucinemia, and at the same time, but resulted in a marked difference in postprandial MPS rates. This would suggest that the kinetics of plasma leucine, and not simply the magnitude and time to reach peak concentration of plasma leucine has an effect on postprandial MPS rates. In support, the ingestion of 60g wheat protein had a similar peak leucinemia to 35g casein, but a more prolonged elevation in plasma leucine, resulting in postprandial MPS rates similar to that of 35g casein (Gorissen *et al.*, 2016). Therefore, prolonging this lower amplitude postprandial plasma concentration of AAs may offer a means of overcoming or minimising the refractory response that muscle exhibits to sustained elevation of AAs. The anabolic potential of a meal may therefore be optimised by appropriate manipulation of postprandial AA plasma kinetics. These findings provide rationale for the use of a targeted-release form of leucine that would be capable of achieving the prolonged low amplitude leucinemia. Interestingly, microencapsulation is an emerging technology that has shown potential for the optimal delivery of drugs and nutrients (Dias, Ferreira and Barreiro, 2015; Champagne and Fustier, 2007). This technique is based on the immobilization of a core ingredient, in this case leucine, in a miniature-sealed capsule which maintains structural integrity until degradation and release of the ingredient at an appropriate time or site in the body (Champagne and Fustier, 2007). Furthermore, through appropriate design, the degradation and release of the leucine can be manipulated to create the desired plasma kinetics. We propose that a targeted-release form of leucine can cause an initial rise in leucinemia, with a more prolonged sustained low amplitude leucinemia, compared to that of 3g free leucine. Such a technology has recently been developed (Brodkorb and Doherty, 2015) and is the subject of investigation in this chapter. The present study will investigate the plasma kinetics of leucine in response to consuming leucine in either its free form or in a novel microencapsulated form, in comparison to that of a maltodextrin control.

5.2 Methods

5.2.1 Participants

The experimental procedure was approved by University College Dublin (UCD) Research Ethics Committee in accordance with the *Declaration of Helsinki*. All participants gave informed written consent before participating in the study. Participants were recruited through information leaflets and social media posts targeted at university students, seeking out healthy active males, between 18-35 years of age, participating in physical activity ≥3 times per week.

5.2.1 Materials

The microencapsulation of leucine was prepared as per **section 3.1.**

5.2.2 Method

Ten healthy active males, [age (26.4±4.1y), height (1.76±0.06m), body mass (76.7±6.9kg), BMI (24.4±1.6kg/m^2), body fat percentage (15.4±4.2%), body fat mass (11.2±3.1kg), lean body mass (61.8±6.9kg)], visited the laboratory in UCD on five separate occasions, with each visit separated by 7 days. Participants were randomly assigned to one of the following five conditions, in a cross-over design (See **figure 5.1**);

- A bolus of 3g free leucine (BOLUS)
- 3g microencapsulated targeted-release leucine (ME)
- 3g maltodextrin (CONTROL)
- 1.5g free leucine consumed at 0 hours and 1.5g consumed 2 hours later (PULSE)
- 1.5g microencapsulated targeted-release leucine + 1.5g free leucine (ME +LEU)

The rationale for using 3g of leucine in BOLUS and ME conditions is that 3g leucine and the equivalent of 3g leucine in a whole protein has been shown to maximise the stimulation of MPS in healthy young males, with greater doses showing no increase in the magnitude of stimulation (Witard *et al.*, 2014; Glynn *et al.,* 2010; Moore *et al.,* 2009; Cuthbertson, 2004). The rationale for using 1.5g of free leucine followed by the same dose 2 hours later in PULSE condition is to mimic the desired sustained release plasma kinetics of the ME leucine. The rationale for using ME+LEU condition, in which 1.5g microencapsulated targeted-release leucine is provided with 1.5g free leucine in one bolus, is to observe the plasma kinetics when both supplements are combined, which represents a similar situation as enriching a protein meal/beverage with additional ME leucine.

During each visit, participants arrived at the laboratory after an overnight fast (approximately 8 hours). A cannula was inserted in the antecubital vein and the line was kept patent with saline. Blood samples (~4ml) were drawn into a vaccutainers containing lithium heparin (BD Vaccutainers, Heparin Tubes), inverted eight to ten times and placed on ice. The blood was centrifuged at 4000 g for 10 min at 4°C and the upper layer of plasma was transferred into three separate 1.5ml tubes and stored at -80°C until further analysis. Participants ingested one of the above conditions, dissolved in a total of 250ml of water. Irrespective of the condition, participants consumed two white opaque bottles at 0 min and 120 min, as per figure 1, to maintain the single-

blind design. Blood samples were taken at rest prior to the first drink (0 min), every 15 min in the first hour, and every half hour for the next three hours, as per **Figure 5.1**.

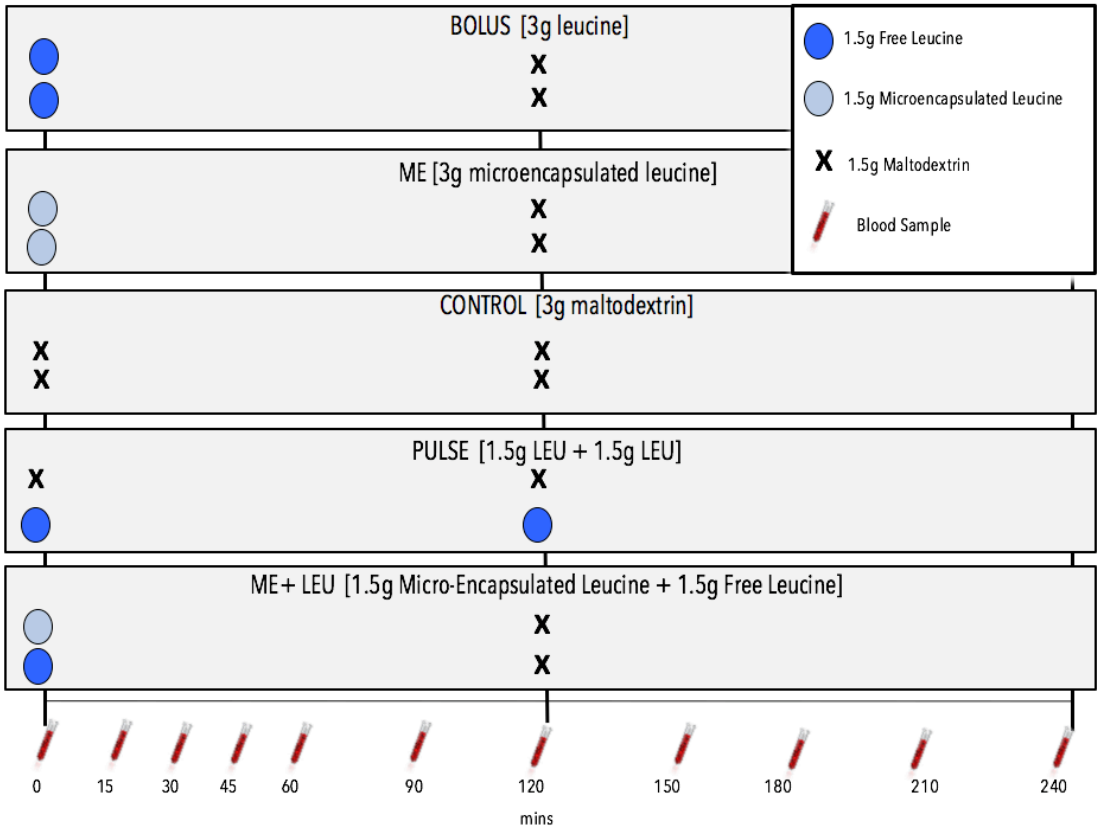


Figure 5.1: Overview of ingestion protocol of five experimental conditions. Blood samples taken every 15 min for the first hour and every 30 min for the subsequent 3 hours.

Participants visited the lab on four subsequent occasions, with the same testing procedure being carried out, with only the supplement condition being different between trials.

On the first visit, participants provided a two-day food diary, detailing their dietary habits over the two days preceding their first laboratory visit. Participants were provided a copy of this diary and asked to repeat the same two-day dietary intake prior to the subsequent four visits to the laboratory.

5.2.3 Data Analysis

High Performance Liquid Chromatography (HPLC) was carried as per **Section 3.3**.

5.2.4 Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS)(IBM SPSS Statistics Version 23). Plasma leucine, branched chain amino acids (BCAAs), total amino acids (AAs), essential amino

acids (EAAs) and non-essential amino acids (NEAAs) are presented as mean±SD. The difference from baseline over time and the difference between conditions was analysed using a two-way (time x condition) repeated measures ANOVA. When a main effect for time was indicated, post-hoc analysis within each condition was performed using one-way repeated measures ANOVA, and pairwise comparisons to 0 min with Dunnett's adjustment. When a main effect for condition was indicated, post-hoc analysis between condition was performed using one-way repeated measures ANOVA, and pairwise comparisons with Bonferroni's adjustment. The area under the curve (AUC) above baseline (ΔAUC_{240}) for each variable was calculated by trapezoidal integration, and is presented as mean±SD. The difference in AUC between conditions was analysed using a one-way repeated measures ANOVA with post-hoc pairwise comparisons using Bonferroni's adjustment. Statistical significance was accepted at $P<0.05$.

5.3 Results

5.3.1 Plasma Leucine

Table 5.1 and **figure 5.2** represents plasma leucine concentrations for each condition over 4 h. For plasma leucine there was an interaction effect, and main effect for both condition and time ($P<0.001$ for all). During BOLUS, plasma leucine peaked at 30 min at 542±83μM, which was significantly different from baseline ($P<0.001$). During ME, leucine peaked at 30 min, 212±38μM, which was significantly different from baseline ($P=0.020$). During PULSE, plasma leucinemia peaked from baseline at 30 min (325±72μM, $P=0.001$), trended towards baseline levels at 120 min (169±23.2μM) and peaked again at 273±81μM 30 min after the second leucine ingestion, which was significantly different from baseline ($P=0.023$). Plasma leucine remained unchanged during CONTROL, in which the peak value, 118±7μM, did not differ significantly from baseline ($P>0.99$). During ME+LEU (1.5g microencapsulated leucine with 1.5g free leucine), leucinemia peaked at 363±77μM, which was not significantly different from 1.5g LEU + 1.5g LEU, the condition in which 1.5g of leucine was ingested alone at 0 min ($P=0.695$). At 30 min, plasma leucine BOLUS, ME, PULSE and ME+LEU was significantly greater than CONTROL ($P<0.001$ for all). BOLUS AUC was 32±7μM.240min^{-1}, which significantly greater than CONTROL (-1±1μM.240min^{-1}, $P<0.001$) (See **figure 5.3A**). PULSE AUC was 24±7μM.240min^{-1}, which was not significantly different from BOLUS AUC ($P=0.116$). ME had an AUC of 6±3μM.240min^{-1}, which was significantly greater than CONTROL AUC ($P=0.001$). The AUC for ME+LEU was 24±7μM.240min^{-1}, which was not significantly different to 1.5g LEU + 1.5g LEU (15±7μM.240min^{-1}, $P=0.06$).

5.3.2 Plasma BCAA

Results for BCAA plasma kinetics are reported in **table 5.2.** For plasma BCAA there was an interaction effect, and main effect for both condition and time ($P<0.001$ for all). For BOLUS, plasma BCAA peaked at 30 min (808±94μM), which was significantly different from baseline ($P<0.001$). For ME, plasma BCAA peaked at 30 min (523±63μM), which was significantly different from baseline ($P=0.001$). Plasma BCAA peaked in PULSE and ME+LEU at 30 min (592±86μM and 640±101μM, respectively, both of which were significantly greater than baseline ($P<0.002$ for both). At 30 min, plasma BCAA was significantly greater in BOLUS, ME, PULSE and ME+LEU compared to CONTROL ($P<0.001$ for all). BCAA AUC is presented in **figure 5.3B**. BCAA AUC for BOLUS, was 16±3μM.240min^{-1}, which was significantly greater than ME and CONTROL (3±1 and -5±1 μM.240min^{-1}, $P=0.003$, $P=0.002$, respectively). AUC for PULSE was 10±3μM.240min^{-1}, which was significantly greater than CONTROL ($P=0.02$).

5.3.3 Plasma AA, EAA and NEAA

Results for plasma AAs, EAAs and NEAAs plasma kinetics are presented in **table 5.3, 5.4** and **5.5**, respectively. For plasma AAs, there was an interaction effect ($P<0.001$), and main effect for condition ($P=0.025$) and time ($P<0.001$). For plasma EAAs there was an interaction effect, and main effect for both condition and time ($P<0.001$ for all). For NEAAs there was an interaction effect ($P=0.002$), a main effect for time ($P=0.043$), but no main effect for condition ($P=0.72$). However, on further post-hoc analysis, no difference over time were apparent for plasma NEAAs. No significant difference existed between condition, for total AAs AUC, EAAs AUC or NEAAs AUC ($P>0.05$ for all).

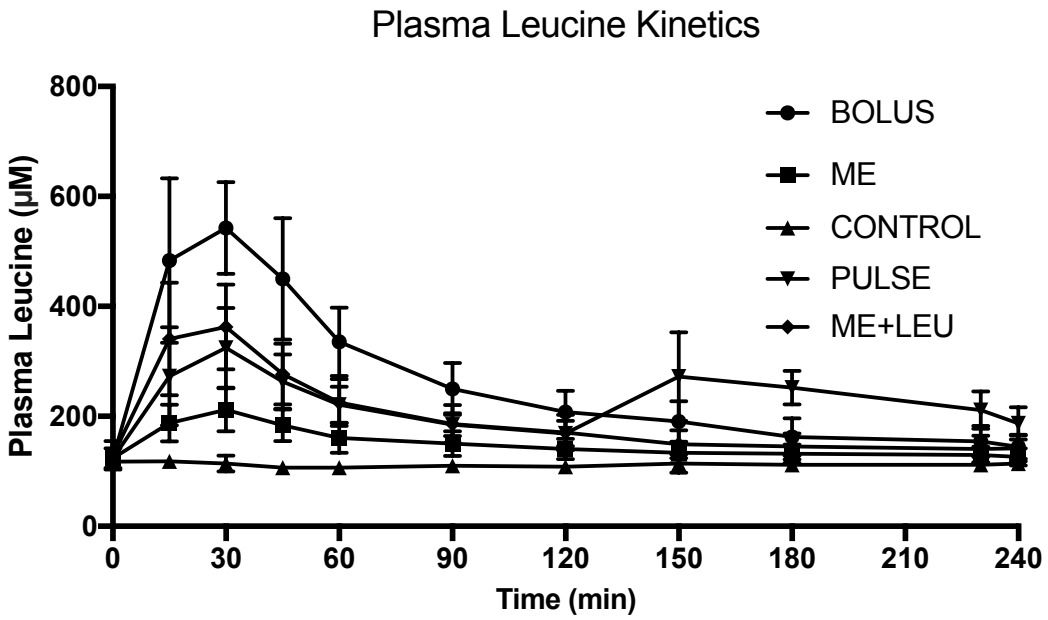


Figure 5.2: Plasma leucine concentration across each condition, expressed in µM. Values are Mean±SD BOLUS, 3g leucine; ME, 3g microencapsulated leucine; CONTROL, 3g maltodextrin; PULSE, 1.5g leucine ingested at 0 hours and 2 hours; ME+LEU, 1.5g microencapsulated leucine and 1.5g leucine. Significance markers are not included on the graph due to the complexity of the comparisons, but are described in detail in the main text.

Time (min)	BOLUS	ME	CONTROL	PULSE	ME + LEU
0	123 ± 12	124 ± 12	118 ± 12	124 ± 18	129 ± 26
15	483 ± 150 [ab]	188 ± 34 [ab]	118 ± 12	273 ± 89 [ab]	341 ± 102 [b]
30	542 ± 84 [ab]	212 ± 39 [ab]	114 ± 15	325 ± 73 [ab]	363 ± 77 [ab]
45	450 ± 111 [ab]	184 ± 29 [ab]	107 ± 12	263 ± 49 [ab]	277 ± 56 [ab]
60	335 ± 62 [ab]	161 ± 27 [ab]	107 ± 11	221 ± 33 [ab]	225 ± 42 [ab]
90	250 ± 47 [ab]	150 ± 23 [ab]	110 ± 13	185 ± 21 [ab]	186 ± 35 [ab]
120	208 ± 39 [ab]	141 ± 18 [b]	109 ± 8	169 ± 23 [ab]	171 ± 33 [ab]
150	191 ± 37 [ab]	134 ± 19	114 ± 17	273 ± 81 [ab]	149 ± 26
180	162 ± 34 [b]	132 ± 17 [b]	112 ± 7	252 ± 30 [ab]	145 ± 24 [b]
210	155 ± 28 [b]	130 ± 15 [b]	112 ± 11	211 ± 34 [ab]	141 ± 24 [b]
240	145 ± 22 [b]	126 ± 16	114 ± 11	187 ± 30 [ab]	142 ± 24 [b]

Table 5.1: Plasma leucine concentrations across each condition; expressed in µM. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points and between conditions. Difference existing from baseline is indicated by [a] $P<0.05$ vs. 0 min and differences existing between conditions is indicated by [b] $P<0.05$ vs. CONTROL.

Time (min)	BOLUS	ME	CONTROL	PULSE	ME + LEU
0	394 ± 31	400 ± 43	375 ± 43	397 ± 64	412 ± 75
15	768 ± 150[ab]	479 ± 55[ab]	375 ± 41	544 ± 111[ab]	621 ± 121[b]
30	808 ± 94[ab]	522 ± 62[ab]	365 ± 49	592 ± 86[ab]	640 ± 102[ab]
45	694 ± 154[ab]	478 ± 48[ab]	343 ± 39	510 ± 74[ab]	536 ± 92[ab]
60	557 ± 98[ab]	433 ± 62[b]	345 ± 36	454 ± 63[b]	459 ± 70[b]
90	447 ± 81[b]	418 ± 60	354 ± 45	399 ± 54	401 ± 60
120	388 ± 60	399 ± 51	345 ± 28	377 ± 56	383 ± 65
150	386 ± 76	382 ± 48	361 ± 64	474 ± 96	345 ± 49
180	332 ± 75	380 ± 46	348 ± 25	443 ± 49	345 ± 49
210	326 ± 65	375 ± 36	347 ± 38	387 ± 57	339 ± 45
240	316 ± 45	365 ± 39	352 ± 37	363 ± 56	346 ± 44

Table 5.2: Plasma branched-chain amino acid concentrations across each condition; expressed in µM. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points and between conditions. Difference existing from baseline is indicated by [a] $P<0.05$ vs. 0 min and differences existing between conditions is indicated by [b] $P<0.05$ vs. CONTROL.

Time (min)	BOLUS	ME	CONTROL	PULSE	ME + LEU
0	2112 ± 159	2026 ± 156	2014 ± 204	2058 ± 223	2135 ± 312
15	2573 ± 235[ab]	2191 ± 225[b]	2047 ± 198	2209 ± 226[a]	2346 ± 320[a]
30	2578 ± 253[ab]	2343 ± 281[ab]	2001 ± 237	2316 ± 241[ab]	2408 ± 353[ab]
45	2444 ± 259[b]	2263 ± 284	1939 ± 235	2212 ± 220[b]	2299 ± 326[b]
60	2285 ± 237	2114 ± 274	1949 ± 211	2158 ± 233	2159 ± 279
90	2132 ± 245	2111 ± 253	2024 ± 265	2019 ± 177	2060 ± 221
120	2064 ± 211	2035 ± 120	1975 ± 177	2016 ± 177	2119 ± 368
150	2145 ± 188	2018 ± 156	2089 ± 267	2108 ± 282	1975 ± 276
180	1985 ± 261	2054 ± 224	2009 ± 153	2084 ± 205	1998 ± 268
210	2026 ± 242	2079 ± 234	1991 ± 236	1977 ± 191	2005 ± 267
240	1994 ± 181	2008 ± 139	2013 ± 200	1995 ± 221	2066 ± 295

Table 5.3: Plasma total amino acid concentrations across each condition, expressed in µM. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points and between conditions. Difference existing from baseline is indicated by [a] $P<0.05$ vs. 0 min and differences existing between conditions is indicated by [b] $P<0.05$ vs. CONTROL.

Time (min)	BOLUS	ME	CONTROL	PULSE	ME + LEU
0	890 ± 51	866 ± 55	840 ± 75	878 ± 116	919 ± 148
15	1296 ± 157 [ab]	979 ± 87 [ab]	851 ± 89	1026 ± 131 [a]	1134 ± 168 [ab]
30	1322 ± 126 [ab]	1056 ± 112 [ab]	830 ± 106	1082 ± 111 [ab]	1164 ± 167 [ab]
45	1196 ± 190 [ab]	996 ± 90 [ab]	793 ± 82	995 ± 112 [ab]	1053 ± 163 [ab]
60	1048 ± 145 [b]	917 ± 114	796 ± 77	935 ± 107 [b]	952 ± 125 [b]
90	923 ± 139	904 ± 116	823 ± 96	859 ± 96	878 ± 102
120	853 ± 103	865 ± 66	805 ± 67	840 ± 94	875 ± 160
150	871 ± 128	845 ± 77	843 ± 122	927 ± 128	803 ± 109
180	789 ± 143	850 ± 85	813 ± 53	900 ± 97	811 ± 108
210	796 ± 126	852 ± 62	806 ± 91	821 ± 82	804 ± 91
240	781 ± 86	827 ± 49	817 ± 82	810 ± 84	826 ± 104

Table 5.4: Plasma total essential amino acid concentrations across each condition, expressed in µM. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points and between conditions. Difference existing from baseline is indicated by [a] $P<0.05$ vs. 0 min and differences existing between conditions is indicated by [b] $P<0.05$ vs. CONTROL.

Time (min)	BOLUS	ME	CONTROL	PULSE	ME + LEU
0	1222 ± 125	1160 ± 121	1174 ± 140	1180 ± 130	1216 ± 169
15	1278 ± 114	1212 ± 147	1197 ± 126	1183 ± 133	1211 ± 169
30	1256 ± 138	1287 ± 181	1171 ± 143	1234 ± 144	1244 ± 193
45	1248 ± 103	1268 ± 201	1147 ± 161	1216 ± 130	1246 ± 177
60	1237 ± 126	1197 ± 175	1153 ± 145	1223 ± 142	1207 ± 167
90	1209 ± 122	1207 ± 156	1202 ± 174	1160 ± 110	1182 ± 139
120	1211 ± 133	1169 ± 111	1170 ± 122	1175 ± 112	1244 ± 214
150	1274 ± 87	1172 ± 117	1246 ± 150	1181 ± 166	1172 ± 179
180	1196 ± 133	1203 ± 166	1196 ± 112	1184 ± 130	1187 ± 172
210	1230 ± 136	1227 ± 185	1186 ± 150	1156 ± 125	1201 ± 186
240	1213 ± 118	1182 ± 123	1196 ± 131	1185 ± 144	1240 ± 197

Table 5.5: Plasma total non-essential amino acid concentrations across each condition, expressed in μM. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points and between conditions. Difference existing from baseline is indicated by [a] $P<0.05$ vs. 0 min and differences existing between conditions is indicated by [b] $P<0.05$ vs. CONTROL.

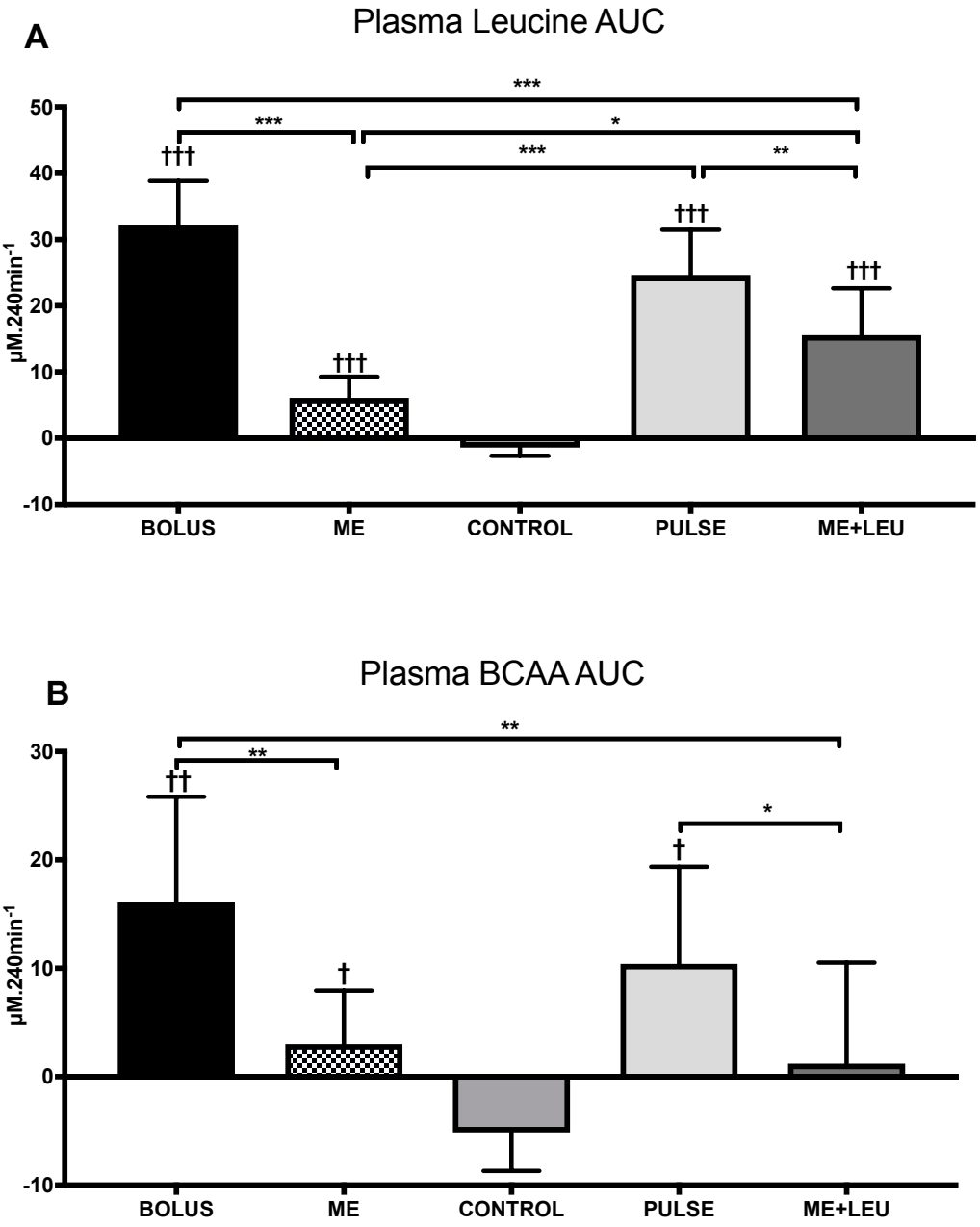


Figure 5.3: AUC for leucine (A) and branched chain amino acids (B) across each condition, expressed in µM.240min^{-1}. BOLUS, 3g leucine; ME, 3g microencapsulated leucine; CONTROL, 3g maltodextrin; PULSE, 1.5g leucine ingested at 0 hours and 2 hours; ME+LEU, 1.5g microencapsulated leucine and 1.5g leucine. Values are Mean±SD. A one-way repeated measures ANOVA by condition was performed. When $P<0.05$, post-hoc pairwise comparisons, with Bonferroni's adjustment for multiple comparisons, were used to determine where differences existed between conditions, as indicated by *=$P<0.05$, **=$P<0.01$, ***=$P<0.001$. Difference from control is indicated by †=$P<0.05$, ††=$P<0.01$, †††=$P<0.001$.

5.4 Discussion

Postprandial plasma kinetics of leucine after the ingestion of a protein-containing meal or supplement is a key regulator of MPS, but few studies have described the plasma kinetics of leucine after the ingestion of different leucine supplementation forms and timing. The aim of this study was to investigate the plasma kinetics of leucine in response to consuming leucine in its free form, in a novel microencapsulated form, in a timed pulsed ingestion protocol and in comparison to that of a maltodextrin control. The microencapsulation technique showed reduced bioavailability in comparison to leucine ingested in its free form. Leucine in its free form causes a greater magnitude of postprandial leucinemia compared to the similar doses of leucine ingested in a whole protein matrix reported in the literature (Areta 2013; Churchward-Venne *et al.* 2012; Moore *et al.,* 2009).

Despite the peak leucinemia and AUC being significantly different from baseline and CONTROL, the microencapsulated leucine technology shows poor bioavailability when compared to free leucine in BOLUS and PULSE conditions. Although a smaller peak was anticipated when compared to 3g free leucine condition, due to the targeted-release characteristic of the condition, the AUC for ME was significantly lower than that of BOLUS, suggesting reduced bioavailability (6±3μM.240min^{-1}, vs to 32±7μM.240min^{-1} respectively). Plausible explanations for this reduced bioavailability are the untimely degradation of the microencapsulation technology and/or impaired absorption in the small intestine. Microencapsulation is a process in which small films are applied to a solid particle to preserve the active ingredient (Jackson and Lee, 1991). Due to the intricate nature of the technology it requires accurate design to ensure the core ingredient's release occurs at the correct site of absorption. Otherwise the lower pH of stomach acid may break it down too quickly, or the outer shell may be incompletely broken down at the absorption site. It is plausible that the microencapsulated leucine was not degraded at the small intestine, and degradation occurred either too early, later in the large intestine, or did not entirely breakdown and passed through the gastrointestinal tract incompletely degraded. This would be the result of sub-optimal development of the microencapsulation technique, suggesting it needs further review. Reduced bioavailability of ME may also be explained by impaired intestinal absorption, resulting in a lower plasma leucinemia. Increased splanchnic utilisation blunts the rise in aminoacidemia after ingestion of an AA mixture. The splanchnic tissues are responsible for absorption of the ingested AAs and their release to the circulatory system, but if the splanchnic tissues utilise these AAs, less AAs will be available for absorption to circulation (Bos *et al.*, 2005, 2003; Fouillet. *et al.,* 2002). However, an increased uptake of AAs by enterocytes is predominately the result of a slower rate of gastric emptying (Boirie *et al.*, 1997). Factors

responsible for a slower rate of gastric emptying include greater food/liquid volume, the addition of carbohydrates, fats, proteins, calories, and/or soluble fibre to the meal (Rondanelli *et al.*, 2017; Holwerda et al., 2017; Have *et al.* 2007; Duranti, 2006; Hunt 1980). While the liquid volume ingested was standardised across conditions, and there were no additional carbohydrate, fats or calories in any conditions, the whey protein component of the microencapsulation matrix may have caused a slowing in gastric emptying. However, it is unlikely that the whey protein matrix is entirely responsible for the reduced bioavailability of leucine bound in the microencapsulation. While impaired intestinal absorption may certainly be a contributing factor to the reduced bioavailability of ME leucine, the untimely breakdown of the microencapsulation technology due to sub optimal formulation, may be a more significant factor in these results.

Despite leucine representing the key AA in initiating signalling cascades that activate MPS, few studies have investigated the leucinemia after ingestion of leucine alone, with most focusing on postprandial leucinemia after the ingestion of milk proteins, plant proteins, AA mixtures, or protein foods. The ingestion of 3g of leucine in its free form produced a steep rise in leucinemia to 540μM, peaking at 30 min after ingestion. This is similar to finding by Matsumoto *et al.*, (2014), who compared the plasma kinetics of leucine after the ingestion of graded doses of crystalline leucine diluted/partially diluted in water. Matsumoto *et al.*, (2014) reported a peak of ~800μM at 30 min after the ingestion of 45mg/kg or ~3g leucine. Similarly, Wilkinson *et al.*, (2013) reported a peak leucinemia of ~500μM at 30 min after the ingestion of 3.42g leucine. Notably, the absorption kinetics of 3g leucine is different to that contained in whole intact proteins reported in the literature. Despite egg and milk protein having the highest Protein Digestibility Corrected Amino Acid Score (PDCAAS), ingestion of a similar quantity of leucine in these intact forms results in an attenuated and delayed peak leucinemia, when compared to free leucine. The ingestion of 40g egg protein, containing 3.4g leucine resulted in a peak in plasma leucinemia of 167μM, 45 min after ingestion (Moore *et al.,* 2009), and the ingestion of 25g whey, containing ~3g leucine, causes a peak in plasma leucinemia of approximately 390μM, 90 min after ingestion (Churchward-Venne *et al.* 2012). The attenuated and delayed peaks in plasma leucinemia values may be explained by the slower rate of gastric emptying due to the increases energy load associated with whole proteins. Indeed, doubling the energy density of food from 0.7 to 1.4 kcal/ml increases gastric emptying from 116 kcal to 176 kcal/30 min, instead of to 232 kcal/30 min had there been no slowing of gastric emptying (Hunt 1980). This 50% reduction in the rate of gastric emptying is because receptors on the duodenum wall determine the osmotic properties of a food stuff, and are considered to be responsible for modulating the rate of gastric emptying. Furthermore, the plasma leucine kinetics after the ingestion of a beverage containing

6.25g whey (containing 0.75g leucine) plus 2.25g of free leucine, which provided a total of 3g of leucine causes a peak in leucinemia of 530μM, 1 hour after ingestion (Churchward-Venne el al. 2012), which is similar in magnitude, although delayed by 30 min, to the results of this current trial after the ingestion of 3g of free leucine. This similarity is potentially due to the low energy and macronutrient contribution of 6.25g of whey protein to the ingested beverage. This suggests that since a large proportion of leucine in the mixed beverage consists of free form leucine, and a small quantity of whole whey, the rate of gastric emptying is slowed less, resulting in a high peak leucinemia when compared to whole form protein such as 40g whey protein (Areta 2013), 40g of egg protein (Moore *et al.,* 2009) or 25g whey (Churchward-Venne *et al.* 2012). Furthermore, a peak postprandial plasma leucinemia of ~500μM is apparent 90 min after the ingestion of 16.6g of whey with an additional 3.4g of free form leucine (Tipton *et al.* 2009). Despite the leucine representing 5.4g of this mixed beverage, it does not result in a greater peak leucinemia when compared to that of 3g leucine in the present trial. This may be explained by the whey protein component of the beverage causing a slower digestion kinetics, and/or the saturation of the transporter proteins carrying leucine across the intestinal wall, and thereby a delayed and decreased peak leucinemia.

Regarding the transport process, the absorption of most AAs from the lumen of the intestine requires an active transport process across the enterocytes. Leucine, being a monoamino-monocarboxylic (neutral) AA, uses the system L transporters via a single saturable pH-independent transporter (Fraga, Serrão and Saores-de-Silva, 2002; Iannoli *et al.,* 1999). Another possible explanation for this discrepancy therefore may be the protein transporter within the membrane of the enterocytes is reaching saturation levels at this concentration of leucine in the lumen, meaning the greater leucine concentration in the intestines does not result in a higher plasma leucine concentration. In support, Matsumoto *et al.*, (2014) reported no further increase in peak leucinemia after the ingestion of ~4g, 5g, and 6g free leucine. PULSE fed leucine,1.5g of leucine, ingested at 0 min and again at 120 min, resulted in a cyclical oscillation of leucinemia, in which plasma leucinemia peaked at 325μM at 30 min, and reached as low as 169μM at 120 min. However, this low concentration was significantly different from baseline values for that condition, suggesting that while leucinemia trends towards baseline values, it does not reach baseline values 120 min after ingestion of 1.5g free leucine. Elsewhere, a similar dose of leucine, in the form of 10g whey protein, delivered at 0 min and every 90 min thereafter for 12 hours, causes an initial peak in plasma leucinemia of ~130μM at 90 and 120 min. This increase in leucinemia continues to rise and reaches its greatest peak of 270μM at 6.5 hours. Thereafter, it begins to trend downwards, despite further ingestion of 10g whey protein every 90 min (Areta *et al.,* 2013). This sustained

hyperaminoacidemia pattern may be related to the slower digestion kinetics of whole protein, and the shorter interval between feeding times compared to the present study.

The superiority of animal-based protein over plant-based protein in stimulating MPS is well-reported and is attributed to the difference in digestion rates and relative leucine content of respective proteins, with animal-based proteins resulting in a greater hyperleucinemia and hyperaminoacidemia when compared to plant-based proteins (Gorissen *et al.*, 2016; Tang *et al.* 2009; Wilkinson *et al.,* 2007). These present results show the potential advantage of using free-form AAs in supplement form, compared to whole proteins such as whey and egg, when the objective is to cause a sharp rise in leucinemia. Due to the importance of leucine in anabolic stimulation, the enrichment of lower leucine meals/supplements with a free-form leucine may have significant impact on anabolism and muscle recovery in those following a plant-based or low animal protein diet, or for elderly who may find it difficult to eat optimal levels of per meal leucine and protein (Gorissen and Witard, 2018; van Vliet, Burd and van Loon, 2015). Indeed, the co-ingestion of 5g of leucine supplement with three main meals enhances integrated 3-day rates of MPS in free-living older men, consuming 0.8 and 1.2g protein/kg per day (Murphy et al. 2016). Therefore, another application of microencapsulated leucine, irrespective of any time-release property, would be in leucine-enrichment of plant-based protein supplements and food matrices, which may add efficacy to nutrition strategies targeting an increase in MPS, and by extension, lean body mass. By increasing plasma leucinemia, this would thereby stimulate MPS to a greater magnitude compared to a plant-based or leucine-poor protein alone, which has been reported elsewhere (Engelen *et al.*, 2007). However, to the best of our knowledge, post prandial plasma leucine kinetics after the ingestion of a leucine-enriched plant-based protein has not been investigated. Since MPS is stimulated in a dose-dependent manner in response to a rise in plasma leucine (Glynn *et al.,* 2010; Moore *et al*., 2009; Cuthbertson, 2004), using free form AAs in supplement form may show promise in recovery from training bouts in the athletic populations, and in ameliorating the age-related decline of muscle mass in elderly populations. Lastly, the use of microencapsulated leucine may also enhance the palatability of leucine-containing protein supplements, as masking the bitter taste properties of free leucine proves difficult (Buttery *et al.*, 1989).

5.5 Conclusion

The microencapsulated leucine technology used in this study, which was designed to achieve a sustained increase in leucinemia through targeted-release, did not produce the desired effect, and its formulation needs

review. The oral ingestion of 3g of leucine in free form resulted in a steep rise in leucinemia to 540μM, peaking at 30 min after ingestion. This peak postprandial leucinemia is notably higher than that of whole protein, such as egg and whey reported in the literature, providing the same dose of leucine. This higher and earlier peak leucinemia may be explained by the faster digestion and absorption kinetics of leucine when provided in its free-form compared to when provided in whole protein. Since change in plasma leucine concentration is a key signal in activating MPS, dietary supplementation with free-form leucine may show promise as a strategy for optimising the plasma kinetics required to maximise MPS. Furthermore, the enrichment of lower leucine meals, beverages and food matrices with a free-form leucine may have significant impact on anabolism in those following a plant-based or low animal protein diet, or elderly populations to ameliorate age-related decline in muscle mass.

Chapter 6

6.0 The plasma leucine kinetics after the ingestion of a plant-based meal enriched with leucine

Background: Leucine-enrichment, in which free leucine is added to a suboptimal protein dose, is an emerging nutrition strategy for increasing the anabolic properties of a meal. Despite plasma leucinemia being a key modulator in the postprandial anabolic response, the plasma kinetics after the ingestion of leucine-enriched meals remain underexplored.

Objective: The aim of the present study was to investigate the plasma kinetics of leucine and amino acids (AAs) in response to the ingestion of a plant-based meal low in leucine, but enriched with free leucine.

Design: Five healthy active males [age (25.6±2.4y)] visited the laboratory on three separate occasions, and in a cross-over design were randomly assigned to one of the following conditions in combination with a mixed meal (845 kcal, 24g protein, 117g carbohydrates, 33g fat): A bolus of 3g leucine (BOLUS); 1.5g free leucine consumed at 0 hours and 1.5g consumed 2 hours later (PULSE); 3g maltodextrin (CONTROL). Blood samples for plasma analysis were taken every 15 min in the first hour, and every half hour for the next three hours. These participants had previously participated in Chapter 5, which also allowed for comparison of the effects of meal co-ingestion on leucine kinetics.

Data Analysis: Concentrations of AAs in plasma were measured using high performance liquid chromatography. Changes in plasma leucine, branched chain amino acids (BCAAs), total AAs, essential amino acids (EAAs) and non-essential amino acids (NEAAs), over time and between conditions was evaluated using a two-way repeated measures ANOVA. Post-hoc analysis was performed using a repeated measured ANOVA and pairwise comparisons with Bonferroni's adjustment for C_{max} between conditions, and Dunnett's adjustment for within condition effects.

Results: The enrichment of a mixed meal with 3g leucine resulted in C_{max} for leucinemia of 350±44μM, which was significantly greater than C_{max} after a mixed meal ingested alone (152±22μM, $P<0.05$). There was a dampened increase in plasma leucinemia when 3g leucine was ingested with a mixed meal compared to the plasma leucinemia achieved after the ingestion of 3g leucine alone in chapter 5 (542±83μM), as well as a smaller AUC (15±5 vs. 32±7 μM.240min^{-1}, respectively). When 1.5 g of leucine was ingested at 120 min, the prior ingestion of a meal at 0 min resulted in a smaller AUC for leucine (4±4 μM.240min^{-1}) in this experiment, when compared to no meal ingested at 0 min (14±5 μM.240min^{-1}) in Chapter 5.

Conclusion: The enrichment of a plant-based meal with 3g leucine results in a greater increase in leucinemia when compared to a plant-based meal alone. The presence of other macronutrients and fibre in the mixed meals compromises postprandial plasma leucinemia. Marked difference in postprandial leucinemia are apparent between 3g leucine ingested with and without a mixed meal. When using leucine-enrichment as a strategy for increasing the anabolic characteristics of a meal, this is an important consideration as addition leucine may be necessary to reach a threshold required to maximise MPS.

6.1 Introduction

Leucine has been identified as the key AA that acts as a trigger for stimulating MPS (Atherton et al., 2010a), and the resultant leucinemia likely explains in part the dose-response increase in MPS observed after the ingestion of a protein-containing meal (Witard *et al.*, 2014; Moore *et al.*, 2009). Consequently, protein sources that are low in leucine, particularly plant-based proteins (USDA National Nutrient Database for Standard Reference, 2009), exhibit an inferior stimulation of postprandial MPS, when compared to an equivalent dose of animal-based protein (Gorissen *et al.*, 2016; Wilkinson *et al.*, 2007; Yang *et al.*, 2012a; 2012b; Tang *et al.*, 2009). The reason for this inferior anabolic response is likely due at least in part to a blunted increase in postprandial leucinemia, and therefore a decreased availability of leucine for delivery to the skeletal muscle (Gorissen *et al.*, 2016; Tang *et al.*, 2009). Indeed, anti-nutritional factors present in plant-based foods, such as fibre and the energy content of carbohydrate and fat, may slow gastric emptying and impede protein digestion and absorption, and therefore attenuate the expected increase in postprandial leucinemia (Gorissen and Witard, 2018).

Efforts have been made to increase the anabolic potential of plant-based meals, such as combining plant-based foods to render a meal complete in all nine EAAs, and increasing the dose of protein in plant-based meals (Gorissen and Witard, 2018; van Vliet, Burd and van Loon, 2015). These approaches are not without their shortcomings, particularly because of the food quantity and therefore energy intake required to attain the desired AA profile to maximise MPS in one meal. This presents challenges particularly for athletic populations and older adults, both of which have protein requirements greater than the PRI (Egan, 2016; Thomas, Erdman and Burke, 2016; Bauer *et al.*, 2013; Phillips, 2012a; Phillips and van Loon, 2011). The energy intake therefore required to reach the purported leucine threshold when consuming low leucine foods may be an issue for athletes whose sport requires close monitoring of body mass and body composition. Furthermore, older adults, who experience a deterioration in appetite with age (Morley, 2001), may find it impractical to eat large volumes of food in one sitting.

Leucine-enrichment, in which free leucine is added to a meal or food matrix, is emerging as a promising means of 'rescuing' the inferior anabolic response apparent after the ingestion of low leucine/plant-based protein sources and meals (Murphy *et al.*, 2016; Bauer et al., 2015; Bukhari *et al.*, 2015; Wilkinson *et al.*, 2017; Casperson *et al.*, 2012). Indeed, the enrichment of whey protein with leucine has yielded promising results in acute studies for changes in MPS (Devries *et al.*, 2018; Kramer *et al.*, 2017; Churchward-Venne *et al.*, 2014), and long-term intervention studies in changes of LBM (Bauer *et al.*, 2015). Despite this interest, the

plasma kinetics after the ingestion of leucine in combination with a mixed meal remain underexplored. In chapter 5, we investigated the plasma AA kinetics of different forms and timing of leucine when ingested alone in a fasted state. While leucine acts as a trigger stimulating MPS above post-absorptive values, other (both non-essential and essential) AAs are required as a substrate for the synthesis of new muscle protein (Churchward-Venne *et al.*, 2012), with the rationale that leucine be ingested with other EAA when the aim is to maximise MPS. Therefore, the aim of this present study was to determine the plasma leucine and AA kinetics after the ingestion of a plant-based mixed meal inherently low in leucine, with or without enrichment with free leucine.

6.2 Methods

6.2.1 Study Design

The experimental procedure was approved by University College Dublin (UCD) Research Ethics Committee in accordance with the *Declaration of Helsinki*. Five healthy males, who had previously been recruited for the chapter 5, agreed to take part in the present study. Participants [age (25.6±2.4y), height (1.78±0.08m), body mass (75.3±8.7kg), BMI (23.6±0.9kg/m^2), body fat percentage (17.3±2.8%), body fat mass (12.46±1.84kg), lean body mass (59.87±7.94kg)], visited the laboratory in UCD on three separate occasions, with each visit separated by seven days. Participants were assigned in random to one of the following three conditions for each visit in a single blind, cross-over design;

- A bolus of 3g free leucine (BOLUS)
- 1.5g free leucine consumed at 0 hours and 1.5g consumed 2 hours later (PULSE)
- 3g maltodextrin (CONTROL)

Test drinks were prepared identically to BOLUS, PULSE and CONTROL in chapter 5. During each visit, participants arrived to the laboratory after an overnight fast (approximately 8 hours). A cannula was inserted in the antecubital vein and the line was kept patent with saline. Blood samples (~4ml) were drawn into vacutainers containing lithium heparin (BD Vaccutainers, Heparin Tubes), inverted eight to ten times and placed on ice. The blood was centrifuged at 4000 g for 10 min at 4°C and the upper layer of plasma was transferred into three separate 1.5ml tubes and stored at -80°C until further analysis. Participants ingested a mixed meal containing a hemp protein shake (Manitoba Hemp 50), almonds, two cereal bars and an electrolyte sports drink (See **table 6.1** for nutritional information of the meal). Hemp protein was chosen as it is one of the lowest leucine-containing protein powders, and we wanted to minimise the interference of leucine from mixed meal with plasma leucine concentrations. Participants ingested the meal, with one of the

above test conditions, dissolved in a total of 150ml of water. Irrespective of the condition the condition, participants consumed two white opaque bottles at 0 min and 120 min, as per **figure 6.1**, to maintain the single-blind design. Blood samples were taken at rest at rest prior to meal (0 min), every 15 min in the first hour, and every half hour for the next three hours. Participants visited the laboratory on two subsequent occasions, with the same testing procedure being carried out, and participants consuming one of the other two conditions. On the first visit, participants provided a two-day food diary, detailing their dietary habits over the two days preceding their first laboratory visit. Participants were provided a copy of this diary and asked to repeat the same two-day dietary intake prior to the subsequent two visits to the laboratory.

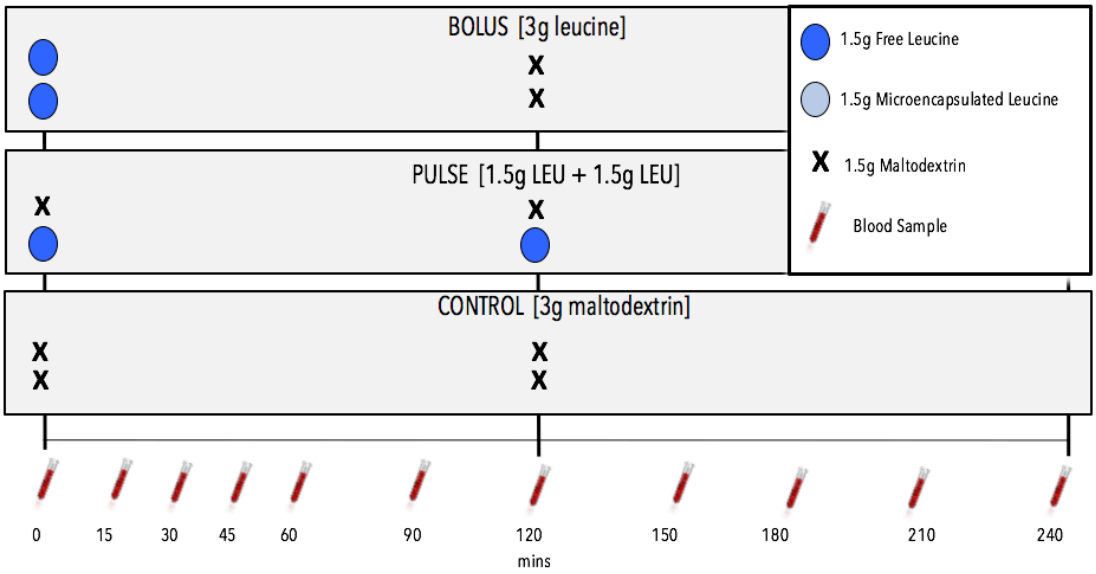


Figure 6.1: Overview of ingestion protocol of each beverage condition.

	Mixed Meal	Free Leucine	Meal + Leucine
Energy (kcal)	854	16	870
Protein (g)	24	3	27
Leucine (g)	1.2	3	6.2
Carbohydrate (g)	117	-	117
Fat (g)	33	-	33

Table 6.1: Nutritional composition of mixed meal, leucine powder and combined (Meal+Leucine).
g, grams; kcal, calories.

6.2.2 Data Analysis

High Performance Liquid Chromatography (HPLC) was carried as per **Section 3.3**.

6.2.3 Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics Version 23). Plasma leucine, branched chain amino acids (BCAAs), arginine (the second most abundant AA in hemp protein), total amino acids (AAs), essential amino acids (EAAs) and non-essential amino acids (NEAAs) are presented as mean±SD. A two-way (condition x time) repeated measures ANOVA was performed to determine differences in plasma concentration of AAs over time and between conditions. The difference in AA concentrations from baseline within each condition was analysed using a one-way ANOVA, and post-hoc testing with Dunnett's adjustment. As PULSE consisted of leucine ingested at 0 min and again at 120 min, $PULSE^1$ defined plasma AA concentrations from 0-120 min, and $PULSE^2$ defined as plasma AA concentrations from 150-240 min. As peak plasma AA concentrations were reached at different time-points for participants, maximum concentration (C_{max}) of AA was determined. The area under the curve (AUC) above baseline (ΔAUC_{240}) for each variable was calculated by trapezoidal integration, and is presented as mean±SD. The difference in C_{max} and AUC between conditions was analysed using a one-way repeated measures ANOVA. Where main effect was found, post-hoc testing consisted of pairwise comparison using Bonferroni's adjustment. Statistical significance for all tests was accepted at $P<0.05$. As the 5 participants used for the present study were also used in chapter 5, rather than compare this chapter's data to the $n=10$ in chapter 5, additional descriptive statistics were performed on the respective $n=5$ for plasma leucine in BOLUS and PULSE from chapter 5. This was done in order to compare the plasma leucine C_{max} and AUC after leucine was ingested alone compared to leucine ingested with a mixed meal in the present study.

6.3 Results

6.3.1 Plasma Leucine

Plasma leucine kinetics are presented in **figure 6.2 and table 6.3**. The time at which plasma leucine reached peak concentration varied between participants. C_{max} for BOLUS was 350±44 μM, which was significantly greater than C_{max} for CONTROL (152±22μM, $P=0.006$). C_{max} for $PULSE^1$ was 217±43μM, which was not different to C_{max} for CONTROL ($P=0.2$). C_{max} for $PULSE^2$ was 209±48μM, which was not different to C_{max} for CONTROL ($P=0.38$). C_{max} for $PULSE^1$ and $PULSE^2$ showed no difference ($P=0.47$). For plasma leucine, there was a main effect for time ($P<0.001$) and condition ($P<0.001$), and an interaction effect ($P<0.001$). During BOLUS, there was a marked increase in plasma leucinemia at 30, 45, 60, 90 and 120 min after ingestion, when compared to baseline ($P<0.05$). During CONTROL, there was a marked decrease in plasma leucinemia at 150, 180, 210 and 240 min after ingestion, when compared to baseline ($P<0.05$). AUC for leucine is

presented in **figure 6.3**. BOLUS AUC was significantly greater than AUC for CONTROL (15±5 vs. -3±1 μM.240min^{-1}, P=0.001). PULSE AUC was 8±9μM.240min^{-1}, which was not significantly different to CONTROL (P=0.13). PULSE^{1} AUC was 4±4μM.120min^{-1}, and PULSE^{2} AUC was 4±4μM.120min^{-1}, which were not significantly different (P=0.82).

	BOLUS	PULSE^{1}	PULSE^{2}	CONTROL
Leucine	350 ± 44[a]	217 ± 43	209 ± 48	152 ± 22
Arginine	130 ± 28	120 ± 30	106 ± 20	122 ± 43
BCAA	632 ± 75	491 ± 22	427 ± 35	446 ± 38
AA	2713 ± 273	2538 ± 134	2330 ± 119	2576 ± 296
EAA	1187 ± 112	1073 ± 41	920 ± 55	1029 ± 111
NEAA	1566 ± 141	1509 ± 67	1418 ± 74	1579 ± 174

Table 6.2: Cmax for amino acids concentrations across each condition, expressed in μM. Values are mean±SD. PULSE^{1} is plasma amino acid concentrations, from 0 to 120 min, after the ingestion of 1.5g leucine with a mixed meal at 0 min. PULSE^{2} is plasma amino acid concentrations, from 150 to 240 min, after the second 1.5g leucine ingestion at 120 min. A one-way repeated measures ANOVA, with Bonferroni's adjustment, was used to determine differences between conditions. Differences are indicated by [a] P<0.05 vs. CONTROL.

Time (min)	BOLUS	PULSE	CONTROL
0	124 ± 25	132 ± 19	130 ± 3
15	262 ± 62	180 ± 32	133 ± 5
30	307 ± 55[a]	182 ± 27	143 ± 29
45	286 ± 54[a]	190 ± 49	139 ± 22
60	266 ± 64[a]	175 ± 37	131 ± 17
90	203 ± 26[a]	155 ± 29	122 ± 13
120	181 ± 13[a]	134 ± 16	109 ± 3[a]
150	159 ± 12	205 ± 52	106 ± 6[a]
180	128 ± 8	173 ± 28	99 ± 8[a]
210	121 ± 9	140 ± 16	99 ± 9[a]
240	111 ± 11	138 ± 13	99 ± 9[a]

Table 6.3: Plasma leucine concentrations across each condition, expressed in μM. Values are mean±SD. A one-way repeated measures ANOVA, with Dunnett's adjustment was used to determine differences from baseline. Differences are indicated by [a] P<0.05 vs. 0 min.

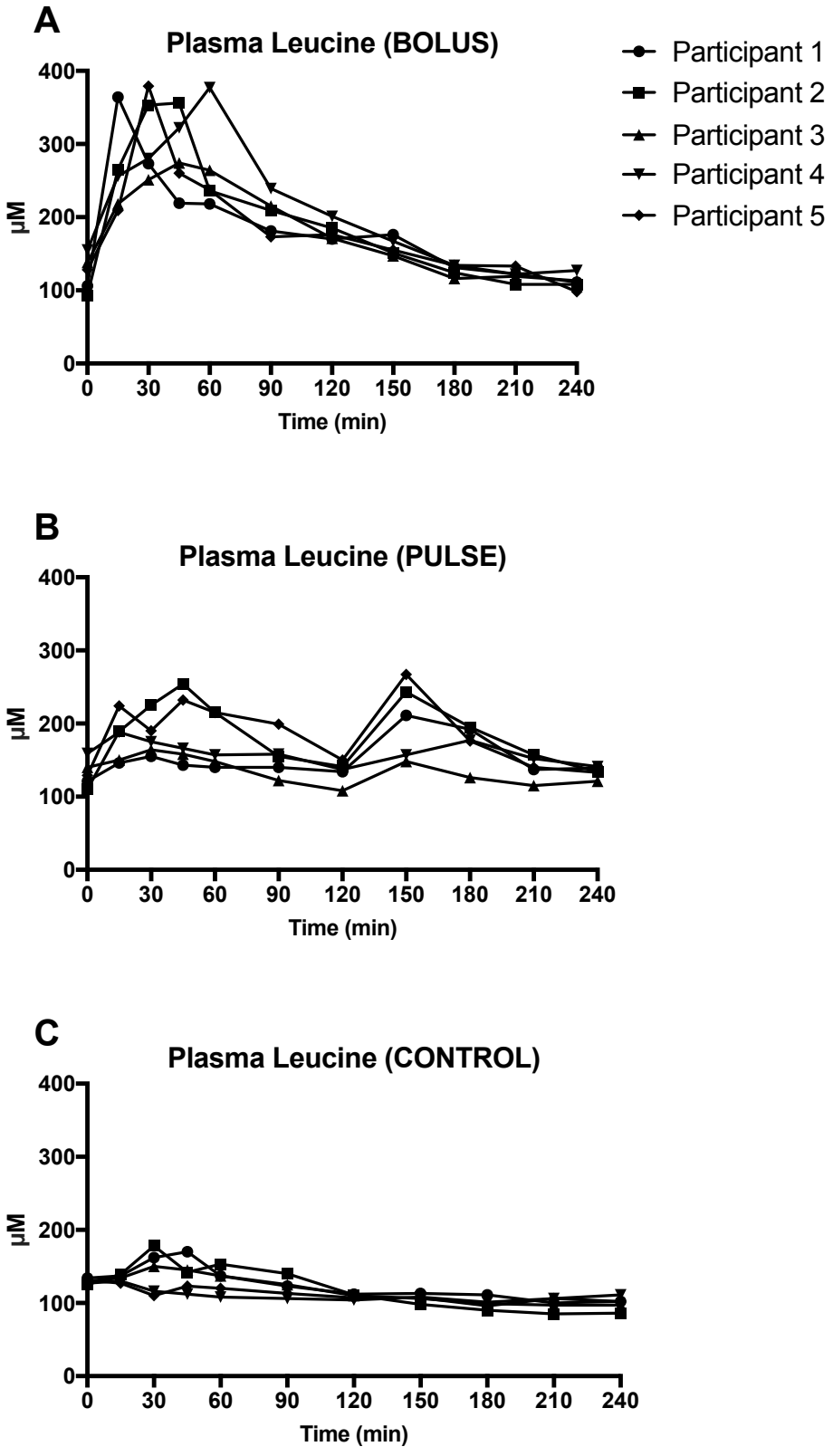


Figure 6.2: Plasma leucine concentration after the ingestion of mixed meal with BOLUS (A), PULSE (B) and CONTROL (C), expressed in μM. Values expressed as individual participant data. BOLUS, 3g leucine; PULSE, 1.5g leucine ingested at 0 hours and 2 hours; CONTROL, 3g maltodextrin.

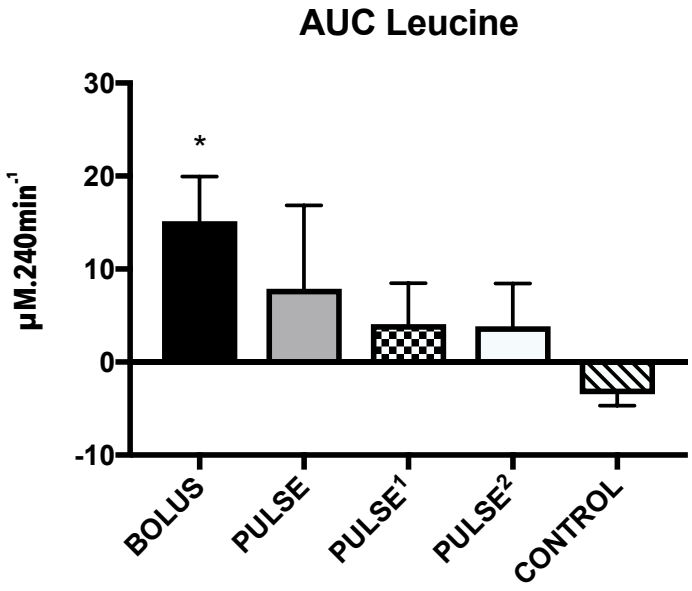


Figure 6.3: Area under the curve for leucine across conditions, expressed in µM.240min^{-1}. Values are mean±SD. BOLUS, 3g leucine; PULSE, 1.5g leucine ingested at 0 hours and 2 hours; PULSE^{1}, plasma amino acid concentrations, from 0 to 120 min, after the ingestion of 1.5g leucine with a mixed meal at 0 min; PULSE^{2}, plasma amino acid concentrations, from 150 to 240 min, after the second 1.5g leucine ingestion at 120 min; CONTROL, 3g maltodextrin. A one-way repeated measures ANOVA by condition was performed, with Bonferroni's adjustment for multiple comparisons. Differences are indicated by $^{*}P<0.05$ vs. CONTROL.

6.3.2 Plasma Arginine

Arginine C_{max} for each condition are presented in **table 6.2** and revealed no significant differences between conditions ($P>0.05$). Plasma arginine kinetics are presented in **table 6.6.** For plasma arginine there was a main effect for time ($P<0.001$). During BOLUS, there was a significant increase in plasma arginine above baseline at 30 and 60 min ($P<0.05$). AUC for arginine was 6±4µM.240min^{-1}, 5±4µM.240min^{-1}and 6±2µM.240min^{-1} for BOLUS, PULSE and CONTROL, respectively. No significant difference existed between condition, for arginine AUC ($P=0.87$).

6.3.3 Plasma BCAAs

BCAA C_{max} for each condition are presented in **table 6.2** and revealed no significant differences between conditions ($P>0.05$). Plasma BCAA kinetics are presented in **table 6.5.** For plasma BCAAs, there was a main effect for time ($P<0.001$) and an interaction effect ($P<0.001$). During BOLUS, there was a significant increase in plasma BCAAs above baseline at 30 min ($P<0.05$). AUC for BCAAs was 4±22µM.240min^{-1}, -4±12µM.240min^{-1}and -6±6µM.240min^{-1} for BOLUS, PULSE and CONTROL, respectively. No significant difference existed for BCAAs AUC between condition ($P=0.59$).

Time (min)	BOLUS	PULSE	CONTROL
0	83 ± 18	80 ± 13	79 ± 20
15	105 ± 17	90 ± 15	87 ± 33
30	123 ± 24[a]	105 ± 23	114 ± 49
45	124 ± 31	116 ± 32	113 ± 39
60	124 ± 27[a]	114 ± 33	115 ± 42
90	116 ± 27	109 ± 16	114 ± 40
120	115 ± 24	101 ± 20	104 ± 26
150	112 ± 21	105 ± 20	105 ± 15
180	97 ± 20	98 ± 19	100 ± 26
210	92 ± 16	88 ± 18	101 ± 8
240	86 ± 22	89 ± 19	101 ± 16

Table 6.4: Plasma arginine concentrations across each condition, expressed in µM. Values are mean±SD. A one-way repeated measures ANOVA, with Dunnett's adjustment, was used to determine differences from baseline. Differences are indicated by [a] $P<0.05$ vs. 0 min.

Time (min)	BOLUS	PULSE	CONTROL
0	343 ± 122	411 ± 75	390 ± 42
15	498 ± 134	446 ± 55	395 ± 29
30	597 ± 70[a]	463 ± 29	416 ± 47
45	561 ± 85	472 ± 34	420 ± 55
60	528 ± 126	438 ± 20	399 ± 17
90	426 ± 60	406 ± 51	386 ± 9
120	311 ± 82	364 ± 34	352 ± 21
150	283 ± 88	424 ± 34	351 ± 37[a]
180	236 ± 80	373 ± 42	334 ± 40[a]
210	229 ± 80	319 ± 42	327 ± 45[a]
240	210 ± 52	319 ± 34	325 ± 44[a]

Table 6.5: Plasma branched chain amino acid concentrations across each condition, expressed in µM. Values are mean±SD. A one-way repeated measures ANOVA, with Dunnett's adjustment, was used to determine differences from baseline. Differences are indicated by [a] $P<0.05$ vs. 0 min.

6.3.4 Plasma AA, EAA and NEAA

Total AA C_{max} for each condition are presented in **table 6.2** and revealed no significant differences between conditions ($P>0.05$). Plasma total AA kinetics are presented in **table 6.6.** For plasma total AAs, there was a main effect for time ($P<0.001$) and condition ($P<0.001$), and an interaction effect ($P=0.011$). During BOLUS, there was a significant increase in plasma AAs above baseline at 30 and 45 min ($P<0.05$). AUC for total AAs was 29±47µM.240min^{-1}, 4±26µM.240min^{-1} and 29±28µM.240min^{-1} for BOLUS, PULSE and CONTROL, respectively. No significant difference existed for total AAs AUC between conditions ($P=0.81$).

EAA C_{max} for each condition are presented in **table 6.2** and revealed no significant differences between conditions ($P>0.05$). Plasma EAA kinetics are presented in **table 6.7.** For plasma EAAs there was a main effect for time ($P<0.001$) and an interaction effect ($P<0.001$). During BOLUS, there was a marked increase in plasma EAAs at 30 min, when compared to baseline ($P<0.05$). During PULSE, there was a marked decrease in plasma EAAs at 210 and 240 min after ingestion, when compared to baseline ($P<0.05$). AUC for EAAs was 1±29μM.240min^{-1}, -11±15μM.240min^{-1} and -5±12μM.240min^{-1} for BOLUS, PULSE and CONTROL, respectively. No significant difference existed for EAAs AUC between conditions ($P=0.46$).

NEAA C_{max} for each condition are presented in **table 6.2** and revealed no significant differences between conditions ($P>0.05$). Plasma NEAA kinetics are presented in **table 6.8.** For plasma NEAAs there was a main effect for condition ($P<0.03$). However, on further post-hoc analysis, no differences were detected. No significant difference existed between conditions for NEAAs AUC ($P=0.063$).

Time (min)	BOLUS	PULSE	CONTROL
0	2129 ± 319	2259 ± 213	2154 ± 47
15	2412 ± 164	2282 ± 158	2237 ± 109
30	2642 ± 215[a]	2450 ± 148	2316 ± 365
45	2581 ± 309[a]	2480 ± 117	2404 ± 306
60	2558 ± 395	2411 ± 198	2390 ± 224
90	2373 ± 246	2371 ± 153	2434 ± 301
120	2248 ± 155	2264 ± 176	2264 ± 93
150	2240 ± 84	2328 ± 115	2288 ± 115
180	2008 ± 118	2176 ± 195	2218 ± 180
210	1965 ± 117	2045 ± 267	2107 ± 155
240	1823 ± 158	2070 ± 211	2142 ± 207

Table 6.6: Plasma total amino acid concentrations across each condition, expressed in μM. Values are mean±SD. A one-way repeated measures ANOVA, with Dunnett's adjustment, was used to determine differences from baseline. Differences are indicated by [a] $P<0.05$ vs. 0 min.

Time (min)	BOLUS	PULSE	CONTROL
0	832 ± 185	940 ± 122	889 ± 41
15	1033 ± 152	974 ± 85	923 ± 39
30	1160 ± 110[a]	1030 ± 68	955 ± 145
45	1102 ± 149	1034 ± 37	972 ± 125
60	1057 ± 208	982 ± 63	941 ± 69
90	918 ± 112	934 ± 85	928 ± 84
120	788 ± 80	865 ± 83	851 ± 16
150	754 ± 84	918 ± 52	847 ± 54
180	659 ± 80	832 ± 98	812 ± 70
210	644 ± 80	751 ± 109[a]	778 ± 68
240	576 ± 69	755 ± 92[a]	780 ± 80

Table 6.7: Plasma essential amino acid concentrations across each condition, expressed in µM. Values are mean±SD. A one-way repeated measures ANOVA, with Dunnett's adjustment, was used to determine differences from baseline. Differences are indicated by [a] $P<0.05$ vs. 0 min.

Time (min)	BOLUS	PULSE	CONTROL
0	1296 ± 173	1319 ± 121	1265 ± 13
15	1379 ± 105	1309 ± 97	1314 ± 73
30	1481 ± 134	1421 ± 92	1361 ± 219
45	1480 ± 173	1446 ± 82	1432 ± 184
60	1501 ± 209	1429 ± 138	1449 ± 157
90	1455 ± 156	1437 ± 75	1507 ± 218
120	1460 ± 141	1399 ± 97	1413 ± 84
150	1486 ± 100[a]	1410 ± 77	1441 ± 73
180	1349 ± 100	1344 ± 103	1406 ± 116
210	1321 ± 103	1294 ± 162	1329 ± 88
240	1247 ± 118	1315 ± 122	1362 ± 130

Table 6.8: Plasma non-essential amino acid concentrations across each condition, expressed in µM. Values are mean±SD. A one-way repeated measures ANOVA, with Dunnett's adjustment, was used to determine differences from baseline. Differences are indicated by [a] $P<0.05$ vs. 0 min.

6. 4 Discussion

Leucine-enrichment, in which free leucine is added to a suboptimal protein dose, is an emerging nutrition strategy for increasing the anabolic properties of a meal. This is particularly relevant for plant-based meals and food matrices, which are inherently low in EAAs, in particular leucine, the critical EAA for stimulating an anabolic response in skeletal muscle. Despite plasma leucinemia being considered a key modulator in the postprandial MPS response, to the best of our knowledge, no previous studies have investigated the postprandial aminoacidemia after the ingestion of a leucine-enriched plant-based meal. As a follow-up study

from previous work in chapter 5, the present study sought to determine the effect of the co-ingestion of free leucine with a plant-based mixed meal on plasma aminoacidemia.

In the present study, despite the mixed meal containing ~1.2g leucine, plasma leucine concentration did not increase significantly from baseline values in CONTROL condition. The enrichment of a plant-based meal with 3g leucine results in a greater increase in leucinemia when compared to a plant-based meal alone. The enrichment of the meal with 3g leucine resulted in a C_{max} for leucine of 350±44μM, which is 2.3-fold greater than C_{max} (152±22μM) for CONTROL, in which only 3g of maltodextrin was ingested with the mixed meal. Since PULSE condition consisted of 1.5g leucine ingested with a mixed meal at 0 min, and another bolus at 120 min without a meal, the condition was separated into two additional variables; $PULSE^1$ and $PULSE^2$, which represents plasma AA concentrations at 0-120 min, and 150-240 min, respectively. $PULSE^1$ C_{max} was 217±43μM, and $PULSE^1$ C_{max} was 209±48μM. Despite these being 42% and 38% higher than CONTROL C_{max}, these were not statistically significant from CONTROL. While there was a main effect for time in PULSE, and from **figure 6.2B** the data shows observational differences from baseline, and marked difference to CONTROL in **figure 6.2C**, post-hoc analysis revealed no significant differences between time-points. The reason for this non-significance is likely due to a low *n*=5, which is a limitation of the study, as well as a large variation in plasma leucinemia between participants when leucine is ingested with a meal. Participant 1 and 3 in particular did not show a large response in plasma leucinemia to the PULSE condition (See **Figure 6.1**), which is increasing the variability in results, particularly at 45 and 60 min, and another reason for failing to reaching a significance from baseline. During CONTROL, there was a significant main effect for time, in which plasma leucinemia is significantly lower at 150, 180, 210 and 240 min, compared to baseline. These results suggest that there is a marked clearance in leucine in the latter postprandial hours, which is detectable when additional leucine is not ingested at baseline. The leucine AUC for CONTROL was -3±1 μM.240min^{-1}, which supports that there is a clearance of leucine from circulation results in concentrations below that of baseline. BOLUS AUC was 15±5μM.240min^{-1}, which was greater than that of CONTROL AUC. PULSE, $PULSE^1$ and $PULSE^2$ AUC were 8±9, 4±4 and 4±4 μM.240min^{-1}, respectively, however, these did not reach statistical significance compared to CONTROL.

When compared to results from the same *n*=5 from chapter 5, it is clear that there was a smaller peak in plasma leucinemia when 3g of leucine was ingested with a mixed meal (350±44μM), compared to the C_{max} achieved after the ingestion of 3g leucine ingested alone (558±45μM). There was also a smaller AUC for

plasma leucinemia after the ingestion of leucine-enriched meal compared to leucine alone (15±5 vs. 38±8μM.240min$^{-1,}$ respectively). These results suggest that the co-ingestion of a mixed meal with free leucine and has a large impact on the bioavailability of addition leucine, in which less AAs are reaching systemic circulation. An explanation for this is the presence of other macronutrients, fibres and anti-nutritional factors in food matrices and mixed meals act to slow gastric emptying (Rondanelli *et al.*, 2017; Holwerda *et al.*, 2017; Have *et al*., 2007; Duranti, 2006; Hunt 1980). A greater meal volume, as well as solid meals compared to liquid meals, also slows the rate of gastric emptying (Achour, Méance and Briend, 2001; Doran *et al.*, 1998). This decrease in the rate of gastric emptying results in a delayed and slowed absorption of AAs, possibly as a result of increased splanchnic extraction of AAs, in which AAs are taken up by splanchnic tissue, reducing the availability of AAs for release into systemic circulation (Boirie *et al.*, 1997). Therefore, the carbohydrate, fat and fibre component of the mixed meal explain the reason for the delayed and attenuated increase in leucinemia in the present study, compared to results established in chapter 5. Similarly, Dangin *et al.* (2001) compared the plasma kinetics of 30g AA mixture, with 30g casein, both of which contained 3g leucine. After AA ingestion, plasma leucinemia peaked at~400μM, while casein ingestion resulted in a modest increase in plasma leucinemia (~180μM). These marked differences in plasma AA appearance can be attributed to slower digestion of casein, due its coagulation in the stomach, which has been reported elsewhere (Veldhorst *et al.*, 2009).

This steep increase in plasma leucinemia after the ingestion of free-form AAs in comparison to its co-ingestion with other macronutrients is reported elsewhere (Wilkinson *et al.*, 2017; Churchward-Venne *et al.*, 2012). Churchward-Venne *et al.*, (2012) demonstrated that the enrichment of 6.25g whey protein to achieve 3g leucine, resulted in a steep increase in leucinemia (~500μM at 60 min). This was greater than peak leucinemia achieved after the ingestion of 25g whey, which also contained 3g leucine (~500μM at 90 min). Wilkinson *et al.*, (2017) demonstrated that a 6g EAA beverage containing 2.4g leucine, resulted in a greater peak leucinemia, when compared to 40g whey, which contained 4g leucine. Of interest, the aforementioned study by Churchward-Venne *et al.*, (2012) reported no differences in the magnitude of increase in MPS between whey and leucine-enriched beverages, under resting conditions.

Observed differences are also apparent in the plasma leucine kinetics of PULSE in the present study when compared to results in chapter 5. In the present study, PULSE AUC was 8±9 μM.240min^{-1} when 3g was ingested with a mixed meal, however, chapter 5 showed an AUC for 25±10 μM.240min^{-1} for n=5, in which

leucine was ingested alone. Interestingly, there are also marked differences between PULSE^2 in the present study, and PULSE^2 in chapter 5, despite leucine being ingested alone at 120 min in both conditions. PULSE^2 AUC was 4±4 μM.240min^{-1} in the present study, and appears much greater in *n*=5 for chapter 5 (14±5 μM.240min^{-1}). This suggests that the mixed meal ingested at 0 min in the present study, has an impact on the digestion and absorption of the second 1.5g bolus ingested at 120 min, despite the boluses being 2 h apart.

Being cognisant of the purported 'leucine threshold' (the plasma leucine concentration required to increase MPS above postabsorptive values), it appears that postprandial leucine concentrations are strongly compromised by the co-ingestion of a mixed meal. These differences should be considered when comparing the resultant MPS after the ingestion of free AAs vs. whole-protein vs. a mixed meal in various studies, as the present results show that each would produce markedly different postprandial plasma leucinemia. When using leucine-enrichment as a strategy for increasing the anabolic characteristics of a meal, this attenuated increase in leucinemia when free leucine is ingested with a mixed meal is also an important consideration. Indeed, a higher dose of leucine may be required when enriching a mixed meal in order to create the desired leucine plasma kinetics associated with maximal postprandial MPS. Contrary to these points, it must be noted that whole foods produced greater MPS in their whole form in some instances, compared to an isolated form, when matched for leucine content (Van Vliet *et al.*, 2017). Therefore, it is important to consider that plasma leucinemia is not the sole driver of the postprandial anabolic response and other factors such as the accompanying AAs and the co-ingestion of macronutrients and micronutrients may also exhibit a stimulatory effect on MPS. It remains to be determined if the plasma leucine kinetics achieved after a leucine-enriched plant-based meal is conducive to positive changes in postprandial MPS, or offer an advantage in recovery from exercise, when compared to a plant-based meal alone.

6. 5 Conclusion

The ingestion of a plant-based mixed meal enriched with 3g leucine results in a greater increase in plasma leucinemia when compared to the ingestion of a plant-based mixed meal alone, which supports the utility of leucine-enrichment of meals and food matrices inherently low in leucine. However, the presence of other macronutrients and fibre in the mixed meals act to slow gastric emptying thereby delaying the absorption of leucine into the circulation and likely explain the delayed and attenuated increase in leucinemia in the present

study compared to free leucine ingested in chapter 5. This is an important consideration when aiming to increase the anabolic potential of a meal through leucine-enrichment.

Chapter 7

7.0 The effect of bolus verus pulse-fed leucine supplementation on delayed onset muscle soreness and recovery of muscle function after intense exercise

Background: The use of essential amino acids (EAAs) in the post-exercise window is emerging as a potential strategy for minimising the deleterious effect of exercise-induced muscle damage (EIMD). In particular, leucine has been identified as the key EAA in increasing postprandial anabolism and timing of leucine ingestion has been seen to also exhibit an influence on the muscle protein synthetic response. However, the efficacy of leucine, and the timing of its ingestion in recovery from EIMD is less understood.

Objective: The aim of the present study was to investigate the efficacy of 12g leucine, bolus and pulse fed, in the 14 h post-exercise period on recovery of muscle function, markers of muscle damage and perceived soreness following an intense resistance exercise bout.

Design: Thirteen healthy, active males were recruited to participate in this study. Participants performed ten sets of ten repetitions on the leg press at 60% of their estimated 1 repetition maximum. In a double-blind, placebo-controlled, randomised cross-over design of three trials, participants followed a low leucine meal plan for the 14 h post-exercise period, supplemented with three different recovery protocols: BOLUS, 3g leucine every 4 h; PULSE; 1.5g leucine every 2 h; CON, 3g maltodextrin fed every 2 h. Blood samples were drawn before (PRE), immediately after (POST), 24 and 48 h POST and later assessed for plasma creatine kinase (CK) and lactate dehydrogenase (LDH). Perceived soreness and recovery using a visual analogue scale was recorded PRE, POST, 24 and 48 h POST. Participants performed a counter movement jump (CMJ) for measurement of jump height and peak power at PRE, POST, 24 and 48 h POST.

Data Analysis: Two-way (condition x time) repeated measures ANOVAs were performed. When main or interaction effects were indicated, a repeated measures one-way ANOVA was used to determine differences between time-points within each supplement condition using post-hoc pair-wise comparisons to PRE with Dunnet's adjustment for multiple comparisons test. For all analyses, statistical significance was accepted at $P<0.05$.

Results: There was no change in CK or LDH over time. For perceived soreness and recovery, there was a main effect for time, in which soreness was increased above PRE at POST, 24 h and 48 h POST. Perceived recovery score was decreased from PRE at POST and 24 h POST. There was a decrement in jump height and peak power immediately POST. There was no main effect for condition, or time x condition interaction effect for any outcome measures.

Conclusion: 12g leucine supplemented in the 14 h post-exercise window, appears to exhibit no beneficial effect on recovery of muscle function, markers of muscle damage and perceived soreness following an intense resistance exercise bout.

7.1 Introduction

Acute, unaccustomed resistance exercise can produce micro-damage as a result of trauma to muscle fibres and connective tissue (Nosaka, Newton and Sacco, 2002). This exercise-induced muscle damage (EIMD) is often accompanied with dull pain, tenderness and stiffness, termed delayed onset muscle soreness (DOMS) (Cheung, Hume and Maxwell, 2003; Connolly, Sayers and Mchugh, 2003; Clarkson, Nosaka and Braun, 1992), and a decrement in muscle function, which can have a detrimental effect on athletic performance (Pearcey *et al.*, 2015).

A nutritional strategy that is efficacious in minimising the deleterious effect of EIMD may be advantageous in minimising the compromised quality of exercise sessions in the subsequent days following this muscle-damaging exercise bout. The use of protein and/or amino acids (AAs) in the post-exercise window is emerging as one such potential strategy. However, the results have been equivocal, with some research suggesting a beneficial effect on recovery from EIMD (Howatson *et al.*, 2012; Cockburn *et al.*, 2008; Greer *et al.*, 2007; Matsumoto *et al.*, 2007) and other suggesting no beneficial effect on recovery (Fouré *et al.*, 2016; Kephart *et al.*, 2016; Ra *et al.*, 2013; Betts *et al.*, 2009).

There are several studies which support the thesis that a single bolus of exogenous AAs, ingested prior to or immediately after exercise, ameliorates the deterioration of muscle function and rise in blood markers of muscle damage, when compared to a placebo (Shimomura *et al.*, 2010; Etheridge, Philp and Watt, 2008). Of note, there is a tendency for longer, rather than acute, supplementation strategies to confer greater benefits in recovery from EIMD (Waldron *et al.*, 2017; Howatson *et al.*, 2012; Matsumoto *et al.,* 2010; Kraemer *et al.*, 2006). In support, Nosaka, Sacco and Mawatari, (2006) demonstrated that extending AA supplementation several days beyond the immediate post-exercise period confers greater benefits in exercise recovery, such as an attenuated elevation in CK and a decrease in perceived soreness in the days following intense exercise, compared to AAs ingested only at pre and post-exercise.

The exact mechanism which underpins the enhancement in recovery after the ingestion of AAs in the post-exercise period is not understood. It has been erroneously suggested that accelerated recovery is due to the increase in MPS apparent after the ingestion of AAs. However, since the magnitude of myofibril protein turnover required to render a physiologically meaningful benefit on recovery is a lengthy process, this likely

does not explain the benefit in the short (several days) post-exercise period. Nonetheless, of the three branched-chain amino acids (BCAAs), leucine is the most evident contributor to postprandial anabolism and anti-catabolism (Atherton et al., 2010a). However, few studies have investigated the effect of leucine, in isolation, on recovery from intense resistance training. Those few that have investigated the effect of leucine supplementation on accelerating recovery from EIMD have deemed leucine alone to elicit little to no benefit when compared to a placebo (Stock *et al.*, 2010). A plausible explanation for the insignificant differences in recovery markers or perceived soreness between treatment groups is that leucine was only consumed before and after exercise (two doses of ~1.9g), on the day of intense exercise bout. Instead, a recovery protocol aimed at providing several boluses of leucine in the hours following the intense exercise bout may accelerate recovery. To the best of our knowledge, no studies have investigated the effect of leucine supplementation on exercise recovery parameters in this way. The primary aim of the present study is to investigate whether 12g leucine, supplemented in the 14 h post-exercise period, exhibits a benefit on recovery of muscle function, markers of muscle damage and perceived soreness following an intense resistance exercise bout. Furthermore, recent research suggest that the timing of AA ingestion exhibits an effect on postprandial MPS, suggesting that BOLUS ingestion, in which large boluses are ingested ~4 h apart, over the course of the day, results in greater cumulative daily rate of MPS (Mamerow *et al.*, 2014; Areta *et al.*, 2013; Moore *et al.*, 2012). For that reason, the two doses used in chapter 5 and 6, BOLUS and PULSE, and their effect on ameliorating the deleterious effects of intense resistance exercise, when applied using two different temporal strategies, are under investigation as a secondary aim.

7. 2 Methods

7. 2. 1 Study Design and Participants

Participants were recruited through information leaflets and social media posts targeted at university students, seeking out healthy active males, between 18-35 years of age, participating in physical activity ≥3 times per week, for the last 6 months. All participants gave informed written consent before participating in the study. The experimental procedure was approved by University College Dublin (UCD) Research Ethics Committee in accordance with the *Declaration of Helsinki*. This study was a double-blind, placebo-controlled randomised cross-over design, comprising three experimental conditions (as per **figure 7.1**) with each separated by approximately 14 days as described in detail in Section 7.2.7. All preparation and recovery strategies and

experimental procedures were identical for each trial with the exception of the supplementation strategy provided in recovery.

7. 2. 2 Baseline Testing and Familiarisation

Thirteen healthy, active males [age (25.5±5.2y), height (1.82±0.07m), body mass (86.4kg ± 13.1kg) and BMI (26.1±3.4kg/m^2)], participated in baseline testing and a familiarisation trial, one week prior to the first experimental trial.

Baseline tests included body mass (to the nearest 0.2 kg) using a calibrated digital scales (SECA, Germany) and height (to the nearest 0.01 m) using a wall-mounted stadiometer (Holtain, UK). Participants were familiarised with a visual analogue scale (VAS) as a method of assessing soreness and perceived recovery status (**Appendix 1**). Counter-movement jump (CMJ) onto a floor-mounted force plate (AMTI, Waterstown, USA) was performed to determine maximum jump height and peak power output. Following a warm-up, participants performed three CMJs at a self-selected depth, separated by two-minute rest intervals. Participants were familiarized with the leg press machine and proper lifting technique. Following a warm-up, the load was increased in single increments until a 3 repetition max (RM) was achieved. A two-minute rest period was allowed between each attempt. The maximum weight lifted for 3 reps was subsequently used to estimate their 1RM. Using 60% of this estimated 1RM, 3 sets of 10 repetitions were performed with a two-minute rest period between sets.

7. 2. 3 Diet and Activity Before Experimental Trial

Participants recorded their food intake for 24 h prior to the start of the first experimental trial. This food record was photocopied and sent to participants in advance of subsequent trials. Participants were instructed to replicate this diet 24 h before the start of the second and third trial. Participants were instructed to continue their habitual training for the duration of their involvement in the study but to refrain from any type of physical exercise for 24 hours before each trial.

7. 2. 4 Pre-Exercise Assessment

On day 0, after an overnight fast, participants arrived at the laboratory. A resting blood sample (~4ml) was taken from the antecubital vein. Blood was drawn into a vacutainer containing lithium heparin (BD Vacutainers, Heparin Tubes), inverted eight to ten times and placed on ice. The blood was centrifuged at 4000 g for 10 min at 4°C and the upper layer of plasma was transferred into three separate 1.5ml tubes and stored at -80°C until further analysis. Participants were asked to rate their perceived muscle soreness on 1-10 Likert pain VAS, with 0 being 'no pain at all' and 10 being 'the worst pain imaginable'. Participants also rated their perceived recovery status using a 1-10 Likert VAS, with 0 being 'very poorly recovered/extremely tired' and 10

being very well recovered/highly energetic' (See **appendix 1**). To minimise the possibility of anchoring bias, participants were not shown any of their previous ratings. Following a four-minute general warm-up on a cycle ergometer and a specific warm-up for the CMJ, participants completed three maximal effort CMJ, with two-minute resting intervals between each jump.

7. 2. 5 Muscle-Damaging Protocol

The participants completed an individualized, incremental warm-up on the leg press based on their previously estimated 1RM. This protocol involved the completion of ten sets of ten (10x10) on the leg press at 60% of the individual's estimated 1RM at a 4111 tempo, with each set separated by a two-minute rest interval. This protocol was based on the method used by Macdonald *et al.*, (2014), which was shown to cause a deterioration in jump height performance, maximal voluntary contraction and increase in perceived muscle soreness in the 24-48 h post-exercise period. The only deviation to their protocol was that we used the leg press exercise in place of barbell back squat exercise. To standardise the range of motion of the movement, a goniometer was used to mark the point on the leg press machine at which the participants produced a knee flexion angle of 60°. A metre stick was placed at this point for the duration of the session to provide the subject with a visual cue for the depth of their movement (See **appendix 2** for diagram). If the participants needed to drop the resistance in order to complete the total repetitions and sets, this was recorded and repeated for the subsequent trials.

7. 2. 6 Post-Exercise Assessment

Within 10-15 minutes of completing the exercise protocol (POST), another blood sample was taken. CMJs were repeated, in which three maximal effort CMJs, with two-minute resting intervals between each jump, were performed. Participants were asked to rate their perceived muscle soreness and perceived recovery status as per **section 7.2.4**.

The participant's first meal, consisting of a hemp protein shake and a pre-prepared carbohydrate and fat meal (See **Section 7.2.8**), was consumed before leaving the laboratory, along with two bottles of the appropriate supplement condition. A bag containing the participants' meal plan and supplements for the subsequent 14 h was provided before departure, along with instructions for the timing of meals and supplement protocol.

At 24 h POST and 48 h POST participants arrived to the laboratory after an overnight fast. A blood sample was taken prior to completion of the same warm-up and CMJ as Day 0. Soreness and perceived recovery were also recorded as described above.

7. 2. 7 Supplementation Protocol

Each participant participated in three experimental trials separated by approximately 14 days. Each participant was randomly assigned to one of three groups in a cross-over design;

- Bolus feeding (BOLUS), 3g free leucine every 4 hours for 14 hours
- Pulse feeding (PULSE), 1.5g free leucine fed every 2 hours for 14 hours
- Control group (CON), 3g maltodextrin fed every 2 hours for 14 hours

Each supplement was provided as powder providing 1.5g of L-leucine (Sigma-Aldrich, Pharmagrade) or maltodextrin (Bulk Powders, UK) in an opaque 150ml bottle, to which plain water was added and the contents shook prior to ingestion. The flavour of the leucine and maltodextrin was masked with sucralose (0.04g per 1.5g leucine/1.5g maltodextrin) to maintain the double-blind design of the study. Regardless of which condition the participant partook in, all participants consumed two bottles every two hours to maintain the double-blind design, as per **figure 7.1**. During the BOLUS condition, participants consumed 3g of leucine every 4 hours, and 3g of maltodextrin on the alternating four h period. During the PULSE condition, participants consumed 1.5g of leucine and 1.5g of maltodextrin every two hours. During the CON condition, participants consumed 3g of maltodextrin every two hours. Therefore, BOLUS and PULSE were isonitrogenous, and all three conditions were isocaloric. Participants were provided with pre-packaged bottles of supplements and instructed to consume each supplement at these specific times for the subsequent 14 h after completing the 10x10 leg press exercise protocol (See **appendix 3** for participant instructions).

7. 2. 8 Diet and Exercise Control

The participants' diets were controlled and standardised on Day 0 of the experimental trial i.e. during the 14 h recovery period, in which they followed a low leucine, but protein-rich meal plan for 14 h. Once the 10 x 10 exercise protocol was completed, participants were provided their first meal of the plan in the laboratory. The remaining three meals were consumed at 4 h intervals throughout the day. Each meal plan was designed to provide the following – Protein 1.2g/kg (13% energy intake (EI)); Carbohydrate 5g/kg (53% EI); and Fat 1.4g/kg (34% EI); and used low leucine plant-based foods such as hemp protein, cereal bars, nuts, isotonic sports drink, rice and oats (See **appendix 4** for sample meal plan). Participants were asked not to partake in any form of organised exercise, foam rolling or mobilisation outside of the experimental trial for Day 0 and Day 1 and were permitted to return to habitual exercise following the 48 h follow-up visit on Day 2.

7. 2. 9 Data Analysis

The concentration of creatine kinase (CK) and lactate dehydrogenase (LDH) in each blood samples was analysed as per **section 3.4.** We were unable to take blood samples from one participant, and another participant had CK values that were out of range for CK. CK and LDH are therefore representative of a $n=11$ and 12, respectively. Jump height (by take-off velocity) and peak power was calculated from CMJ force plate data, as per Reiser, Rocheford and Armstrong (2006).

7. 2. 10 Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics Version 23). In general, the distribution of the data approximated normality, or was transformed as appropriate to approximate normality. Two-way (condition x time) repeated measures ANOVAs was performed to determine changes, if any, in response to the exercise bout over time, and differences, if any, between supplement conditions at these time-points. When main or interaction effects were indicated, a repeated measures one-way ANOVA was used to determine differences between time-points within each supplement condition, using post-hoc pair-wise comparisons to PRE with Dunnet's adjustment for multiple comparisons test. For all analyses, statistical significance was accepted at $P<0.05$. Independent of traditional null hypothesis statistical testing, standardised differences in the mean were used to assess magnitudes of effects for differences at 24 h and 48 h POST, compared to PRE. These effect sizes were calculated using Cohen's *d* and interpreted using thresholds of 0.2, 0.5, and 0.8 for small, moderate, and large, respectively.

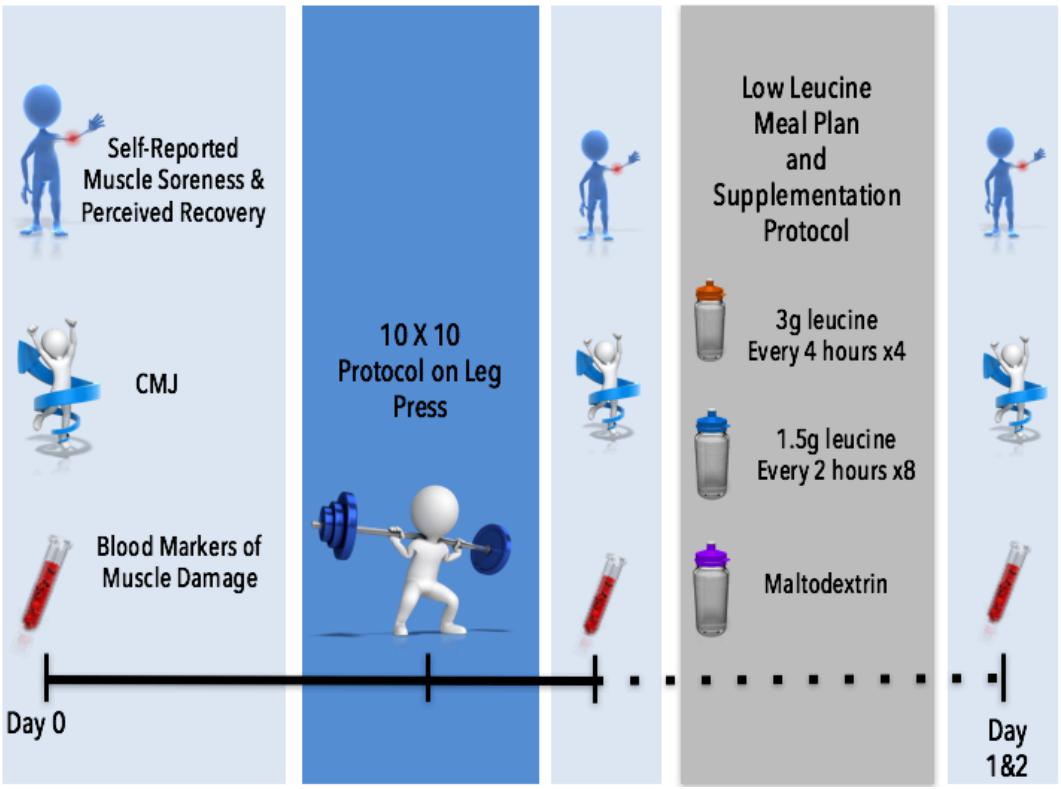


Figure 7.1: Overview of study design.

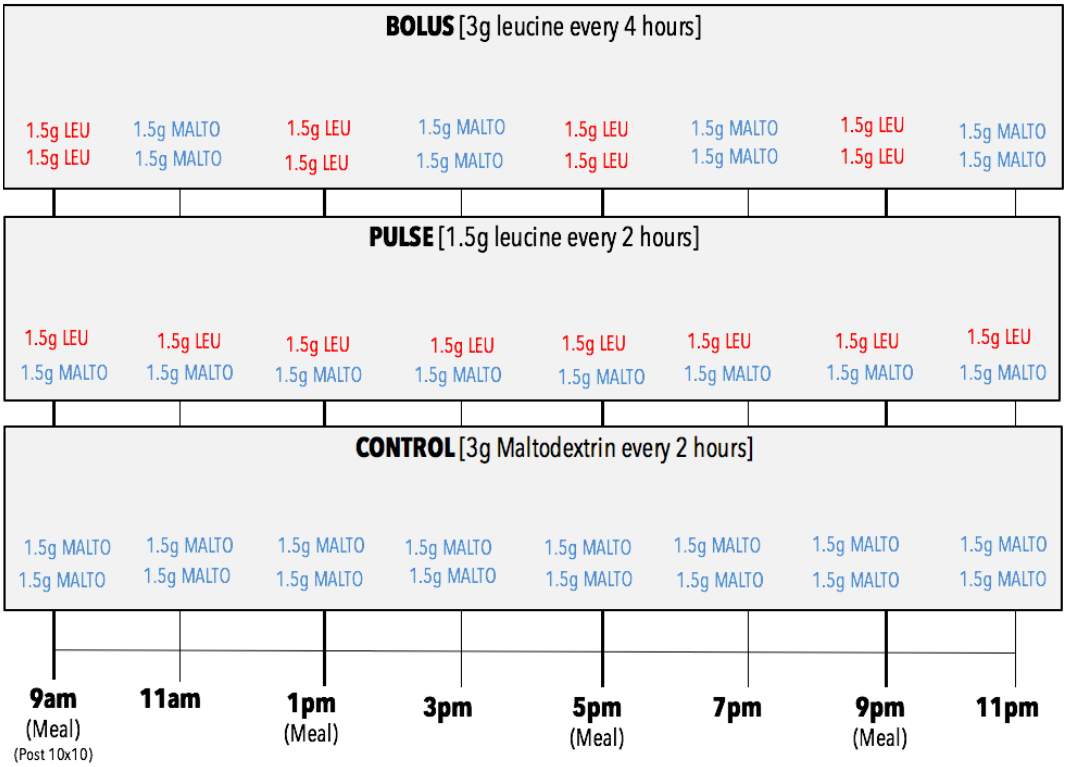


Figure 7.2: Overview of the supplement protocol in the 14-hour post-exercise period. LEU, leucine; MALTO, maltodextrin.

7. 3 Results

7.3.1 Creatine Kinase and Lactate Dehydrogenase

Figure 7.3A shows PRE CK values between each trial (u/L). **Figure 7.4A** represents percentage change from PRE for CK, at each time-point for each supplement condition (expressed as mean and SD). For CK, the two-way ANOVA revealed no main effect for condition (P=0.898), but a main effect for time (P=0.02). However, on further analysis, the post-hoc analysis revealed there was no difference in CK values between time-points (P>0.05 for all). Cohen's d for differences in CK at POST, 24 h and 48 h compared to PRE were 1.66 (large), 0.85 (large) and 0.57 (medium) for BOLUS respectively; 1.73 (large), 0.93 (large) and 0.39 (small), for PULSE respectively; and 1.90 (small), 1.00 (small) and 0.61 (medium), for CONTROL respectively.

Figure 7.3B shows PRE LDH values between each trial (u/L). **Figure 7.3B** represents percentage change from PRE for LDH, at each time-point for each supplement condition (expressed as mean and SD). For LDH, the two-way ANOVA revealed no main effect supplement condition (P=0.468), or for time-point (P=0.163). Cohen's d for differences at POST, 24 h and 48 h compared to PRE were 1.35 (large), 0.78 (medium) and 0.67 (medium) for BOLUS respectively; 0.23 (small), -0.27 (small) and 0.08 (trivial), for PULSE respectively; and 0.07 (trivial), 0.10 (trivial) and -0.11 (trivial), for CONTROL respectively.

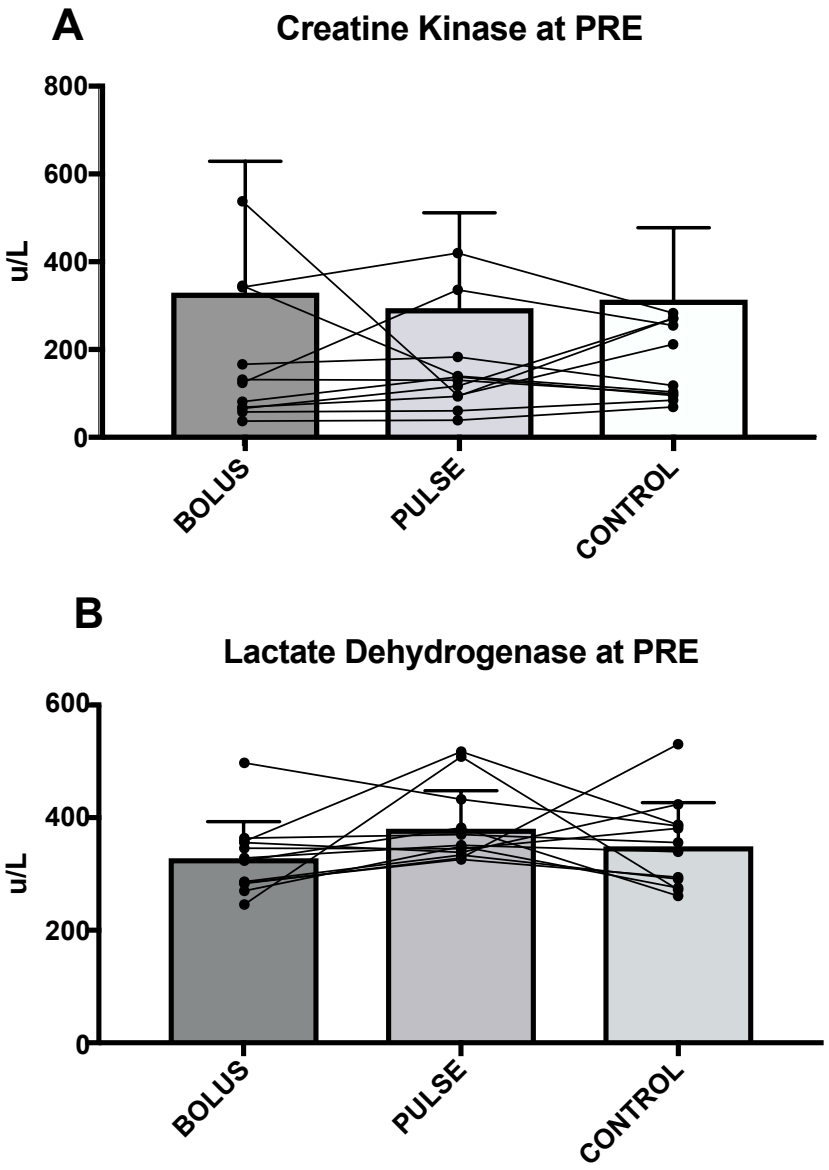


Figure 7.3: Creatine kinase values (A) and lactate dehydrogenase (B) at PRE between each trial. Plotted as mean±SD (bars) and individual data points (lines and symbols). Expressed as units per per litre (u/L). *n*=11 for creatine kinase; *n*=12 for lactate dehydrogenase.

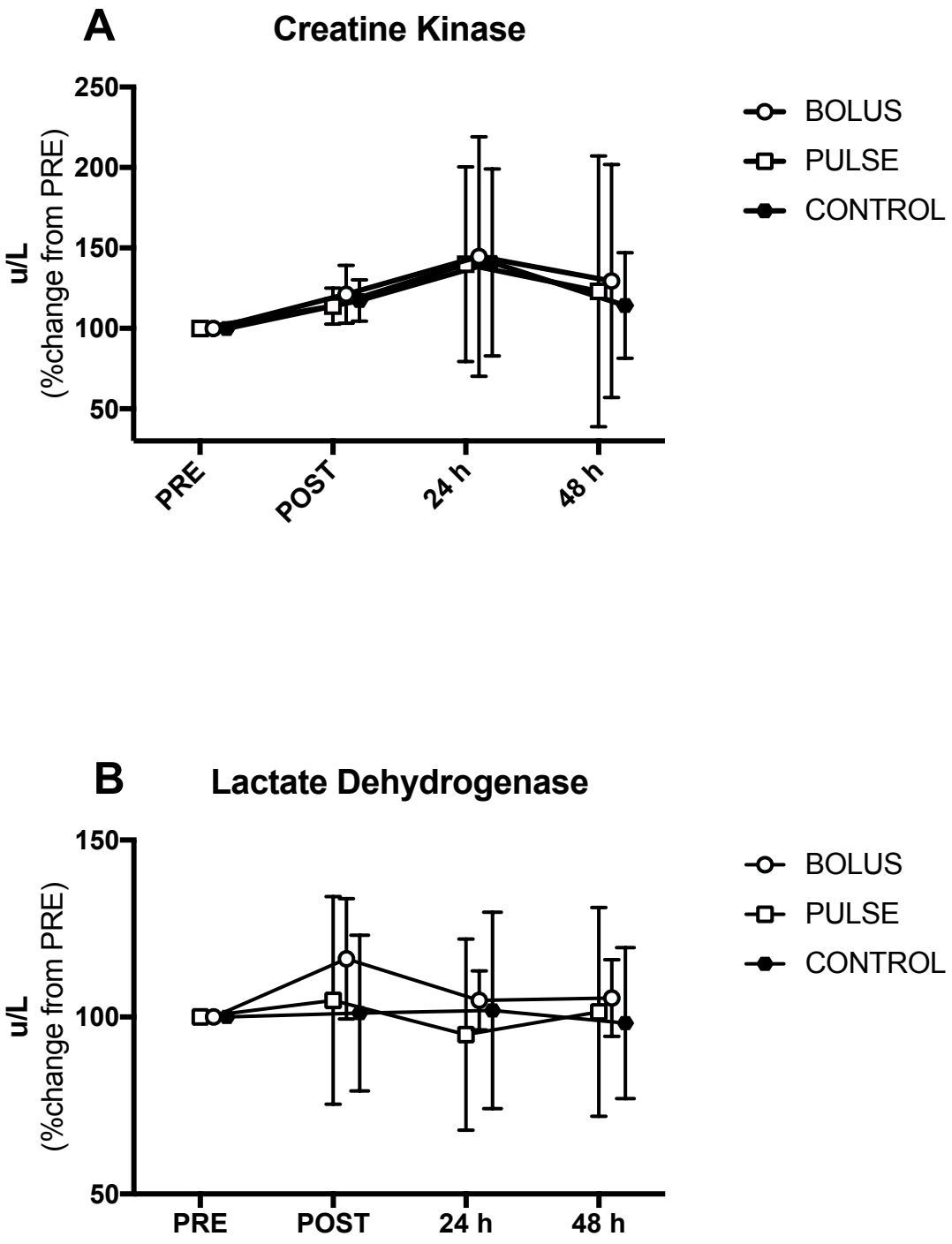


Figure 7.4: Percentage change in creatine kinase (A) and lactate dehydrogenase (B) expressed in units per litre (u/L), across each supplement condition, pre-exercise, immediately post-exercise, 24 hours and 48 hours post-exercise. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time-point). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points. Difference existing from PRE is indicated by $^{*}P<0.05$.

7.3.2 Perceived Muscle Soreness and Recovery

Figure 7.5A represents perceived muscle soreness, at each time-point for each supplement condition (expressed as mean and SD). For perceived muscle soreness, the two-way ANOVA revealed a main effect for time ($P<0.001$), but no main effect for condition ($P=0.678$). Perceived soreness experienced an increase in the immediate post-exercise period for each groups. Mean values increased from 1.2±1.0, 0.9±0.8 and 1.0±1.1 at PRE, to 3.6±1.9, 3.8±2.2 and 3.2±2.1 at POST in BOLUS, PULSE and CONTROL, respectively ($P<0.05$ for all, Cohen's d = 2.08 (large), 2.52 (large) and 1.86 (large), respectively). At 24 h POST, perceived soreness increased to 3.9±1.9, 4.2±1.7 and 4.6±2.5 in BOLUS, PULSE and CONTROL condition, ($P<0.05$ for all, Cohen's d = 2.08 (large), 2.52 (large) and1.86 (large) respectively). At 48 h POST, perceived soreness remained elevated above PRE (3.5±2.2, 3.6±2.1 and 4.5±2.8 for BOLUS, PULSE and CONTROL, respectively) ($P<0.05$ for all, Cohen's d = 1.36 (large), 1.74 (large) and 1.69 (large) respectively).

Figure 7.5B represents perceived recovery at each time-point for each supplement condition (expressed as mean and SD). For perceived recovery, the two-way ANOVA revealed a main effect for time ($P<0.001$), but no main effect for condition ($P=0.757$). Perceived recovery decreased in the immediate post-exercise period for each group. Mean values decrease from 7.6±1.6, 8.1±1 and 7.9±1.1 at PRE, to 4.4±1.8, 4.1±2.1 and 3.9±1.9 at POST, in BOLUS, PULSE and CONTROL, respectively ($P<0.05$ for all, Cohen's d = -1.28 (large), -0.93 (large) and -0.85 (large), respectively). At 24 h POST, perceived recovery decreased to 5.1±2.3, 5.1±2.4 and 5.4±2.7 for BOLUS, PULSE and CONTROL, respectively ($P<0.05$ for all, Cohen's d = -1.28 (large), -1.64 (large) and -1.22 (large), respectively). At 48 h POST, perceived recovery remained unchanged from PRE for all conditions 5.8±2.3, 6.4±2.4 and 6.3±2.5 for BOLUS, PULSE and CONTROL, respectively ($P>0.2$ for all, Cohen's d =-0.86 (large), -0.93 (large) and -0.85 (large), respectively).

7.3.3 Muscle Function

Figure 7.6A represents jump height in centimetres, at each time-point for each supplement condition (expressed as mean and SD). For jump height, the two-way ANOVA revealed a main effect for time ($P<0.001$), but no main effect for condition ($P=0.583$). The post-hoc analysis revealed that jump height was lower immediately POST, in which it decreased from 35±6, 35±5 and 35±6 at PRE to 31±4, 30±5 and 31±5 for BOLUS, PULSE and CONTROL ($P<0.05$ for all, Cohen's d = -0.83 (large), -0.93 (large) and -0.67 (medium), respectively). No further decrement in jump height was apparent at 24 h POST ($P>0.99$ for all). Jump height for BOLUS, PULSE and CONTROL at 24 h POST was 34±6, 34±7 and 35±5, respectively. Cohen's d for jump

height at 24 h compared to PRE were -0.16 (trivial), -0.92 (large) and -0.02 (trivial), respectively. No further decrement in jump height was apparent 48 h POST (P>0.99 for all). Jump height for BOLUS, PULSE and CONTROL at 48 h POST was 35±5, 34±6 and 35±7, respectively. Cohen's d for jump height at 48 h compared to PRE were -0.12 (trivial), -0.08 (trivial) and -0.01 (trivial), respectively.

Figure 7.6B represents peak power in Watts, at each time-point for each supplement condition (expressed as mean and SD). For peak power, the two-way ANOVA revealed a main effect for time (P<0.001), but no main effect for condition (P=0.352). The post-hoc analysis revealed that peak power was lower immediately POST, in which it decreased from 4244±476, 4306±671 and 4280±684 at PRE to 3971±526, 3934±561 and 4017±540 for BOLUS, PULSE and CONTROL (P<0.05 for all, Cohen's d = -0.57 (medium), 0.60 (medium) and -0.43 (small) respectively). No further decrement in peak power was apparent at 24 h POST (P>0.99 for all). Jump height for BOLUS, PULSE and CONTROL at 24 h POST was 4223±662, 4209±656 and 4292±590, respectively. Cohen's d for peak power at 24 h compared to PRE were -0.03 (trivial), -0.15 (trivial) and -0.02 (trivial) respectively. No further decrement in peak power was apparent 48 h POST (P>0.99 for all). Peak power for BOLUS, PULSE and CONTROL at 48 h POST was 4247±709, 4196±633 and 4316±720, respectively. Cohen's d for peak power at 48 h compared to PRE were 0.01 (trivial), 0.01 (trivial) and -0.17 (trivial), respectively.

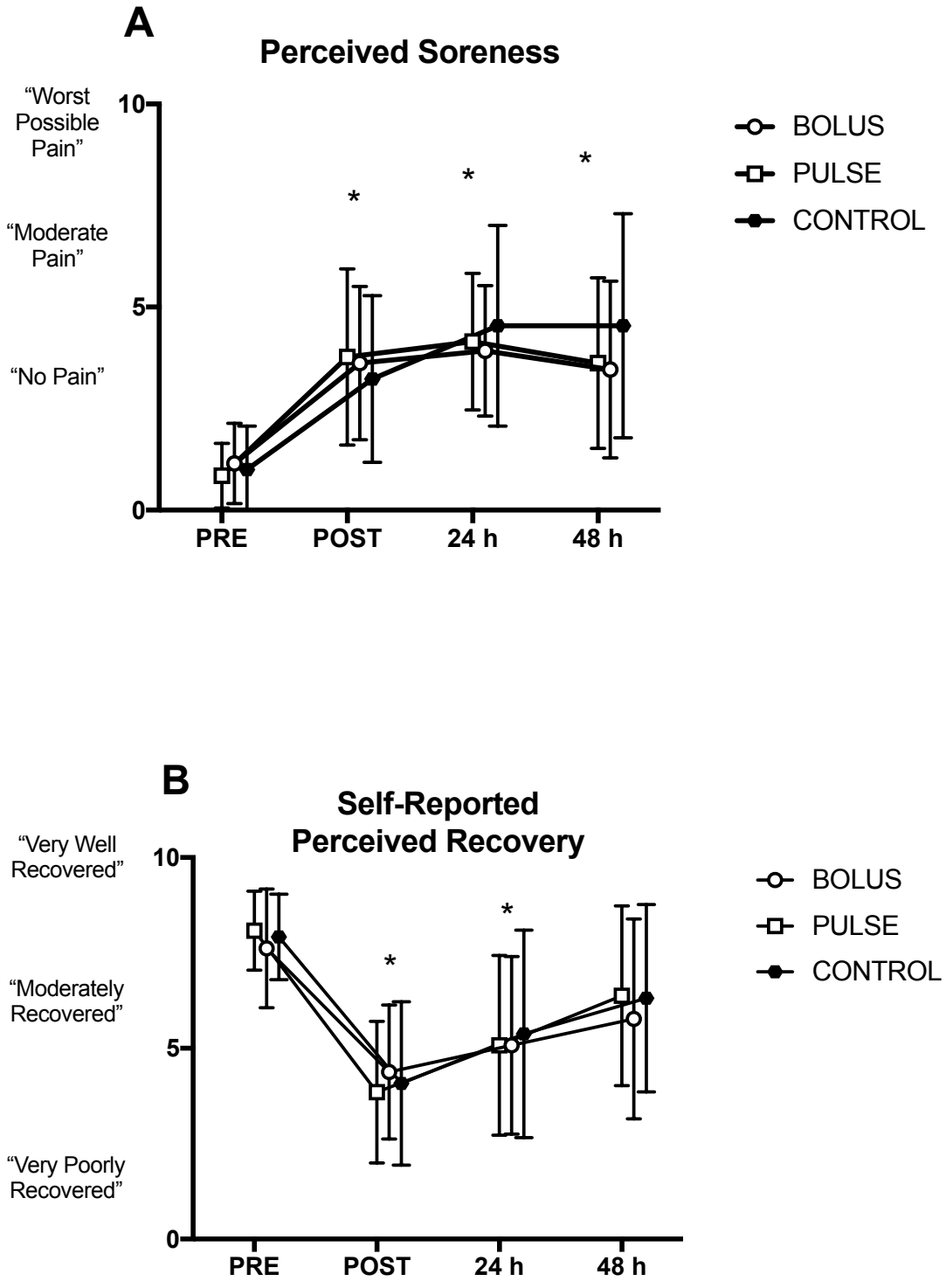


Figure 7.5: Perceived soreness (A) and perceived recovery (B) using a 1-10 Likert visual analogous scale, across each supplement condition, pre-exercise, immediately post-exercise, 24 hours and 48 hours post-exercise. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time-point). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points. Difference existing from PRE is indicated by $^{*}P<0.05$.

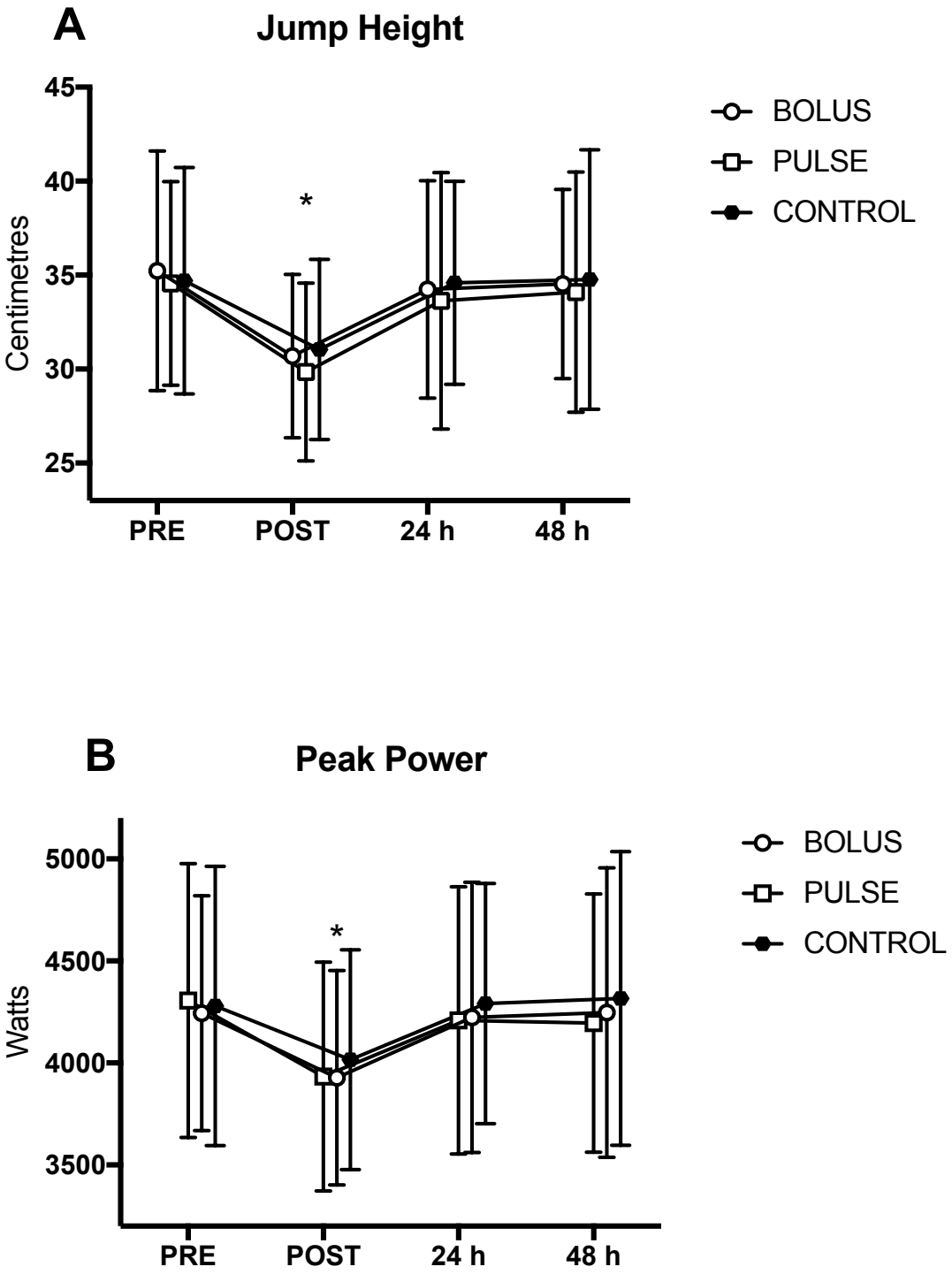


Figure 7.6: Jump height, expressed in centimetres (A) and peak power, expressed in Watts (B), across each supplement condition, pre-exercise, immediately post-exercise, 24 hours and 48 hours post-exercise. Values are Mean±SD. A two-way repeated measures ANOVA was performed (Condition*Time-point). When $P<0.05$, post-hoc one-way repeated measures ANOVA were used to determine where differences existed between time-points. Difference existing from PRE is indicated by $^{*}P<0.05$.

7.4 Discussion

The aim of the current study was to investigate if 12g leucine, supplemented in the 14 h post-exercise period in either BOLUS or PULSE form, would ameliorate changes in blood markers of muscle damage, muscle function and perceived soreness and recovery, at 24 h and 48 h after an intense resistance exercise bout. Our results conclude that leucine supplementation of a protein-rich meal plan offers no enhancement in recovery from intense resistance exercise in the selected markers under investigation.

Current literature suggests that the supplementation of AAs in the post-exercise period minimises the deleterious effects of intense resistance exercise. Greer *et al.*, (2007) demonstrated that 2.5g BCAA pre-exercise and 60 min post-exercise, resulted in attenuated increase in CK, LDH and soreness following 90 min cycle at 55% $V0_{2peak}$ in untrained males. Waldron *et al.*, (2017) demonstrated that ~8g BCAAs was superior to dextrose placebo in attenuating the decrease in CMJ performance and perceived soreness following an intense resistance exercise bout. Howatson *et al.* (2012) also concluded that 10g BCAAs x2 per day in the 4 days post-exercise reduced the rise in CK concentrations and perceived soreness, compared to placebo, following 100 drop jumps. Results from the present trial are in contrast with the aforementioned findings, in which no difference were apparent in CK, LDH, CMJ performance or perceived soreness, between leucine supplement groups, compared to a maltodextrin control. This is agreement with finding by Betts *et al.*, (2009), in which 21g protein was added to a carbohydrate drink, and fed in eight boluses every 30 min following 90 min intermittent shuttle runs. Authors found no difference in CK, LDH or soreness when protein was added to a carbohydrate drink. Similarly, Ra *et al.*, (2013) concluded that BCAA supplementation three times a day, for 2 weeks prior and 3 days after intense resistance exercise had no added benefits on CK, LDH or perceived soreness, compared to control. The present findings suggest that 12g leucine ingested over the 14 hour post-exercise period exhibits no further enhancement in recovery, when compared to a placebo, following intense resistance exercise. Importantly, we prescribed a meal plan for the day of the experimental protocol, which provided 1.2g/kg BM protein, with carbohydrate, fat and energy intake matched to the individuals BM and predicted energy requirements based on activity-adjusted metabolic rate. Of note, the dietary control of the trial was a strength of the study design, particularly as previous studies investigating the benefit of leucine supplementation on recovery from intense exercise allowed participant to follow their habitual diet (Kirby *et al.,* 2012; Stock *et al.,* 2010). The ad libitum dietary intake resulted in an intake of ~1g and ~1.4-1.9g/kg body mass protein respectively (Kirby *et al.,* 2012; Stock *et al.,* 2010). The higher intake of protein and particularly the lack of control over types of protein (i.e. the allowance of animal-based proteins), would result in a high

leucine intake post-exercise, and therefore potentially negate any benefit of additional supplementary leucine. In the present study it is possible that the meal plan provided sufficient nutrients to recover from intense exercise, and the additional leucine offered no further advantage on recovery. However, another explanation for the lack of differences, is that the markers of muscle damage and performance measures were not sensitive to the intense exercise bout, and therefore, any differences in measures as a result of the treatment group was unable to be detected.

In relation to the latter point, our results suggest that 1) there is large inter-subject and intra-subject variability (See **figure 7.3**) in CK values following exercise; and 2) plasma CK is a poor marker of muscle damage in the conditions under study herein, both of which have been reported elsewhere (Waldron *et al.*, 2017; Nosaka, Newton and Sacco, 2002; Malm *et al.*, 2000). Moreover, the large variability at PRE due to our limited control of the participants' own training outside the intervention may have contributed to variability in CK results in response to the intervention. Five participants showed particularly large variability in PRE CK values for the three trials (>380u/L between trials), which suggests that they were more/less rested for some exercise sessions than others (See **figure 7.3**). This highlights that asking participants to take 24 h rest from exercise before each exercise session was not sufficient time to facilitate full recovery from their own training, and emphasises the limitation of recruiting participants from different training backgrounds who are not participating in the same training programme (i.e. as part of the same sport/team). This is a plausible explanation for the large variation in CK concentrations in response to the exercise sessions (with some CK concentrations showing a decline 24 h and 48 h POST), as completing the trial with already elevated CK concentrations pre-intervention may have masked the exercise-induced increase in CK. In support, CK may recede to baseline values only 7 to 14 days following exercise cessation (Lieber and Friden, 2002), which would suggest that rest beyond the 24 h window before commencing the exercise trial is warranted. LDH followed a similar pattern to CK, in that there was a high degree of inter-subject and intra-subject variability in results. There was no apparent difference in LDH for at any time-point or between conditions. This suggests that LDH is a poor marker of muscle damage or the outside training of participants interfered with LDH, as in the case of CK.

Another explanation that should not be discounted is that the exercise protocol was unable to elicit sufficient muscle damage that could be detected in the blood markers chosen. The protocol was adapted from Macdonald *et al.*, (2014) in which participants performed 10x10 in the back squat movement at 60% 1RM. This protocol resulted in deterioration in jump height performance, knee extension MVC and increase in perceived

muscle soreness in the 24-48 h post-exercise period. Furthermore, difference between treatment groups (presence or absence of foam rolling) for these parameters was also detected. While the protocol employed was similar to Macdonald *et al.*, (2014), the leg press was used in place of a squat movement, as range of motion was easier to control with a leg press movement. The perceived soreness and recovery results suggest however that our modified exercise protocol was indeed successful in eliciting some degree of muscle damage. Perceived soreness increased at 24 h POST (240%-391% increase across conditions compared to PRE) and remained elevated at 48 h POST (200%-354% increase across conditions compared to PRE). This is consistent with finding by others assessing perceived soreness using a pain VAS at 24 and 48 h POST (Philpott *et al.*, 2018; Macdonald *et al.*, 2014; Cockburn *et al.*, 2012; Howatson *et al.*, 2012). Perceived recovery followed a similar trend, in which feelings of recovery decreased immediately post-exercise. A decrement in feeling of recovery and readiness to perform was apparent at 24 h; POST (33%-37% decrease across conditions) and remained apparent at 48 h POST (21%-24% decrease across conditions). It is partly for this reason that we speculate above that CK and LDH are not appropriate biomarkers of muscle damage in this study. In any case, there was no difference in perceived soreness or recovery between placebo and both leucine conditions, suggesting that supplementing with leucine in the 14 h post-exercise does not confer a benefit to alleviating sensations of DOMS. Similarly, Cockburn *et al.*, (2012) demonstrated that the ingestion of 500ml and 1000ml milk post-exercise had no effect on change of perceived soreness, compared to when water was ingested following 6 sets of 10 repetitions knee flexion exercise. However, a positive attenuation of CK and recovery of knee flexion peak torque was apparent for the 1000ml milk group in the days following exercise. Etheridge, Philp and Watt, (2008) also demonstrated that the ingestion of 100g milk protein concentrate post-exercise had no effect on change of perceived soreness, compared to a placebo, following 30 min downhill running. However, a positive influence on recovery of MVC during an isometric knee extension, and 5 second peak power output (PPO) on a cycle ergometer, was apparent for the milk group. These results suggest that perceived soreness may be an insensitive measure of recovery, as the latter studies demonstrate that the treatment condition exhibited a positive effect on blood markers and performance measures, with no different for soreness measures.

The use of a closed chain movement such as the leg press, as opposed to the open chain movement of the back squat, may not be appropriate for the current study to create sufficient muscle damage to elicit a robust decline in muscle function. While the premise for using large muscle groups to create muscle damage was to make the protocol specific to a real world setting in which complex movements are often performed in training (e.g.

deadlifts, squats, and bench press), an isolated movement (such as a leg extension or bicep curl) may have been more appropriate. A MVC assessment, in which muscle function in the movement used in the damaging protocol was assessed, may have given more of an insight into the extent of performance decrement in the exact muscle groups trained in the muscle-damaging protocol. While performance in CMJ showed a decrement immediately post-exercise, this decrement was not apparent 24 and 48 h POST for any condition. This in contrast with finding by Waldron *et al.*, (2017) who found a decrement in CMJ performance at 24 h POST intense resistance training. Etheridge, Philp and Watt, (2008) also found a decrease in PPO at 24 h POST. However, in this study three maximal 5 second sprints on a cycle ergometer were used as an assessment of peak power. CMJ may potentially be an inappropriate measure of fatigue and recovery, and a MVC may have provided a better insight into fatigue and recovery status. Indeed, protocols in which an isolated movement was used to elicit muscle damage showed a marked decrease in MVC in the same movement 24 h and 48 h POST (Philpott *et al.*, 2018; Howatson *et al.*, 2012; Etheridge, Philp and Watt, 2008).

The present study used a cross-over design, which while a strength in most study designs, is a potential caveat in the present study. The reason for using a cross-over design was that participants acted as their own control, which is particularly beneficial since participants were from different training backgrounds, a factor that may influence the severity of their response to the exercise bout. For instance, those individuals routinely exposed to a similar training stimulus to the movement and/or repetition range being used in the muscle-damaging protocol will have a blunted response in muscle damage to the protocol (McHugh, 2003). However, since we were attempting to investigate EIMD, a repeated bout effect may have interfered with the results, whereby the first trial created the most amount of muscle damage resulting in a substantial adaptation and rendering an attenuated response to subsequent trials (Howatson and Van Someren, 2008). To minimise the interference of the repeated bout effect with our results, we therefore adopted a randomised trial order for each participant. This method has been used elsewhere, however, exercise trials were separated by a 12-week interval to minimise the interference of a repeated bout effect (Shimomura *et al.*, 2006). It remains a speculation as to what effect the cross-over design may have had on our results.

In summary, it was difficult to interpret the true effect of leucine supplementation on recovery from EIMD due to multiple factors interfering with our outcome variables. Recommendations for future research in assessing the impact of a supplement strategy on ameliorating the negative implications of DOMS are as follows; Recruit participants from a team who are therefore partaking in a similar training program, thereby minimising the interference of different training programs with the response to the intense resistance exercise bout. Ask

participants to rest for 72 h before each exercise bout in order to ensure that they are rested from their own training and thereby establish a true baseline for 'resting' CK, or other biomarker, concentrations. If participants are recruited from a team in this way, opt for a parallel design, to minimise the interference of the repeated bout effect. Use an isolated movement such as a knee extension to elicit muscle damage, and use a MVC in the knee extension to assess muscle function at 24 h and 48 h POST.

7.5 Conclusion

Our results conclude that leucine supplementation offers no enhancement in recovery from EIMD.

Chapter 8

8.0 The Effect of a Nutrition Intervention Targeting Leucine-Rich Meals on Changes in Calorie, Macronutrient and Micronutrient Intake, and Protein Distribution in Older Adults

Background: Inadequate dietary protein intake is a key contributor to the decline in skeletal muscle mass and function with advancing age. Protein, and in particular the amino acid leucine, acts as an anabolic stimulus and stimulates skeletal muscle protein synthesis (MPS), and has therefore been emphasised in nutrition strategies targeting sarcopenia.

Objective: The aim of the present study was to determine if instruction and support for a high protein diet, prescribing the equivalent of 3g of leucine per meal, eaten at breakfast, lunch and dinner, is efficacious in augmenting protein intake and protein distribution in older adults.

Design: Participants aged ≥65 were recruited to participate in this study. Participants (n=56) were randomly assigned to one of the following three groups for 12 weeks, as part of a larger study that also included an exercise training intervention: nutrition and exercise group (NUTR+EX); non-exercise nutrition only group (NUTR) and exercise only group (EX). NUTR+EX and NUTR were provided with instructions and weekly support to follow a high protein diet that was rich in leucine. Meals equating to 3g of leucine were translated to user-friendly portion sizes and support was provided through weekly emails and fortnightly phone calls. EX were asked not to change their diet for the duration of the study. All participants completed a 3-day portion estimate food diary at week 0 (PRE), week 6 (MID) and week 12 (POST).

Data Analysis: Food diaries were analysed using Nutritics Nutrition Analysis Software. Changes in calorie, macronutrient and micronutrient intake, and protein distribution over time and between groups was evaluated using a mixed ANOVA. Post-hoc analysis was performed using one-way ANOVA, repeated measured ANOVA, and pairwise comparisons with Bonferroni's adjustment.

Results: At MID, daily protein intake increased in NUTR+EX (65.8± 3.8 to 117.8±23.7g, $P<0.01$) and NUTR (73.4±25.7 to 119.9±30.5g, $P<0.01$). At MID, Relative protein intake increased in NUTR+EX (0.90±0.20 vs. 1.57±0.49g/kg body mass, $P<0.05$) and NUTR (0.99±0.34 to 1.43±0.39 g/kg body mass, $P<0.05$). At MID and POST, average protein intakes for breakfast, lunch and dinner were ≥30g or ≥0.4g/kg protein, in both nutrition intervention groups. There was no difference between values at MID and POST in both nutrition intervention groups, whereas there was no change in any dietary parameter in EX from PRE to MID and POST.

Conclusion: The nutrition intervention was efficacious in increasing protein intake and achieving a more even pattern of daily protein distribution. At MID and POST in both nutrition intervention groups, daily and per meal protein intake reflected an intake that is considered optimal for maximising MPS in older adults. Prescribing and providing support for a whole food-based diet, specifically targeting 3g leucine at breakfast, lunch and dinner, is an effective means of increasing protein intake and optimising daily protein distribution in older adults.

8.1 Introduction

The age-related decline in skeletal muscle mass and function represents a fundamental threat to healthy ageing. This deterioration in muscle mass and function is associated with functional impairment, physical disability, increased frailty and a decrease in quality of life (Fielding *et al.*, 2011; Janssen, 2010). This is a multifaceted issue and an effective prevention and treatment remains to be established (Janssen, 2010). Inadequate dietary intake, in particular protein, is a key contributor to this decline in muscle mass and function with advancing age (Phillips, 2015; Bauer *et al.*, 2013; Malafarina *et al.*, 2013). Protein and essential amino acid (EAA) supplementation, and/or exercise, which each act as an anabolic stimulus and stimulate MPS have been emphasised in preventing and treating age-related decline in muscle mass and function (Phillips, 2015; Bauer *et al.*, 2013; Malafarina *et al.*, 2013).

Habitual protein intake tends to declines with advancing age. Fulgoni, (2008) reported total protein intake of 91g/day/1.3g/kg in young adults aged 19-30 years, and 66g/day 1.0g/kg in older adults aged >71 years. In chapter 4, we showed that habitual dietary protein intake amongst Irish adults decreases from 1.3±0.4 g/kg/d in adults aged 18-35 years, to 1.1g±0.3 g/kg/d in adults aged >65 years. Furthermore, protein intake followed a skewed pattern, in which 15±10g, 30±15 and 44±17g was eaten and breakfast, lunch and dinner (See Chapter 4, **Figure 4.1**). This skewed pattern is considered to be suboptimal for maximising diurnal MPS rates (Paddon-Jones *et al.*, 2015; Mamerow *et al.*, 2014). In this instance, while protein intake at dinner is sufficient to elicit a robust postprandial anabolic response, low protein intake at breakfast and lunch represent a missed opportunity to maximise MPS for that mealtime. Distributing protein intake more equally throughout the day, in which each meal has a sufficient protein dose, has been shown to be most optimal to maximise MPS (Areta *et al.*, 2013; Moore *et al.*, 2012). Indeed, cross-sectional and epidemiological data demonstrate that a more even protein distribution throughout the day is related to a decrease in frailty (Bollwein *et al.*, 2013), and consuming 1-2 meals ≥30g protein per day is associated with greater lean mass and strength (Loenneke *et al.*, 2016). Moreover, interventions to increase protein intake specifically at breakfast and lunch have resulted in positive changes in LBM in middle-aged adults (Norton *et al.*, 2016; Bauer *et al.,* 2015).

While the current recommended daily intake of protein for all adults, regardless of age and sex, is 0.83g per kg body mass per day (g/kg/d) (European Food Safety Authority, 2012), older adults have a higher protein requirement than young (Nowson and O'Connell, 2015). There is a growing acceptance that older adults require >1.2g/kg/d protein to maintain muscle mass and function (Traylor, Gorissen and Phillips, 2018), with intakes up to 1.5g/kg/d required for those suffering from acute or chronic disease (Bauer *et al.*, 2013). This

higher requirement for dietary protein is related to the dampened anabolic response to anabolic stimuli, such as exogenous essential amino acids (EAAs) and exercise, which is apparent in older adults and is termed 'anabolic resistance' (Morton *et al.*, 2018). However, this inferior anabolic response in older adults can be 'rescued' by increasing the dose of EAA ingested (Paddon-Jones *et al.*, 2004) and in particular the quantity of the AA leucine (Katsanos *et al.*, 2006). Indeed, there is growing evidence to support the recommendation of protein on a 'per meal' basis. 0.40 g/kg per meal of a high-quality protein (Moore *et al.,* 2015) or >20-30 g per meal, containing about 2.5 to 2.8g leucine (Layman *et al.*, 2015; Bauer *et al.,* 2013; Symons *et al.*, 2009; Katsanos *et al.*, 2006) represents the optimal per meal protein consumption to elicit a maximal anabolic response in elderly adults. Notably, leucine is well established as the key amino acid which triggers this postprandial anabolic response (Pasiakos, 2012; Atherton et al., 2010a). Despite the importance of leucine in the anabolic response, to date, the effect of specifically targeting an optimal dose of leucine on per meal basis, over an extended period, has not been explored for effects on muscle mass and function in older adults. However, prior to examining effects on functional outcomes, it is necessary to consider the effects of such a dietary strategy on changes in daily protein intake and distribution.

The consumption of whole food meals, as opposed to the consumption of supplements via powder or capsule form, is representative of a normal diet (van Vliet *et al.*, 2018). To date, much of the research aimed at increasing dietary protein intake in older adults has focus on the use of protein powders, such as whey and casein protein, and AA mixtures (Thomas *et al.*, 2016; Norton *et al.*, 2016; Bauer *et al.,* 2015; Malafarina *et al.*, 2013) . While these studies show promise, there remains a significant gap in the literature for the use of a whole food-based nutrition intervention. Moreover, foods consumed in their whole-form, such as whole egg and whole-milk, when compared to egg white and skimmed milk, respectively, show superiority in stimulating postprandial anabolism (van Vliet *et al.*, 2017; Elliot *et al.*, 2006). Therefore, a whole-food intervention may offer an advantage to skeletal muscle accretion over time. Interventions targeting the consumption of beef twice daily (Daly *et al.*, 2014), whole-milk twice daily (Tieland *et al.*, 2012a) and a range of dairy products twice daily (Iuliano, Woods and Robbins, 2013), have proven efficacious in increasing dietary protein intake. However, a dietary strategy that does not rely on one food or food group for additional protein, and focuses on a mixture of protein sources, may represent a long-term, sustainable approach to increasing per meal protein intake in older adults.

The aim of the present study was to determine if instruction and support for a high protein diet, prescribing the equivalent of 3g of leucine per meal, eaten at breakfast, lunch and dinner, is efficacious in augmenting protein intake and protein distribution in older adults.

8.2 Methods

8.2.1 Study Design and Participants

Participants were recruited primarily through a UCD Alumni newsletter seeking males and females aged ≥65 years who were medically stable, and who were free-living, fully mobile and capable of completing the proposed intervention. Participants were excluded if they reported a history of myocardial infarction, cardiac illness, vascular disease, uncontrolled metabolic disease, stroke, or major systemic disease; or if already engaging in two or more structured exercise sessions per week. All participants gave informed written consent before participating in the study, which was approved by the Office of Research Ethics at University College Dublin in accordance with the *Declaration of Helsinki*. Upon entry to the study, which was part of a larger exercise training intervention study (chapter 9) participants were randomly assigned to one of the following three groups: nutrition and exercise group (NUTR+EX); exercise only (EX); non-exercise nutrition only group (NUTR). Five participants from NUTR were excluded from final analysis due to non-compliance and two participants dropped out of EX due to failure to commit to training frequency, leaving a final n size of 56 (NUTR+EX, n=21; NUTR, n=16; EX, n=19).

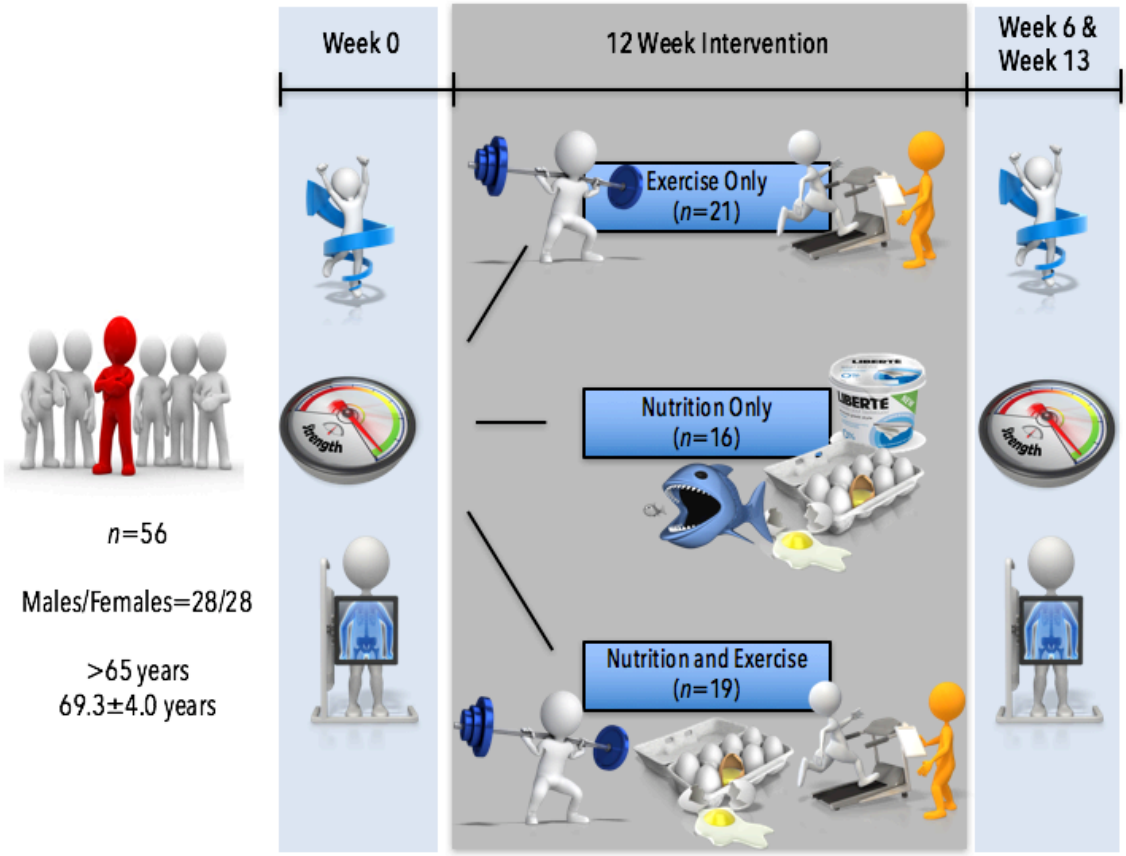


Figure 8.1: Overview of study design.

8.2.2 Nutrition Intervention

All participants completed a Food Frequency Questionnaires before the intervention commenced, to capture habitual dietary intake. NUTR+EX and NUTR were instructed to follow a high protein diet, which focussed on the prescription of providing 3g leucine per meal, for the 12-week period (See **appendix 5** for instructions on nutrition intervention). The nutrition strategy was designed using the USDA Food Composition Database, in which animal-based protein sources, equating to 3g of leucine, were translated to user-friendly portion sizes and meals. Participants from NUTR+EX and NUTR were brought into the laboratory in small groups of 4-6 and the nutrition strategy was explained in detail to the group. Participants were instructed to consume a leucine-rich (≥3g) meal at breakfast, lunch and dinner, every day for the 12-week period. Participants were given a list of foods and the portion size of each food to choose from at each main meal. Participants were required to consume the specified portion in one sitting, and were asked not to split the portion over different eating occasions. No restriction was placed on energy intake. Participants were given a check-list diary to track their adherence daily (See **appendix 6**). A weekly newsletter was sent to both NUTR+EX and NUTR groups, detailing leucine-rich recipe ideas. Weekly contact was kept with NUTR+EX during supervised exercise sessions, while fortnightly phone call contact was kept with NUTR, in which continued support and advice was

provided to encourage participants to adhere to the intervention. EX were asked not to change their habitual dietary intake for the duration of the trial. All participants completed a 3-day portion estimate food diary at PRE, MID and POST. Participants were asked to estimate food-weight based on food packaging, and if this was not possible, to quantify and describe food size as accurately as possible. For mixed meals and recipes, participants were asked to record each meal component and/or ingredient separately (See **appendix 7** for food diary instructions).

8.2.3 Data Analysis

Food diaries were analysed using Nutritics Nutrition Analysis Software (Version 5.029). The log of each meal was separated into eating occasions (EO), defined as any energy-containing food or fluid separated by more than 30 minutes. For intake and distribution, EO were assessed to determine the following:

- Protein Distribution Score^{20}(PDS^{20}): The number of EO per day containing over 20g of protein, averaged across the 3 days.
- Protein Distribution Score^{30}(PDS^{30}): The number of EO per day containing over 30g of protein, averaged across the 3 days.
- Individual protein target (IPT): 0.4g x body mass (kg) per meal
- Protein Distribution Score^{IPT}(PDS^{IPT}): The number of EO per day containing over the IPT, averaged over the 3 days.

A score of 1 was given to each meal reaching the 20g protein, 30g protein and 0.4g/kg body mass protein threshold, for $PDS^{20,}$ PDS^{30} and PDS^{IPT}, respectively. This scoring system is adapted from (MacKenzie *et al.*, (2015) and based on the current recommendation of >20-30g protein and/or 0.4g/kg per meal in maximising MPS in older adults (Layman *et al.*, 2015; Moore *et al.*, 2015; Bauer *et al.,* 2013; Symons *et al.*, 2009).

8.2.4 Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics Version 23). All data are presented as mean±SD. In general, the distribution of the data approximated normality, or was log transformed as appropriate to approximate normality, to allow detection of significant differences between and within groups. The difference from baseline over time within groups, and the difference between treatment groups at the same time-points, for all variables was analysed using a two-way (group x time) mixed ANOVA. When main or interaction effects were indicated, post-hoc analysis was performed using one-way ANOVA, repeated measured

ANOVA, and pairwise comparisons with Bonferroni's adjustment for multiple comparisons. Statistical significance was accepted at $P<0.05$.

8.3 Results

8.3.2 Calorie and Macronutrient Intake

Table 8.1 details energy and macronutrient intake in each group PRE, MID and POST. At PRE, EX group had a greater calorie intake compared to NUTR+EX (1823±344 vs. 1466±371 kcal, $P<0.05$, respectively). There was no change in dietary intake in EX throughout the intervention period, whereas the dietary intervention was successful in increasing daily protein intake, and consequently energy intake, in NUTR+EX and NUTR. At MID, protein intake increased in NUTR+EX (65.8±13.8g to 117.8±23.7g, $P<0.001$) and NUTR (73.4±25.7g to 119.9±30.5g, $P<0.001$), compared to PRE, with no difference between values from MID to POST in both groups. **Table 8.2** details calorie and macronutrient intake relative to body mass in each group at PRE, MID and POST. Relative protein intake increased in NUTR+EX (0.90±0.20 to 1.57±0.49g/kg, $P<0.001$) and NUTR (0.99±0.34 to 1.43±0.39 g/kg, $P<0.001$), between PRE and MID, with no change in EX. Fat intake increased at MID in NUTR+EX (56.2±19.7 to 73.4±22.7, $P<0.05$). Carbohydrate intake did not differ between groups and remained similar over time.

	Energy (kcal)	Protein (g)	CHO (g)	Fat (g)	Alcohol (g)
NUTR+EX					
Pre	1466 ± 371	65.8 ± 13.8	152.0 ± 50.7	56.2 ± 19.7	13.4 ± 13.6
Mid	1873 ± 449**	117.8 ± 23.7***	151.6 ± 43.6	73.4 ± 22.7*	19.4 ± 20.3
Post	1971 ±837**	117.1 ± 39.3***	151.4 ± 51.2	70.6 ± 23.9	19.7 ± 18.0
NUTR					
Pre	1648 ± 441	73.4 ± 25.7	173.6 ± 59.0	64.3 ± 22.3	12.7 ± 16.6
Mid	1949 ± 428**	119.9 ± 30.5***	154.6 ± 43.4	78.4 ± 23.9	20.3 ± 20.9
Post	1989 ± 439**	113.1 ± 29.3***	168.5 ±51.9	79.8 ± 27.1	20.2 ± 21.6
EX					
Pre	1823 ± 344#	80.0 ± 18.1	188.0 ± 38.1	69.6 ± 18.1	17.1 ± 19.2
Mid	1777 ± 437	77.4 ± 18.1#†	175.9 ± 53.4	67.6 ± 23.4	21.7 ± 22.7
Post	1793 ± 421	75.6 ± 23.6#†	185.2 ± 63.7	66.6 ± 16.8	21.2 ± 20.7

Table 8.1: Calorie and macronutrient intakes at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Data are mean±SD. CHO, carbohydrate. Statistical analysis was performed using two-way ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *$P<0.05$, **$P<0.01$, ***$P<0.001$ for the annotated time-point. Differences between groups are indicated by # to denote differences from NUTR+EX group, and † to denote differences from NUTR group, for the time-point ($P<0.05$).

	Protein (g/kg)	CHO (g/kg)	Fats (g/kg)	Alcohol (g/kg)	Protein (% EI)	CHO (% EI)	Fat (% EI)	Alcohol (% EI)
NUTR +EX								
PRE	0.90±0.20	2.1±0.7	0.8±0.3	0.2±0.2	18.5±3.7	41.1±6.7	34.4±7.6	6.6±6.4
MID	1.59±0.28***	2.1±0.6	1.0±0.3	0.3±0.3	25.7±4.2***	32.7±6.8***	35.1±5.7	6.7±6.2
POST	1.59±0.51***	2.0±0.7	1.0±0.3	0.3±0.2	25.4±3.2***	32.6±5.9***	34.8±4.9	6.8±4.7
NUTR								
PRE	0.99±0.34	2.3±0.9	0.9±0.3	0.2±0.2	17.9±3.1	41.7±7.7	34.6±5.3	6.1±8.8
MID	1.52±0.45***	2.0±0.6	1.0±0.4	0.3±0.3	24.6±3.6***	31.7±5.7***	36.0±6.2	7.5±7.8
POST	1.43±0.39**	2.1±0.8	1.0±0.4	0.3±0.3	22.9±4.4**	34.0±7.5**	35.6±6.6	7.4±8
EX								
PRE	1.14±0.35	2.6±0.7	1.0±0.4	0.2±0.3	17.8±3.7	41.7±7.2	34.2±6.5	6.0±6.5
MID	1.10±0.30#†	2.5±0.8	1.0±0.4	0.3±0.3	18±4.1#†	40.0±9.2#†	33.9±5.5	7.9±8.5
POST	1.05±0.28#†	2.6±0.8	0.9±0.3	0.3±0.3	16.9±3.6#†	41.0±7.8#†	33.9±6.4	8.0±7.8

Table 8.2: Relative macronutrient intakes at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Data are mean±SD. CHO, carbohydrates; EI, energy intake. Statistical analysis was performed using two-way between-within ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *P<0.05, **P<0.01 and ***P<0.001, for the annotated time-point. Differences between groups are indicated by # to denote differences from NUTR+EX group, and † to denote differences from NUTR group, for the time-point (P<0.05).

8.3.3 Protein Distribution

Table 8.3 presents PDS scores for meals reaching the 20g, 30g and 0.4g/kg protein target. At baseline PDS^{20} was greater in EX compared to NUTR+EX (1.65±0.67 vs. 1.14±0.52, P=0.025). The nutrition intervention was successful in increasing PDS^{20} in NUTR+EX and NUTR. At MID, PDS^{20} increased in NUTR+EX (1.14±0.52 to 2.68±0.41, P<0.001) and NUTR (1.50±0.54 to 2.69±0.48, P<0.001), with no difference between values from MID to POST in both groups. PDS^{20} was greater in NUTR+EX and NUTR, compared to EX, at both MID and POST (P<0.001 for both). The nutrition intervention was successful in increasing PDS^{30} in NUTR+EX and NUTR. At MID, PDS^{30} increased in NUTR+EX (0.63±0.42 to 2.05±0.55, P<0.001) and NUTR (1.00±0.64 to 2.17±0.69, P<0.001), with no difference between values from MID to POST in both groups. PDS^{30} was greater in NUTR+EX and NUTR, compared to EX, at both MID and POST (P<0.001 for both). There was no change in PDS^{20} or PDS^{30} in EX throughout the intervention period.

The nutrition intervention was successful in increasing PDS^{IPT} in NUTR+EX and NUTR. At MID, PDS^{IPT} increased in NUTR+EX (0.68±0.4 to 2.00±0.63, P<0.001) and in NUTR (0.92±0.66 to 1.94±0.79, P<0.001), compared to PRE, with no difference between values from MID to POST in both groups. PDS^{IPT} was greater in

NUTR+EX and NUTR, compared to EX, at both MID and POST ($P<0.001$ for both). There was no change in PDS^{IPT} in EX throughout the intervention period.

	PDS^{20}	PDS^{30}	PDS^{IPT}
NUTR+EX			
PRE	1.14±0.52	0.63±0.42	0.68±0.40
MID	2.68±0.41**	2.05±0.55***	2.00±0.63***
POST	2.60±0.68***	1.97±0.76***	2.05±0.60***
NUTR			
PRE	1.50±0.54	1.00±0.64	0.92±0.66
MID	2.69±0.48***	2.17±0.69***	1.94±0.79***
POST	2.65±0.56***	2.04±0.83***	1.81±0.85***
EX			
PRE	1.65±0.67#	0.84±0.41	1.05±0.67
MID	1.58±0.60#†	0.77±0.27#†	0.91±0.54#†
POST	1.49±0.61#†	0.70±0.41#†	0.81±0.39#†

Table 8.3: The average number of meals per day reaching ≥20g, ≥30g and 0.4g/kg (PDSIPT) protein, at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Data are mean±SD. CHO, carbohydrates. Statistical analysis was performed using two-way ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *$P<0.05$, **$P<0.01$ and ***$P<0.001$, for the annotated time-point. Differences between groups are indicated by # to denote differences from NUTR+EX group, and † to denote differences from NUTR group, for the time-point ($P<0.05$).

Figure 8.2 represent total protein intake at each eating occasion, in each group at PRE, MID and POST. At PRE, there was no difference between groups for protein intake at any eating occasion. There was no change in protein intake for any eating occasion in EX over time. In NUTR+EX, protein intake at breakfast increased from 12.4±4.9 to 33.1±10.2g at MID ($P<0.001$). A similar increase occurred in NUTR from PRE to MID (12.7±9.5 to 32.7±13.9g, $P<0.001$). This increase in protein intake at breakfast was maintained at POST for NUTR+EX and NUTR and was not different to MID (32.0±12.9g, $P<0.99$; 32.1±13.4g, respectively, $P<0.99$ for both). AT MID, protein intake at lunch increased from 17.2±6.4 to 33.1±5.6g in NUTR+EX ($P<0.001$). A similar increase occurred in NUTR from PRE to MID (24.4±10 to 32.9±8.7g, $P<0.018$). This increase in protein intake at lunch was maintained at POST for NUTR+EX and NUTR and was not different to MID (31.5±12g, $P<0.99$; 34.9±9.3g, respectively, $P<0.99$ for both). AT MID, protein intake at dinner increased from 31.0±13.4 to 44.5±15.4g in NUTR+EX ($P=0.025$). A similar increase occurred in NUTR from PRE to MID (35.9±12.8 to 48.5±15.1g, $P=0.045$). This increase in protein intake at dinner was maintained at POST for NUTR+EX and NUTR and was not different to MID (46.2±22.1g, $P<0.99$; 42.2±15.9g, $P=0.25$, respectively).

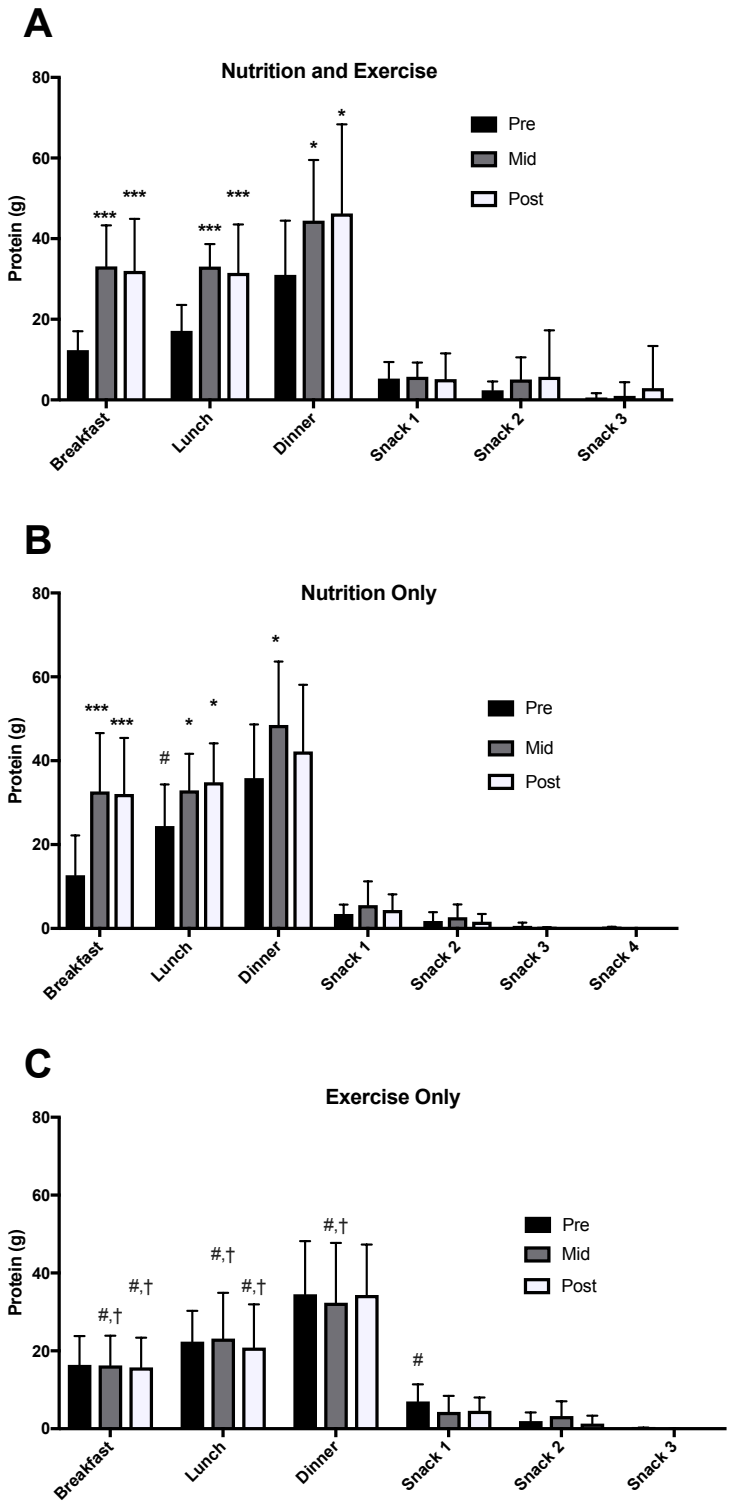


Figure 8.2: Protein intake at breakfast lunch, dinner and snacks, at at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (A), nutrition only group (B) and exercise only group (C). Statistical analysis was performed using two-way between-within ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by **P*<0.05, ***P*<0.01 and ****P*<0.001. Differences between groups are indicated by # to denote differences from NUTR+EX group, and † to denote differences from Nutrition Only group, for the time-point (*P*<0.05).

Table 8.4 represents protein intake relative to body mass at breakfast, lunch and dinner in each group PRE, MID and POST. At PRE, there was no difference between groups in relative protein intake for breakfast, lunch and dinner. There was no change in relative protein intake for any eating occasion in EX over time. AT MID, relative protein intake at breakfast increased from 0.17±0.06 to 0.44±0.11g/kg, $P<0.001$. A similar increase occurred in NUTR from PRE to MID in NUTR+EX (0.16±0.13 to 0.42±0.2g/kg, $P<0.001$). AT MID, relative protein intake at lunch increased from 0.23±0.08 to 0.45±0.08g/kg in NUTR+EX ($P<0.001$). A similar increase occurred in NUTR from PRE to MID (0.32±0.14 to 0.42±0.14g, $P=0.044$). AT MID, relative protein intake at dinner increased from 0.41±0.17 to 0.60±0.22g/kg in NUTR+EX ($P=0.016$). Relative protein intake also increased in NUTR from PRE to MID (0.46±0.16 to 0.61±0.20g/kg, $P=0.049$).

		Breakfast	Lunch	Dinner
Protein (g/kg)	**NUTR+EX**			
	PRE	0.17 ± 0.06	0.23 ± 0.08	0.41 ± 0.17
	MID	0.44 ± 0.11***	0.45 ± 0.08***	0.60 ± 0.22*
	POST	0.41 ± 0.20***	0.40 ± 0.23*	0.63 ± 0.31*
	NUTR			
	PRE	0.16 ± 0.13	0.32 ± 0.14	0.46 ± 0.16
	MID	0.42 ± 0.20***	0.42 ± 0.14*	0.61 ± 0.20*
	POST	0.41 ± 0.21**	0.44 ± 0.17*	0.59 ± 0.29
	EX			
	PRE	0.23 ± 0.12	0.32 ± 0.14	0.49 ± 0.21
	MID	0.23 ± 0.11#†	0.33 ± 0.17#	0.46 ± 0.21
	POST	0.22 ± 0.12#†	0.24 ± 0.22†	0.55† ± 0.34

Table 8.4: Body mass relative protein intake at breakfast, lunch and dinner at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Data are mean±SD. Statistical analysis was performed using two-way ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *$P<0.05$, **$P<0.01$ and ***$P<0.001$ for the annotated time-point. Differences between groups are indicated by # to denote differences from NUTR+EX group, and † to denote differences from NUTR group, for the time-point ($P<0.05$).

8.3.4 Dietary Components

Table 8.5- 8.7 represent average dietary components in each group at PRE, MID and POST. In NUTR+EX, there was in an increase in lactose, saturated fat, monounsaturated fats, trans-fatty acids and cholesterol at MID and POST ($P<0.05$ for all). There was an increase across all B vitamins and vitamin D at MID and POST for NUTR+EX ($P<0.05$ for all). There was an increase in sodium, potassium, chloride, calcium, phosphorus,

magnesium, zinc, selenium and iodine intake at MID and POST for NUTR+EX ($P<0.05$ for all). In NUTR, cholesterol intake increased at MID and POST ($P<0.05$ for all) Lactose intake increase at POST in NUTR ($P<0.05$). Vitamin B2 and B12 intake increased at MID and POST in NUTR ($P<0.05$ for all). Vitamin B3, B5 and B7 increased at POST in NUTR ($P<0.05$ for all). There was an increase in selenium and iodine intake at MID and POST for NUTR ($P<0.05$ for all). There were no changes in micronutrient intakes in EX at any time-point.

	NUTR+EX			NUTR			EX		
	PRE	MID	POST	PRE	MID	POST	PRE	MID	POST
Starch (g)	90.1±35.0	83.0±35.5	87.4±30.2	101.5±31.2	81.3±30.2	85.8±32.9	108.3±23.9	98.9±23.5	109.9±44.4
Oligosaccharide (g)	0.4±0.4	0.5±0.5	0.5±0.5	0.5±0.5	0.5±0.7	0.8±1.1	0.4±0.4	0.6±0.6	0.5±0.7
Fibre (g)	14.3±3.8	15.4±3.7	15.6±4.0	17.4±5.9	15.3±6.1	15.0±5.1	20.9±7.5	19.4±7.8	19.8±7.9
NSP (g)	10.9±3.3	11.6±3.2	11.6±3.2	13.2±4.5	12.0±4.8	11.5±4.2	16.6±6.8	15.4±7.1	15.7±6.8
Sugars (g)	59.4±23.1	66.4±21.6	67.2±20.5	71.9±31.4	63.0±31.2	69.4±30.1	76.7±21.1	73.6±38.3	74.3±37.9
Free Sugars (g)	19.1±12.3	19.6±10.7	16.9±10.1	29.0±18.8	19.9±10.3	24.7±13.8	22.2±12.9	20.6±13.8	26.3±16.5
Glucose (g)	8.8±4.6	7.7±2.6	9.6±4.7	13.5±6.9	9.6±6.3	8.6±5.8	14.2±6.7	13.2±10.0	13.2±9.0
Galactose (g)	0.7±1.3	0.1±0.1	0.1±0.3#	0.3±0.9	0.1±0.4	0.0±0.0#	0.2±0.5	0.3±0.8	0.4±0.6
Fructose (g)	10.1±5.2	9.4±4.2	11.7±5.9	14.2±8.5	11.1±7.8	9.1±6.3	17.6±9.3	18.2±22.4	17.3±20.4
Sucrose (g)	17.8±11.5	15.0±8.4	15.2±8.1	23.3±13.6	16.9±10.2	23.6±11.7	19.3±6.6	20.3±12.3	19.9±10.7
Maltose (g)	2.7±1.3	2.4±1.5	2.7±1.5	4.6±2.7	2.7±1.2	3.6±2.1	5.0±3.6	4.7±3.6	5.7±4.4
Lactose (g)	9.0±4.6	18.7±10.0*#	19.2±8.5*#	7.9±6.3	16.0±13.2	17.5±13.9*#	8.1±6.5	7.9±7.0	7.8±6.9
Saturated Fat (g)	21.9±9.9	28.0±11.3*	29.0±8.0*	25.8±9.6	31.4±13.7	31.8±15.7	26.2±9.1	24.5±6.9	24.4±8.1
MUFAS (g)	18.7±6.2	24.8±8.4*	26.1±6.8*	22.2±8.6	26.4±9.5	27.0±11.3	24.7±8.3	24.5±12.1	24.3±7.1
PUFAS (g)	8.7±3.8	9.3±3.0	11.2±3.5	10.1±3.5	9.5±3.7	10.2±4.4	10.9±5.1	11.3±8.7	11.1±5.2
Omega 3 (g)	1.2±0.8	1.4±1.1	2.1±1.5*	1.4±1.0	1.4±1.1	1.9±1.6	1.4±1.1	1.9±2.1	1.8±1.6
Omega 6 (g)	4.8±3.2	4.6±2.0	5.3±1.7	4.9±2.8	5.2±2.6	5.4±2.8	5.1±3.8	5.2±5.9	5.0±3.5
Trans-fatty acids (g)	0.7±0.5	1.2±0.6*	1.2±0.5*	0.9±0.4	1.2±0.7	1.3±0.8	1.0±0.6	0.9±0.5	0.9±0.5
Cholesterol (mg)	246±79.7	447±149.9*#	464±152*#	293±175	497±221*#	475±216*#	261±106	269±95.8	253±115

Table 8.5: Dietary nutrient intake at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). NSP, non-starch polysaccharides; MUFAs, monounsaturated fatty acids; PUFAs, poly-unsaturated fatty acids. Data are mean±SD. Statistical analysis was performed using two-way ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *$P<0.05$. Differences between groups are indicated by # to denote differences from EX group for the time-point, #$P<0.05$.

	NUTR+EX			NUTR			EX		
	PRE	MID	POST	PRE	MID	POST	PRE	MID	POST
Vitamin A (μg)	743±559#	842±482	928±983	990±620	1077±623	768±420	1519±1294	759±447	794±442
Vitamin C (mg)	55±33	81±43*	81±40	60±41	80±54	74±50	78±48	83±43	81±36
Vitamin D (μg)	2.9±1.9	5.5±3.8*	8.2±5.4*#	4.6±2.8	5.8±4.2	7.0±5.1	3.7±3.1	4.0±2.5	4.3±3.3
Vitamin E (mg)	6.4±3.9	7.5±3.1	7.8±3.0	7.7±2.9	7.2±3.1	7.1±2.9	7.7±3.8	7.7±5.3	7.6±3.5
Vitamin K1 (μg)	11±8	35±34*	34±46	15±15	34±35	22±33	44±81	26±30	19±18
Vitamin B1 (mg)	1.3±0.4	1.7±0.4*	1.7±0.6	1.4±0.6	1.7±0.7	1.8±0.7	1.5±0.4	1.5±0.5	1.6±0.8
Vitamin B2 (mg)	1.2±0.4	2.2±0.5*#	2.4±0.6*#	1.3±0.5	2.2±1.0*#	2.3±1.1*#	1.6±0.7	1.5±0.5	1.5±0.6
Vitamin B3 (mg)	29±9#	43±13*	50±17*#	34±10	46±15	46±18*	37±10	37±9	34±12
Vitamin B5 (mg)	4.6±1.6	6.7±1.5*#	7.5±1.8*#	4.9±1.7	7.1±2.6#	7.2±2.7*#	5.3±1.3	5.2±1.3	4.9±1.3
Vitamin B6 (mg)	1.5±0.5#	2.0±0.5*	2.3±0.8*	1.7±0.6	2.3±1.0	2.2±0.9	2.0±0.6	1.9±0.6	1.9±0.7
Vitamin B7 (μg)	26±6#	44±13*	45±11*#	28±11#	43±17	43±18*	41±18	37±22	33±10
Vitamin B9 (μg)	195±77	242±58*	263±84*	187±57#	238±102	226±92	250±81	251±82	237±97
Vitamin B12 (μg)	4.4±1.6	8.8±3.1*#	11.3±4.9*#	5.3±2.8*	9.5±5.0*#	9.6±5.2#	6.7±6.4	5.3±2.7#	5.2±2.8#
GL	88±34	83±28	89±27	100±31	81±28	90±31	103±22	96±29	106±34
PRAL	14±12	31±12*#	34±16*#	13±13	33±14*#	33±19*#	9±14	7±19	6±16

Table 8.6: Dietary vitamin, GL and PRAL intake, at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). GL, glycaemic load; PRAL, potential renal acid load. Data are mean±SD. Statistical analysis was performed using two-way ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *$P<0.05$. Differences between groups are indicated by # to denote differences from EX group for the time-point, #$P<0.05$.

	NUTR+EX			NUTR			EX		
	PRE	MID	POST	PRE	MID	POST	PRE	MID	POST
Sodium (mg)	1491±542	2250±816*#	2184±809*	1825±785	2166±734	2397±1013	1790±502	1653±434	1776±502
Potassium (mg)	2186±468#	3120±653*	3404±762*	2503±694	3164±1198	3084±1093	2885±773	2886±943	2919±980
Chloride (mg)	2408±962	3527±1251*	3394±1021*#	2923±1146	3423±1120	3707±1484	3157±899	2806±744	2698±662
Calcium (mg)	657±192#	1166±341*	1179±279*	805±297	1091±527	1140±600	902±270	882±479	889±433
Phosphorus (mg)	1129±244#	1727±298*#	1872±451*#	1217±396	1732±630#	1746±680*	1365±269	1368±367	1354±438
Magnesium (mg)	227±51#	325±118*	331±122*	243±72#	328±187	339±157	304±89	324±149	317±133
Iron (mg)	8.9±2.6	10.3±2.3	12.0±3.7	9.3±2.7	10.8±3.5	10.6±4.0	12.5±5.2	16.4±24.6	16.9±22.2
Zinc (mg)	7.1±2.2#	11.5±2.8*	12.0±4.3*	8.6±3.8	11.7±5.0	10.9±4.9	10.0±3.4	9.7±4.5	9.8±4.5
Copper (mg)	0.8±0.2	1.1±0.3	1.1±0.5	1.0±0.3	1.0±0.4	1.0±0.4	1.5±1.0	1.3±0.9	1.3±0.7
Manganese (mg)	3.0±1.2	2.9±1.1	2.8±1.0	3.0±1.1	2.7±1.0	2.7±1.2	3.8±1.4	4.0±2.2	4.0±2.2
Selenium (μg)	45±16	70±27*#	77±29*#	48±19	77±27*#	76±36*#	53±22	52±18	48±18
Iodine (μg)	141±68	250±92*#	268±89*#	117±66	250±144*#	224±132*#	138±70	121±53	127±101

Table 8.7: Dietary mineral intake at PRE (week 0), MID (week 6) and POST (week 12), in nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Data are mean±SD. Statistical analysis was performed using two-way ANOVA. Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *P<0.05. Differences between groups are indicated by # to denote differences from EX group for the time-point, #P<0.05.

8.4 Discussion

The current study demonstrates that intervention with a dietary strategy focussed on whole foods to provide a high protein intake, and specifically targeting 3g leucine at three meals per day, is efficacious in increasing protein intake and achieving a more even daily protein distribution in older adults. Since protein intake is a strong influence on skeletal muscle accretion (Norton *et al.*, 2016; Daly *et al.*, 2014), this nutrition intervention shows promise as a dietary strategy for targeting the age-related decline in muscle mass and function, either alone or in support of exercise training. The results of such an investigation are presented in chapter 9.

Prior to the intervention, which represents the participants' habitual dietary intake, daily protein intake was 66±14g, 73±26g and 80±18g, for NUTR+EX, NUTR and EX, respectively, with no differences between groups. These are similar intakes to those in community-dwelling older adults in Ireland (Chapter 4) and elsewhere (Tieland *et al.*, 2015). Relative to body mass, pre-intervention habitual protein intake was 0.90±0.20 g/kg/d, 0.99±0.34 g/kg/d, 1.14±0.35 g/kg/d for NUTR+EX, NUTR and EX, respectively, with no differences between groups. While these habitual protein intakes exceed the current PRI for protein intake in adults, there is a growing consensus that this recommendation is outdated, and protein requirements for older adults are from 1.2 to 1.5g/kg/d (Bauer *et al.*, 2013).

The nutrition intervention was successful in increasing daily protein intake in NUTR+EX and NUTR. At MID, protein intake increased by 79% and 63% in NUTR+EX and NUTR, respectively. Relative protein intake increased from 0.90±0.20 to 1.57±0.49g/kg/d in NUTR+EX, and 0.99±0.34 to 1.43±0.39g/kg/d NUTR after 6 weeks (MID). There were no differences between values from at MID and POST in both groups, suggesting that the participants successfully continued on the dietary strategy for the latter 6 weeks of the intervention. At MID, fat intake increased by 31% in NUTR+EX. This is likely due to the participants consuming foods which are higher in fat, such as eggs, dairy, oily fish and red meat, all of which were encouraged in the dietary strategy (See **appendix 5** for instructions on nutrition intervention). In support, there was an increase in saturated fat, monounsaturated fat, omega 3 fatty acid intake and cholesterol in NUTR+EX, and lactose and cholesterol in NUTR, throughout the intervention. Carbohydrate intake did not differ between groups and remained the similar over time. Previous nutrition interventions, in which a protein enriched diet is derived from one food or a food group, have also been successful in increasing protein intake. For example, a food-based intervention targeting two servings of dairy protein per day resulted in a 25±12g increase in daily protein intake over 4 weeks in ambulatory, aged care residents (Iuliano, Woods and Robbins, 2013). Total protein intake increased from 1.0g/kg/d to 1.3g/kg/d when 15g protein from whole milk were consumed directly after breakfast and

lunch (Tieland, *et al.*, 2012a). 80g red meat consumed at lunch and dinner, 6 days a week for 16 weeks, caused dietary protein intake to increase from 73.4±23.2 to 88.3±17.5 g/d in older adults (Daly *et al.*, 2014). Beelen, de Roos and de Groot, (2017) investigated the effect of protein enrichment of regular foods and drinks on protein intake in institutionalised older adults. To reflect a real world setting, alternative breads, fruit juices, soups and potatoes, which had been enriched with protein, were given as an option that participants could choose from. The nutrition intervention was successful in increasing protein intake from 0.96 to 1.14 g/kg/d, which represented an extra 11.8g/d of protein. However, participants still fell below the recommendation of ≥1.2g/kg/d to preserve LBM in older adults. Results from the present study show a greater increase in protein intake than this previous work and is analogous to that achieved by powdered protein supplementation in middle-aged to older adults (Norton *et al.*, 2016). Similar to finding in the present study, a nutrition intervention which targeted breakfast and lunch enrichment with milk-based protein supplement, resulted an increase in total protein intake of 83±19 to 106±20g and 1.2±0.3 to 1.6±0.3g/kg/d over 24 weeks (Norton *et al.*, 2016). A unique aspect to this present study was that participants were provided instruction and support for protein intakes that specifically translated to 3g leucine at three main meals. To attain 3g leucine per meal, ≥30g high quality protein is often required; with a 'high quality protein', in this instance, referring to a protein that provides a large dose of EAAs, with animal-based proteins in particular considered to be of a higher quality protein when compared to a plant-based proteins (van Vliet, Burd and van Loon, 2015). Therefore, a plausible explanation for the greater changes seen during the present intervention is that participants were simply required to choose foods that were inherently higher in protein, resulting in a greater daily intake.

Across all groups, pre-intervention protein intake followed a skewed pattern across meals, in which breakfast and lunch contained small amounts of protein, and the highest amount of protein is consumed at dinner. Breakfast, lunch and dinner accounted for 18%, 25%, 45% of protein intake in NUTR+EX; 16%, 31%, 46% in NUTR and 20%, 27%, 42% in EX, respectively. This skewed intake is reported elsewhere (Cardon-Thomas *et al.*, 2017; Tieland *et al.*, 2015; Almoosawi *et al.*, 2013; Bollwein *et al.*, 2013; Ruiz Valenzuela *et al.*, 2013), including Irish adults in Chapter 4. Relative to body mass, prior to intervention, breakfast, lunch and dinner were 0.17, 0.23, 0.41g/kg of protein intake in NUTR+EX; 0.16, 0.32, 0.46g/kg in NUTR; and 0.23, 0.32 and 0.49g/kg in EX, respectively. 0.4g/kg per meal is theorised as the required protein dose to maximise MPS in older adults (Moore *et al.,* 2015), while in absolute values, >20-30g high quality protein is purported as the required protein dose to maximise postprandial MPS in older adults (Layman *et al.*, 2015; Bauer *et al.,* 2013; Symons *et al.*, 2009). Therefore, prior to intervention, while dinner in NUTR+EX and NUTR reached this meal

threshold, breakfast and lunch failed to reach this threshold. The suboptimal protein consumed at these two meals represents a missed opportunity to initiate a robust postprandial anabolic response. At MID, daily protein intake was more evenly distributed between breakfast, lunch and dinner in NUTR+EX and NUTR. While distribution between these meals was skewed, in which dinner represented the greatest protein intake; protein intake across breakfast and lunch improved significantly in NUTR+EX and NUTR (See **Table 8.4**). At MID and POST, average intakes at each of the three main meals in both nutrition intervention groups were ≥0.4g/kg and 30g. Since all food options suggested to participants as part of the dietary strategy were animal-based and rich in leucine (eggs, poultry, beef, fish, yoghurt, milk, cheese, etc.), these foods were representative of a high quality protein sources. Similar to the present findings, Norton *et al.*, (2016) achieved an increase from 0.23±0.1 to 0.4± 0.1g/kg for breakfast and from 0.31±0.2 to 0.47±2g/kg lunch with the enrichment of breakfast and lunch with 0.165g/kg of protein from powdered protein. These data suggest that the nutrition intervention successfully resulted in a protein intake and distribution pattern that is considered optimal for maximising cumulative daily MPS rates in older adults.

Being cognisant of the 20 to 30g protein threshold per meal required to maximise postprandial anabolism, we sought to determine the number of meals reaching these two thresholds over each 3-day food diary period. The number of meals reaching ≥20g protein increased from ~1 per day, to >2.5 in both NUTR+EX and NUTR. This suggests that the nutrition intervention was successful in achieving a more optimal per meal protein intake. PDS^{30} increased in NUTR+EX, from ~0.6 to ~2 servings per day; and from ~1 to >2 servings per day in NUTR. This higher threshold is more stringent for assessing per meal protein intake, and shows that meals falling between the 20-30g threshold in NUTR+EX and NUTR account for this discrepancy. Although breakfast, lunch and dinner attained a mean group intake of 0.4g/kg per meal, the PDS^{IPT} which counts the average number of meals per day which reached the 0.4g/kg threshold, revealed that on average, not all three the main meals reached this threshold. Nonetheless, there was an improvement in PDS^{IPT} from PRE to MID in both nutrition intervention groups (An increase from ~0.7 to 2 in NUTR+EX, and an increase from ~0.9 to 2 servings per day containing ≥0.4g/kg protein in NUTR).

At the beginning of the study, the two nutrition intervention groups received the same instructions on the dietary intervention, and also received the same weekly newsletters detailing leucine-rich recipes and further support material. However, because NUTR+EX were also involved in the exercise intervention throughout, they potentially received additional support and counselling due to contact with the lead researcher during the

supervised exercise sessions (three times per week). Participants in NUTR instead received a fortnightly phone-call from the lead researcher. Despite NUTR+EX and NUTR receiving different contact time and support, there was no difference in energy intake, protein intake, and protein distribution between groups. This suggests that an initial meeting in a small group, weekly newsletters and a fortnightly follow-up phone-calls are sufficient contact time for achieving adherence to the dietary intervention. This is in contrast to strategies used previously to achieve dietary adherence. In a 12-month weight-loss intervention, Gardner *et al.*, (2018) used weekly face-to-face counselling session in the initial 8 weeks to attain adherence to a low-carbohydrate or low-fat diet, in which no restriction was place on energy intake. Arguable, the use of a weekly newsletters and fortnightly phone-calls in the present study is less time-consuming and labour intensive when compared to strategies used by Gardner *et al.*, (2018). However, the intervention by Gardner *et al.*, (2018) was a over longer period, and also weight-loss focused, and additional support may be warranted for dietary interventions of this duration and type.

A unique aspect to this study was that participants followed a whole food-based nutrition plan, for breakfast, lunch and dinner, rather than consuming powdered protein/AA supplements. Of note, there was an increase in micronutrient intake in NUTR+EX and NUTR, with no change in micronutrient intake in EX throughout the intervention period. This suggests that increasing protein intake through animal-based foods increases the micronutrient content of the diet, which is supported elsewhere (Phillips *et al.*, 2015).

As a consequence of increasing protein and fat intake, energy intake increase by 28% and 18% in NUTR+EX and NUTR, respectively. Energy intake was not restricted, and therefore intake was ad libitum. This is contrast with finding by Weigle *et al.*, (2005) who provide high protein meals to participants, and placed no restriction on energy intake, over 12 weeks. Authors found a spontaneous decrease in energy intake due to an increase in satiety. The reason for an increase in calorie intake in the present study, is that instructions of protein intake was more regimented with participants required to consume ~30g protein from animal sources, with these sources often being high in fat, and therefore high in calories. We did not collect data on the ease or difficulty with which the participants followed the nutrition intervention. However, anecdotally, we noted that more than half of the participants in the study verbally reported struggling with consuming all three portions of protein per day at least one time throughout the intervention. Indeed, there were two dropouts in the NUTR group due to appetite difficulties. This is not surprising, as deterioration of appetite with age is well documented (Morley, 2001), and protein is a satiating macronutrient (Astrup, 2005). Other contributors to inadequate intake of energy and protein in older adults include the cost of more nutrient-dense foods, difficulty chewing fibrous

foods and fear of eating too much fat and cholesterol in foods (Bauer *et al.*, 2013; Malafarina *et al.*, 2013; Chernoff 2004). While this nutrition intervention was successful in increasing protein intake, and achieving an average per meal protein threshold of ≥30g and/or 0.4g/kg, these aforementioned factors need considering when designing a long-term, sustainable dietary strategy that targets preservation of LBM in older adults. Furthermore, deriving this quantity of daily protein predominantly from animal sources may pose a threat to environmental sustainability (de Vries and de Boer, 2010). For these reason, there are merits in the use of supplementation with powdered protein and EAAs, or leucine-enrichment of low leucine foods, or a combination of whole food and supplementation in ameliorating the age-related decline in muscle mass and function.

8.5 Conclusion

A nutrition intervention targeting a high daily protein intake and 3g leucine at breakfast, lunch and dinner from whole foods is efficacious in increasing protein intake and achieving a more even distribution of protein intake in community-dwelling older adults. The nutrition intervention successfully resulted in participants consuming ≥30g and/or 0.4g/kg per meal of high quality protein at three main meals, which is purported to be optimal for maximising cumulative rates of MPS in older adults. A unique aspect of the current intervention was that participants consumed additional dietary protein at each meal from whole foods as opposed to powdered protein supplements. While there are benefits to using whole foods over supplements, such as increase in micronutrient intake, a well-established issue with advancing age is the deterioration of appetite. For that reason, protein supplementation, and particularly leucine-enrichment of lower leucine or plant-based foods, should not be discounted as a strategy for achieving recommended protein intake and distribution patterns in older adults. Indeed, a combination of both whole foods and leucine-enrichment, to achieve the desired 3g leucine per meal, may be the most feasible nutrition strategy in older adults.

Chapter 9

9.0 The Effect of Concurrent Exercise Training and/or a Nutrition Intervention Targeting Leucine-Rich Meals on Body Composition and Physical Function in Older Adults

Background: The age-related decline in muscle mass and function can contribute to an increased risk of morbidity and mortality, and a decrease in quality of life in older adults. A combination of exercise training and a high protein diet shows potential for prevention and treatment of this progressive deterioration. The majority of studies in older adults have employed powdered protein and oral nutrition solutions to increase protein intake, but recent evidence suggests whole food sources of protein may have added value for stimulating muscle protein synthesis and preventing the age related decline in muscle mass.

Objective: The aim of the present study is to determine if a nutrition intervention, providing the equivalent of 3g of leucine per meal, eaten at breakfast, lunch and dinner, can augment exercise training-mediated effects on body composition and physical function in older adults.

Design: Participants aged ≥65 years were recruited to participate in this study. Participants (n=56) were randomly assigned to one of the following three groups: nutrition and group (NUTR+EX); non-exercise nutrition only group (NUTR); and exercise only (EX). NUTR+EX and NUTR were provided with instructions and weekly support to follow a nutrition intervention targeting leucine-rich meals. Meals equating to 3g of leucine were translated to user-friendly portion sizes and support was provided through weekly emails and fortnightly phone calls. Exercise training consisted of 24 min of combined resistance and aerobic exercise performed three times per week for 12 weeks. Body composition, by DXA scan, and physical function outcomes were assessed at week 0 (PRE), week 6 (MID) and week 12 (POST).

Data Analysis: A Two-way (group x time) mixed ANOVA was performed to determine changes in response to the intervention within each group, and differences between intervention groups at each time-point. When main or interaction effects were indicated, post-hoc pair-wise comparisons using Bonferroni's adjustment was performed.

Results: Protein intake increased by 79% and 63% in NUTR+EX and NUTR, respectively, and average protein intakes for breakfast, lunch and dinner were ≥30g or ≥0.4g/kg protein, in both groups. Lean body mass (LBM) increased in NUTR+EX only (1.1±1.7%; $P<0.05$) and fat mass increased in NUTR only (3.3±5.5%; $P<0.05$) at POST. 1RM leg press increased across both exercise groups, with the largest increase observed in NUTR+EX POST (33.4±37.7%), which was greater than EX at POST (12.8±16.6%, $P<0.05$). 1RM chest press increased in NUTR+EX (18.1±14.9%; $P<0.05$) and EX (19.2±14.5%; $P<0.05$) at POST, which were both greater than changes in NUTR (7.4±11.0%; $P<0.05$) (both $P<0.05$).

Conclusion: Concurrent exercise training, combined with a leucine-rich nutrition intervention produces a gain in LBM and further augments improvements in lower limb strength, compared to exercise alone. The combination of exercise and nutrition should be strongly emphasised when targeting the prevention and/or treatment of age-related decline in muscle mass and function in older adults.

9.1 Introduction

Ageing is associated with a progressive decline of muscle mass and muscle function, which are major contributors to decrease in quality of life, morbidity and mortality (Fielding *et al.*, 2011; Janssen, 2010). Muscle mass experiences a modest 10% loss between the second and fifth decade of life, however, thereafter, this process is accelerated, in which a 30% loss typically occurs between the fifth and eight decade of life (Lexell, Taylor and Sjostrom 1988). Reduced physical activity with age is considered a strong contributor to the age-related decline of muscle mass, and the manifestation of a reduced sensitivity to the anabolic properties of exercise and exogenous protein, termed 'anabolic resistance', further exacerbates the matter (Burd, Gorissen and Van Loon, 2013; Malafarina *et al.*, 2013; Thompson, 2007; Latham *et al.*, 2004). Exercise and appropriate nutrition intervention remain two of the most modifiable and promising strategies in the prevention and treatment of age-related decline in muscle mass and function.

Resistance exercise training (RET) is established as an effective approach to maintain or improve LBM and strength in older adults (Malafarina *et al.,* 2013; Verdijk *et al.,* 2009). Previous work from our laboratory has demonstrated the efficacy of concurrent exercise training (CET) (a combination of resistance and aerobic exercise) in improving a range of health-related parameters (Timmons *et al.,* In Press). Indeed, concurrent training resulted in greater improvements in gait speed, lower limb strength and a reduction in trunk fat, when compared to aerobic exercise training alone or RET alone. However, in the absence of changes in LBM after 12 weeks of CET, an intervention which combines this training mode with a nutrition intervention that favour muscle accretion in older adults, is of interest in the present study.

Age-related decline of muscle is related to the blunted response to anabolic stimuli (Katsanos *et al.*, 2006; Katsanos *et al.,* 2005; Cuthbertson, 2004), a phenomenon known as 'anabolic resistance'. After protein ingestion, elderly show a decreased sensitivity to the anabolic stimulatory effects of EAAs. However, at a higher protein dose this deficit can be rectified and stimulation MPS above postabsorptive levels is apparent (Katsanos *et al.*, 2006). Indeed, there is now a large body of evidence which supports the thesis that older adults have higher protein requirements when compared to young (Traylor, Gorissen and Phillips, 2018; Nowson and O'Connell, 2015; Bauer *et al.*, 2013). Moreover, a higher quantity of leucine, the key AA in triggering MPS, is required to stimulate MPS above baseline in elderly populations (Katsanos *et al.*, 2006; Katsanos *et al.,* 2005). >20-30g of high quality protein, or that containing ~2.5 to 2.8g leucine per meal

(Layman *et al.*, 2015; Bauer *et al.,* 2013; Symons *et al.*, 2009; Katsanos *et al.*, 2006) is considered the requirement to elicit a near maximal anabolic response in older adults.

Considering the anabolic properties of exercise training and appropriately timed protein/EAA ingestion, it would appear promising that combining both strategies has the potential to elicit greater improvement in muscle mass and function than exercise training alone. Furthermore, exercise increases the sensitivity of skeletal muscle to the anabolic stimulus of exogenous protein and EAA ingestion in young and old (Yang *et al.*, 2012a). While the combined effect of a protein and exercise interventions has shown positive changes on LBM in older adults (Finger *et al.*, 2015), there are also inconsistent findings (Thomas, 2016; Verdijk *et* al., 2009; Leenders *et al.,* 2013). Hence, there is still ambiguity as to the efficacy of protein ingestion alongside exercise training in older adults. At this time, an effective combined nutrition and exercise intervention that has the potential to ameliorate this decline in age-related muscle mass still remains unclear. The combined effect of a concurrent exercise and nutrition intervention, targeting an optimal leucine dose per meal in older adults, represents an obvious gap in the literature. The aim of the present study is to determine if a nutrition intervention, targeting the equivalent of 3g of leucine per meal, eaten at breakfast, lunch and dinner, can augment exercise training-mediated effects on lean body mass and physical function in older adults.

9.2 Methods

9.2.1 Study Design and Participants

Participant recruitment and the broad study design are described in Chapter 8.

9.2.2 Exercise Intervention

NUTR+EX and EX performed three supervised exercise training sessions per week (Monday, Wednesday and Friday) for 12 weeks. This consisted of 12 min of resistance exercise training and 12 min of aerobic exercise training per session. All training sessions were supervised and performed on the Milon Circle (Milon, Germany), a smart card-enabled circuit featuring a combination of eight fully-automated strength (six); leg press, seated row, chest press, lat pulldown, leg extension and triceps dip; and aerobic (two) exercise machines; cross-trainer and cycle ergometer. The exercise session is described in **figure 9.1**. On week 1, resistance exercises were performed for 15 repetitions, for 1 minute, with 30 seconds rest between each station at ~60% 1RM. On week 4, resistance exercises were performed for 12 repetitions. On week 8 resistance exercises were performed for 12 repetitions. Resistance was increased weekly. Aerobic exercise was

maintained at an intensity of 80% predicted heart rate max, for 4 min on the cross-trainer and 4 min on the cycle ergometer. Heart rate was monitored continuously throughout each training session (Polar H7, Finland). NUTR were instructed not to change their habitual physical activity for the duration of the trial.

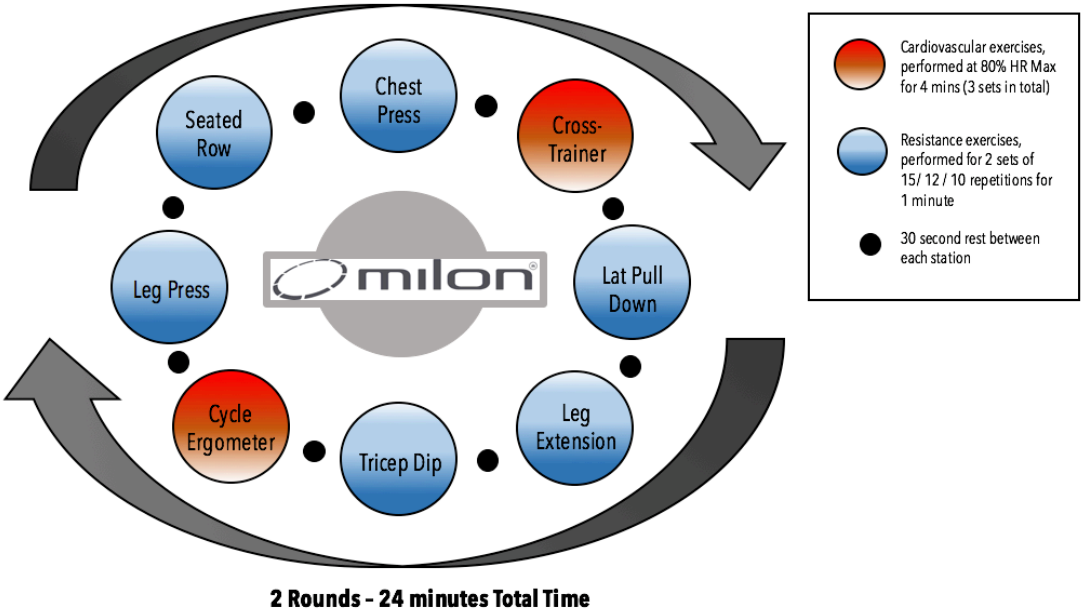


Figure 9.1: Graphic representation of the Milon Circuit. Resistance exercise (blue) were performed for 1 minute, with each exercise performed twice (12 minutes). Cardiovascular exercise (red) were performed for 4 minutes, with participants completing 3 sets of cardiovascular stations (12 minutes). The total time of exercise was 24 minutes. HR, heart rate.

9.2.3 Nutrition Intervention

NUTR+EX and NUTR were instructed to follow a high leucine nutrition intervention, as per chapter 8, for the 12-week period (See **Chapter 8, section 8.2.2** for details on nutrition intervention). EX were asked not to change their habitual dietary intake for the duration of the intervention.

9.2.4 Physical Assessment

The assessment procedure was identical to that carried out in (Timmons *et al.,* In Press). This assessment, which took place over two consecutive days, was carried out at PRE (week 0), MID (week 6) and POST (week 12). On day 1, participants arrived to the lab after an overnight fast. Body mass (to the nearest 0.2 kg) using a calibrated digital scales (SECA, Germany), height (to the nearest 0.01 m) using a wall-mounted stadiometer (Holtain, UK), and body composition by dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, USA) were measured. Supine resting heart rate and blood pressure were then measured in duplicate using an automated blood pressure monitor (Omron, USA). Participants then consumed a small snack (a cereal bar and a banana) and were allowed water ad libitum. Handgrip strength of the dominant hand was then measured to

the nearest 0.5 kg using a hydraulic hand dynamometer (JAMAR, USA). Lower body physical function assessed using the 2.4m timed-up-and-go test (TUGT) and short physical performance battery (SPPB) consisting of habitual gait speed (3m), standing balance (non-tandem and tandem), and five repetition sit-to-stand. Cognitive function was then assessed using Montreal cognitive assessment test (MoCA). Lastly, aerobic fitness was assessed using the Chester step test. On day 2, participants reported to the exercise training facility (Medfit Proactive Healthcare) for the assessment of leg power by stair climbing test (SCT) and lower and upper limb strength by 1 repetition maximum (1RM) on leg press and chest press machines, respectively (Milon, Germany). Prior to the assessment at PRE, a first familiarisation session was performed wherein the correct lifting technique was demonstrated and practiced, after which maximum strength was estimated using the multiple repetitions testing procedure. This informed the assessment of 1RM, which was performed in a second session undertaken one week after the familiarisation session.

9.2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc., USA), and are presented as mean±SD. Differences between groups at baseline (PRE) for all parameters were compared using a one-way ANOVA. A two-way (group x time) between-within ANOVA was performed to determine changes in response to the 12-week intervention within each group, and the differences between groups at each time-point. When main or interaction effects were indicated, post-hoc pair-wise comparisons using Bonferroni's adjustment was performed. Statistical significance was accepted at $P<0.05$.

9.3 Results

9.3.1 Attendance

Attendance at the exercise training sessions averaged 87.4±7.9% throughout the 12-week intervention, and did not differ by training group at 86.3±10.2% and 88.7±4.1%, for NUTR+EX and EX, respectively.

9.3.2 Anthropometric Measures

Anthropometric measures at baseline in each group is presented in **table 9.1**. There were no differences in anthropometric measures between groups at baseline. **Table 9.2** represents percentage change in anthropometric measures from baseline at MID and POST. Body mass increased by ~1% at post in both NUTR+EX and NUTR (both $P<0.05$) but this was in the form of LBM (1.1±1.7%; $P<0.05$) in NUTR+EX (See **figure 9.2B**), and fat mass (3.3±5.5%; $P<0.05$) in NUTR (See **figure 9.2A**). Trunk fat and trunk LBM were unchanged. Arm fat was unchanged in EX and NUTR+EX but increased at POST in NUTR (7.4±10.4%, $P<0.05$).

Arm LBM increased in EX and NUTR+EX at MID (3.0±4.9%; 3.0±3.9%; $P<0.05$) and POST (4.0±5.8%; 3.4±5.5%; $P<0.05$) but was not statistically different in NUTR, as per **figure 9.3A**. Leg fat mass remained unchanged in NUTR+EX but decreased in EX at POST (-2.4±4.3%, $P<0.05$). However, leg fat mass significantly increased in NUTR compared to EX at MID (2.1±4.1%, $P<0.05$) and compared to both EX and NUTR+EX at POST (3.9±5.3%, $P<0.05$). Leg LBM remained statistically unchanged in all three groups, as per **figure 9.3B**.

	NUTR+EX (n=21)	**NUTR (n=16)**	**EX (n=19)**	**ALL (n=56)**	***P* value**
Anthropometric Measures					
M/F (n/n)	11/10	8/8	9/10	28/28	
Age (y)	69.7±4.6	69.3±3.4	68.8±3.8	69.3±4.0	0.769
Height (m)	168.5±8.5	168.7±9.7	167.6±10.4	168.2±9.4	0.933
Body mass (kg)	75.1±13.0	79.0±8.8	72.5±11.6	75.3±11.5	0.255
BMI (kg m^{-2})	26.3±3.0	28.0±4.4	25.8±3.6	26.6±3.7	0.197
Body fat (%)	34.0±5.8	33.8±11.7	33.4±7.5	33.8±8.2	0.978
Fat mass (kg)	24.47±5.83	26.36±11.13	23.28±6.5	24.61±7.84	0.519
LBM (kg)	47.66±9.34	49.92±7.29	46.22±9.05	47.82±8.67	0.459
Arm fat mass (kg)	2.50±0.53	2.59±1.01	2.51±0.75	2.53±0.75	0.923
Arm LBM (kg)	5.38±1.58	5.42±1.27	5.32±1.73	5.37±1.53	0.983
Leg fat mass (kg)	7.40±1.92	8.54±4.33	7.61±2.27	7.80±2.90	0.474
Leg LBM (kg)	15.73±3.30	16.86±2.39	15.20±3.06	15.87±3.01	0.258
Trunk fat mass (kg)	13.63±4.65	14.29±6.45	12.26±4.60	13.35±5.18	0.398
Trunk LBM (kg)	23.13±4.28	24.28±3.73	22.40±4.02	23.21±4.04	0.497
Muscle and Cognitive Function					
RHR (bpm)	63.2±10.2	62.3±8.0	62.4±9.7	62.7±9.3	0.939
SBP (mmHg)	141.6±13.1	133.7±11.8	136.8±15.3	137.7±13.7	0.207
DBP (mmHg)	85.7±10.1	79.4±7.4	81.3±7.1	82.4±8.7	0.069
Hand-grip strength (kg)	31.7±9.1	31.9±11.9	32.3±11.7	32.0±10.6	0.984
Gait speed (m s^{-1})	1.96±0.32	1.97±0.44	1.72±0.35	1.88±0.38	0.077
Sit-to-stand (sec)	10.85±1.94	10.64±3.71	12.67±4.55	11.41±3.57	0.165
TUGT (sec)	5.32±0.94	5.42±1.11	5.59±1.21	5.44±1.07	0.740
SCT (W)	403.5±87.1	385.3±85.9	387.6±92.7	392.9±87.5	0.765
1RM Leg press (kg)	125.9±39.9	129.9±32.5	129.6±56.1	128.3±43.6	0.951
1RM chest press (kg)	41.9±16.0	40.8±16.8	39.4±15.4	40.7±15.8	0.887
Chester step test (bpm)	124.4±11.3	121.4±9.7	125.3±11.3	123.8±10.8	0.548
MoCA	27.0±2.5	26.2±2.3	26.6±1.6	26.7±2.2	0.497

Table 9.1: Participant characteristics at baseline (PRE). 1RM, one-repetition maximum; BMI, body mass index; DBP, diastolic blood pressure; LBM, lean body mass; M/F, male/female; MoCA, Montreal cognitive assessment; RHR, resting heart rate; SBP, systolic blood pressure; SCT, stair climbing test; TUGT, timed up and go test. *P* values are reported from one-way ANOVA by group.

	NUTR+EX (n=21)	NUTR (n=16)	EX (n=19)
Body mass			
%change from PRE to MID	0.3±1.3	1.1±1.8**	-0.1±1.5
%change from PRE to POST	0.7±1.5**	1.2±1.9**	0.1±1.7
% body fat			
%change from PRE to MID	-1.1±2.6	0.6±3.3	-0.2±3.1
%change from PRE to POST	-1.0±3.3	2.4±4.1**$^{\#,\wedge}$	-1.0±3.3
Arm fat mass			
%change from PRE to MID	3.3±9.1	6.0±15.5	0.5±12.1
%change from PRE to POST	1.0±9.6	7.4±10.4*	1.2±9.0
Leg fat mass			
%change from PRE to MID	0.0±3.0	2.1±4.1$^{\#}$	-1.7±3.5
%change from PRE to POST	-0.3±3.6	3.9±5.3***$^{\#,\wedge}$	-2.4±4.3*
Trunk fat mass			
%change from PRE to MID	-2.2±4.0	0.3±6.3	1.8±7.1
%change from PRE to POST	-0.4±5.3	3.4±8.8	1.1±10.7
Trunk lean body mass			
%change from PRE to MID	0.5±2.0	0.0±3.4	0.6±3.2
%change from PRE to POST	0.4±2.0	-0.2±3.7	-3.8±17.7

Table 9.2: Changes in anthropometric measures in response to 12-week intervention for nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *P<0.05, **P<0.01, ***P<0.001 for the annotated time-point and $^{\&}P$<0.05 vs. MID, and between groups at the respective time-point indicated by $^{\S}P$<0.05 vs. NUTR, $^{\#}P$<0.05 vs. EX, and ^P<0.05 vs. NUTR+EX.

	NUTR+EX (n=21)	NUTR (n=16)	EX (n=19)
RHR			
%change from PRE to MID	-1.7±9.9	4.6±14.9	2.1±14.0
%change from PRE to POST	-1.6±11.2	-1.9±9.2	-5.3±7.3
SBP			
%change from PRE to MID	-5.3±9.9	1.5±13.6	-0.9±6.2
%change from PRE to POST	-3.6±11.1	0.6±12.5	-1.1±7.3
DBP			
%change from PRE to MID	-4.7±9.3	-0.5±11.3	-1.9±7.8
%change from PRE to POST	-4.2±9.0	-2.1±9.3	-3.3±5.1
Hand-grip strength			
%change from PRE to MID	9.9±13.7***	8.0±9.8*	7.5±9.5*
%change from PRE to POST	10.4±13.2***	13.6±13.7***	10.8±10.1***
Sit-to-stand			
%change from PRE to MID	-20.2±12.5***	-12.3±13.0***	-14.7±13.1***
%change from PRE to POST	-26.3±9.8***,&	-22.0±12.5***,&	-26.2±14.1***,&
TUGT			
%change from PRE to MID	-9.0±8.5***	-7.8±9.1**	-9.6±10.8***
%change from PRE to POST	-13.6±8.9***	-12.7±10.8***	-10.4±14.5***
Gait speed			
%change from PRE to MID	1.0±9.7	-1.8±13.6	10.2±13.1**
%change from PRE to POST	12.7±14.5***,&	9.3±16.3*,&	21.9±17.8***,&
SCT			
%change from PRE to MID	4.0±10.1	4.5±6.2	1.3±6.9
%change from PRE to POST	10.1±10.3***,&	14.2±12.5***,&	13.5±15.1***,&
Chester step test			
%change from PRE to MID	-9.6±5.8***,§	0.3±7.9	-6.0±4.8***,§
%change from PRE to POST	-10.1±6.5***,§	-5.0±6.8**,&	-11.4±5.5***,&,§
MoCA			
%change from PRE to MID	3.4±10.0	3.5±8.6	2.0±8.0
%change from PRE to POST	5.7±10.7**	6.2±8.1**	3.0±6.1

Table 9.3: Changes in muscle function and cognitive function in response to 12-week intervention for nutrition and exercise group (NUTR+EX), nutrition only group (NUTR) and exercise only group (EX). Post-hoc pairwise comparisons with Bonferroni's adjustment were used to determine where differences existed within groups compared to PRE as indicated by *P<0.05, **P<0.01, ***P<0.001 for the annotated time-point and $^{\&}P$<0.05 vs. MID, and between groups at the respective time-point indicated by $^{\S}P$<0.05 vs. NUTR, $^{\#}P$<0.05 vs. EX, and ^P<0.05 vs. NUTR+EX.

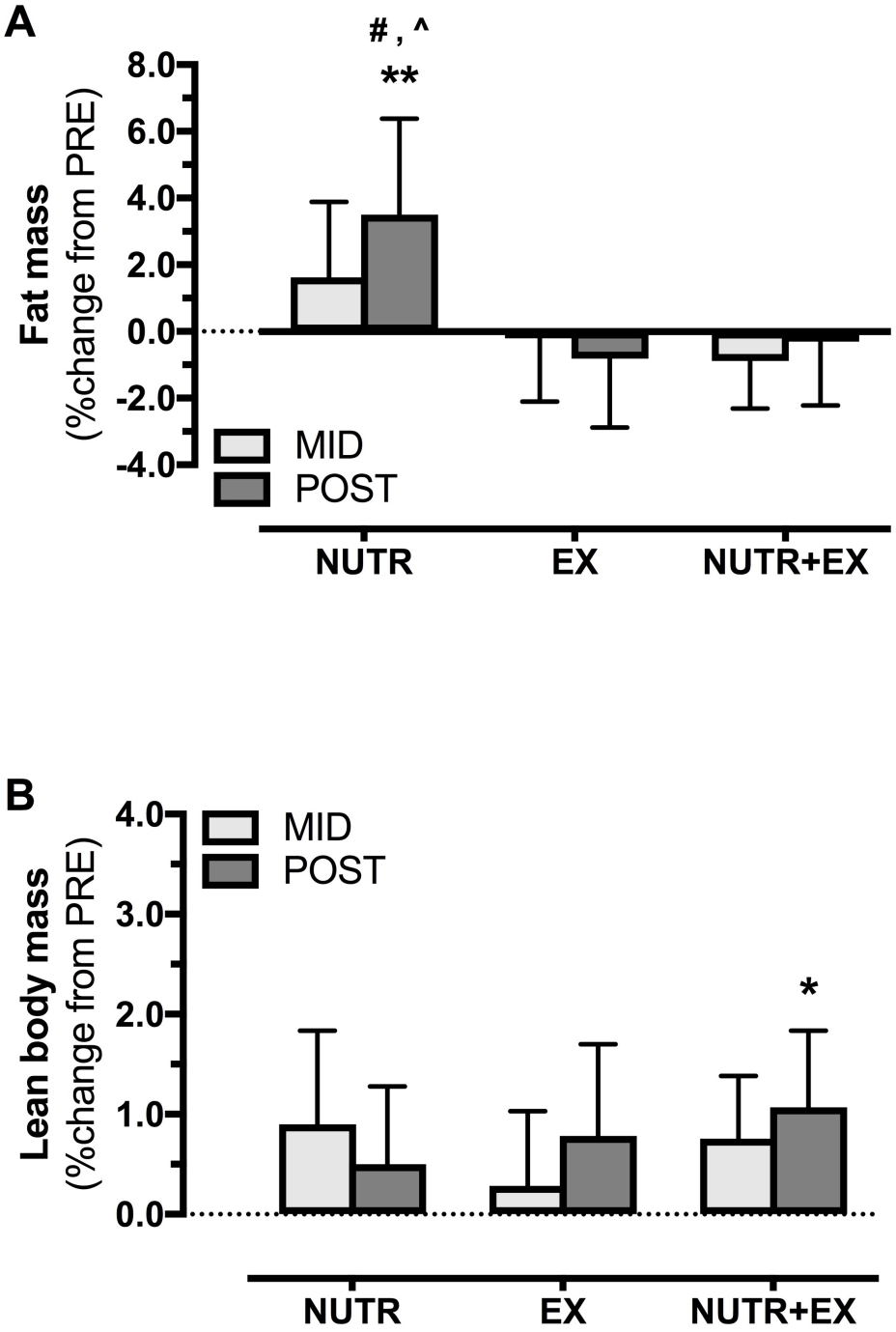


Figure 9.2: Changes in fat mass (A) LBM (B) assessed by DXA scan in response to 12 weeks of nutrition intervention with or without concurrent exercise training; (A) Fat mass; (B), Lean body mass (LBM). Data are mean±SD, representing %change from baseline (PRE) at 6 weeks (MID) and 12 weeks (POST). ***** symbols denote significant difference from PRE for the respective training group; *, $P<0.05$; **, $P<0.01$. # symbol denotes significant difference compared to EX at the respective time-point, $P<0.05$.; ^ symbol denotes significant difference compared to NUTR+EX at the respective time-point, $P<0.05$.

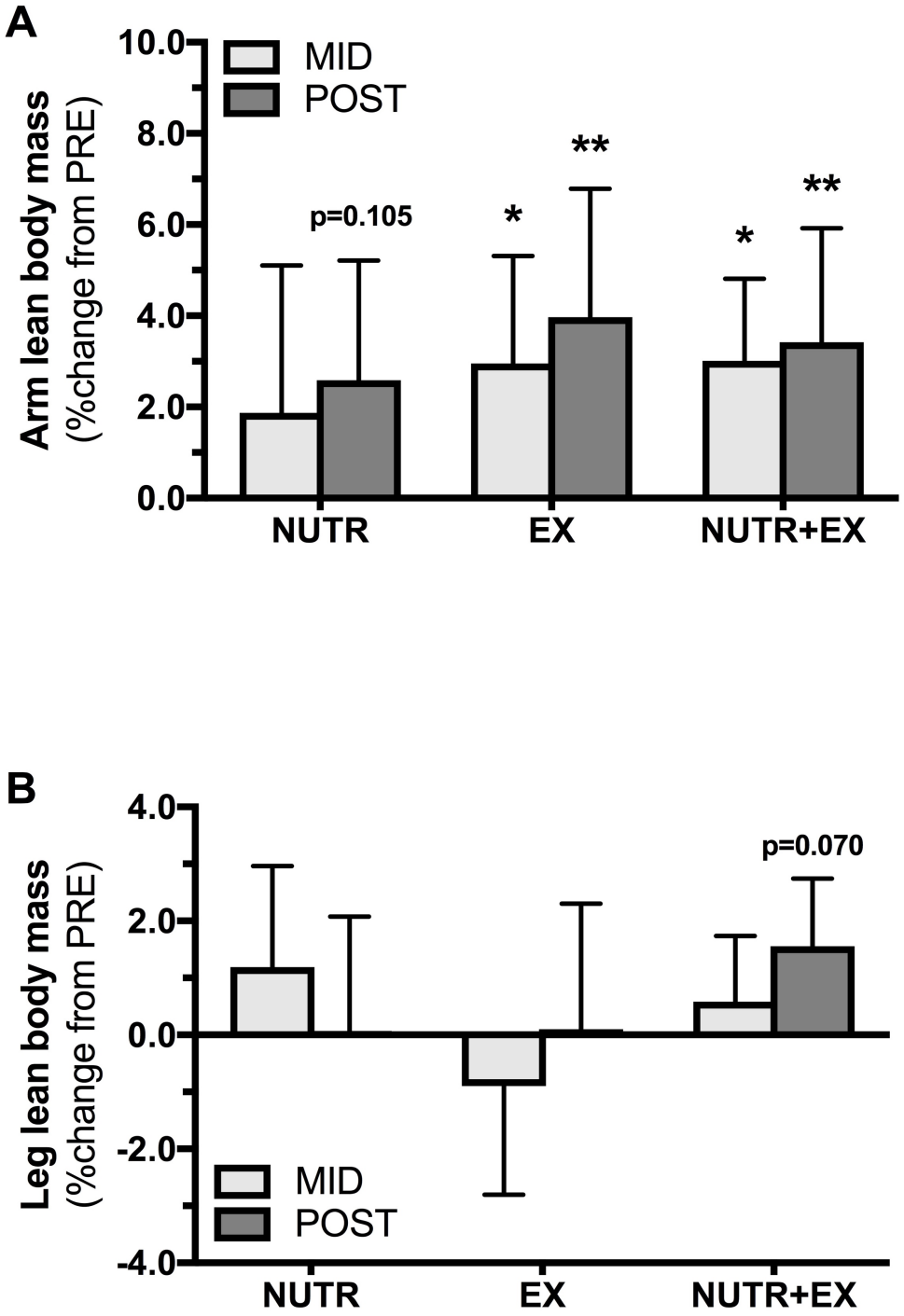


Figure 9.3: Changes in lean body mass of arms (A) and legs (B) assessed by DXA scan in response to 12 weeks of nutrition intervention with or without concurrent exercise training; (A), Arm lean body mass (LBM); (B), Leg lean body mass. Data are mean±SD, representing %change from baseline (PRE) at 6 weeks (MID) and 12 weeks (POST). ***** symbols denote significant difference from PRE for the respective training group; *, $P<0.05$; **, $P<0.01$.

9.3.3 Strength Outcomes

There were no differences between groups at baseline for any strength parameter measured, as per **table 9.1**. **Table 9.3** includes percentage change in strength outcomes from baseline at MID and POST. For upper limb strength, 1RM chest press was increased in NUTR+EX at both MID (9.2±12.3%; $P<0.05$) and POST (18.1±14.9%; $P<0.05$), and was increased at POST in both NUTR (7.4±11.0%; $P<0.05$) and EX (19.2±14.5%; $P<0.05$)(See **figure 9.3A**). These increases observed at POST in both exercise training groups were greater than NUTR (both $P<0.05$). For lower limb strength, 1RM leg press remained unchanged in NUTR but was increased in the two exercise training groups at POST (both $P<0.05$), with the largest increase observed at NUTR+EX POST (33.4±37.7%) being greater (both $P<0.05$) than NUTR+EX MID (13.5±20.8%) and EX POST (12.8±16.6%)(See **figure 9.3B**). Leg power was unchanged at MID but improved at POST in all groups (NUTR, EX and NUTR+EX as 14.2±12.5%, 13.5±15.1%, and 10.1±10.3%, respectively; all $P<0.05$).

9.3.4 Physical Function

Table 9.3 includes percentage change in physical outcome measures from baseline at MID and POST. Handgrip strength increased in all three groups by MID (8.0±9.8%, 7.5±9.5%, and 9.9±13.7% for NUTR, EX and NUTR+EX, respectively; all $P<0.05$), and POST (13.6±13.7%, 10.8±10.1%, and 10.4±13.2% for NUTR, EX and NUTR+EX respectively; all $P<0.05$). Performance in the sit-to-stand test was improved at MID (with completion times decreasing by -12.3±13.0%, -14.7±13.1%, and -20.2±12.5% for NUTR, EX and NUTR+EX, respectively; all $P<0.05$) and improved further at POST compared to MID (with completion times compared to PRE decreasing by -22.0±12.5%, -26.2±14.1%, and -26.3±9.8% for NUTR, EX and NUTR+EX, respectively; all $P<0.05$). Performance in the TUGT was improved at MID (with completion times decreasing by -7.8±9.1%, -9.6±10.8%, and -9.0±8.5% for NUTR, EX and NUTR+EX, respectively; all $P<0.05$) and POST (with completion times decreasing by -12.7±10.8%, -10.4±14.5%, and -13.6±8.9% for NUTR, EX and NUTR+EX, respectively; all $P<0.05$). Gait speed was only increased in EX by MID (10.2±13.1%; $P<0.05$) but was increased for all three groups at POST compared to both PRE and MID (9.3±16.3%, 21.9±17.8%, and 12.7±14.5% vs. PRE for NUTR, EX and NUTR+EX, respectively; all $P<0.05$).

9.3.5 Aerobic Fitness

Table 9.3 includes percentage change in aerobic fitness from baseline at MID and POST. The changes in resting heart rate, systolic and diastolic blood pressure did not reach statistical significance in any group. Aerobic fitness improved at MID in EX and NUTR+EX compared to NUTR (with beats per minute decreasing by -6.0±4.8%; -9.6±5.8%; $P<0.05$). At POST, aerobic fitness was significantly increased in NUTR compared to PRE

and MID (with beats per minute decreasing by -5.0±6.8% vs. PRE; $P<0.05$). However, increases in aerobic fitness in EX and NUTR+EX were significant at both MID and POST (with beats per minute at POST decreasing by -11.4±5.5% and -10.1±6.5% for EX and NUTR+EX respectively, both $P<0.05$), and both exercise training groups resulting in larger improvements in aerobic fitness compared to NUTR at both MID and POST (all $P<0.05$).

9.3.6 Cognitive Function

Table 9.3 includes percentage change in cognitive function from baseline at MID and POST. Cognitive function improved at POST in NUTR and NUTR+EX (6.2±8.1% and 5.7±10.7%; $P<0.05$), with EX remaining unchanged.

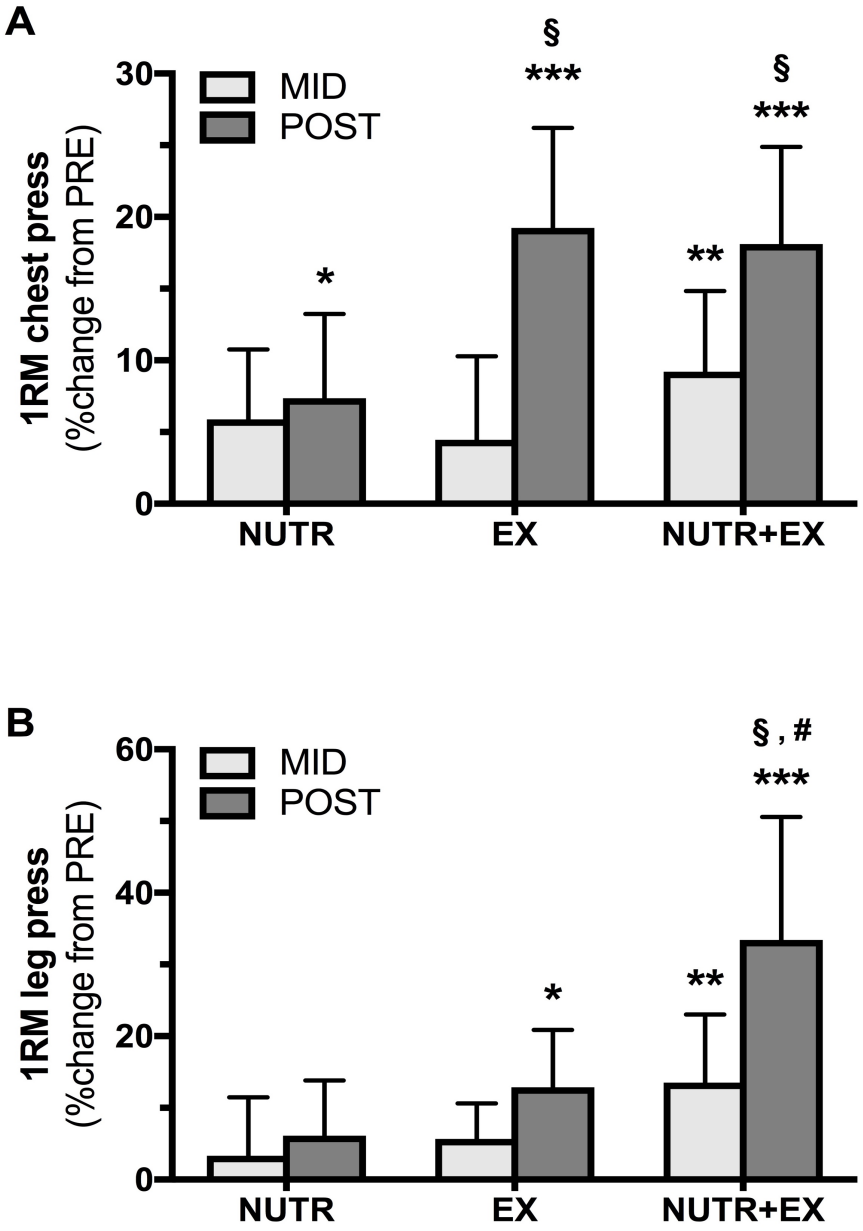


Figure 9.4: Changes in upper (A) and lower (B) limb muscle strength assessed by 1RM in response to 12 weeks of nutrition supplementation with or without concurrent exercise training; (A), Chest press; (B), Leg press. Data are mean±SD, representing %change from baseline (PRE) at 6 weeks (MID) and 12 weeks (POST). ***** symbols denote significant difference from PRE for the respective training group; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. § symbol denotes significant difference compared to NUTR at the respective time-point, $P<0.05$.; # symbol denotes significant difference compared to EX at the respective time-point, $P<0.05$.

9.4 Discussion

This present study confirms the efficacy of concurrent aerobic and resistance exercise training in improving a range of parameters related to body composition, physical and cognitive function in older adults. This is in agreement with previous findings from our laboratory (Timmons *et al.,* In Press), which showed that concurrent aerobic and resistance exercise resulted in changes in physical and cognitive function over 12 weeks, and was superior to either aerobic or resistance exercise training alone across a range of parameters. Furthermore, co-intervention with a high protein diet, targeting the equivalent of 3g of leucine per meal, eaten at breakfast, lunch and dinner, augmented some, but not all, training outcomes. Most notably, the nutrition intervention further improved training-mediated increases in LBM and lower limb strength. This same nutrition intervention, in the absence of exercise training, resulted in some improvements in physical function, but notably resulted in an increase in body fat mass with no changes in LBM.

The effects of protein supplementation in combination with exercise, on changes in muscle mass and function, in older adults have been widely examined, with conflicting findings (Liao *et al.,* 2017; Thomas *et al.,* 2016; Finger *et al.,* 2015; Cermak *et al.,* 2012). Potential explanations for these discrepancies are divergent inclusion criteria for analyses, in particular, the inclusion of health and/or non-healthy, active and/or ambulatory individuals and different age cut-offs. The potential to benefit from a nutrition and exercise intervention is often greater for those who are unhealthy or have low habitual physical activity. Meanwhile, the inclusion of adults aged <65 years may be misrepresentative of an 'elderly' cohort who exhibit anabolic resistance (Morton and Phillips, 2018). The present study is novel in that the effect of a whole food-based nutrition intervention, targeting leucine-rich meals, with concurrent exercise training in older adults has not been previously explored. Both NUTR+EX and NUTR groups were instructed and supported in consuming a nutrition strategy, providing the equivalent of 3 g leucine at three main meals, with the aim of enhancing the exercise training-mediated changes in LBM and physical function. This is consistent with guidelines which advocate consuming ~3g leucine per meal to elicit a maximal anabolic response in older adults (Phillips, 2015; Katsanos *et al.*, 2006). The nutrition intervention resulted in an increase in daily protein intake of both nutrition groups from ~1.0 g/kg/d to ~1.5 g/kg/d, and a more even distribution of protein intake across three meals, in which average protein intake at breakfast, lunch and dinner were ≥30g and/or ≥0.4g/kg per meal threshold to maximise postprandial MPS in older adults (Moore *et al.*, 2015; Symons *et al.*, 2009). Further details of the nutrition intervention and changes in dietary intakes are described in Chapter 8.

NUTR+EX produced a 0.53±0.86kg increase in LBM. This is in contrast to finding by Iglay *et al.*, (2009), in which 12 weeks RET x3 days per week was combined with either a low (LP) or high protein (HP) diet consisting of eggs, meat and dairy, in older adults and no change in LBM occurred. Although the aim in the low protein and high protein group was to achieve 0.8g/kg/d and 1.6g/kg/d protein, respectively, the dietary protein intake achieved by the intervention was 0.9±0.1g/kg/d (LP) or 1.2±0.0g/kg/d (HP). Baseline protein intake across both groups was 1.1±0.1g/kg, therefore, it is likely that the modest ~0.1g/kg/d increase in protein intake in HP throughout the intervention was insufficient to elicit a greater increase in LBM. Our results are also in contrast with previous findings (Arnarson *et al.*, 2013; Verdijk *et al.*, 2009; Godard, Williamson and Trappe, 2002), in which protein supplementation resulted in no further enhancements in LBM and muscle strength when combined with RET. However, a noticeable trend in the latter studies are the use of one single bolus supplement (Godard, Williamson and Trappe, 2002), or the administration of the supplement only on training days (Arnarson *et al.*, 2013: Verdijk *et al.*, 2009). Therefore, a plausible explanation for the positive outcomes on LBM and strength in the present study is that protein distribution was optimised, and all three main meals reached the meal threshold purported to maximise MPS (See Chapter 8), thereby resulting in muscle accretion over time. In support, Daly *et al.* (2014) reported similar improvements in LBM and strength when 45g protein from red meat was split between two daily meals, and combined with RET over 12 weeks. Similarly, Tieland, *et al.*, (2012a) reported positives changes in LBM when breakfast and lunch were enriched with 15g protein. Multiple adequate protein meals (4 x 20g) throughout the day can result in a greater cumulative anabolic response compared to smaller and frequent meals, or larger and less-frequent protein meals in young (Areta *et al.*, 2013; Moore *et al.*, 2012). Therefore, focusing on achieving >1 meal over the anabolic threshold may result in more beneficial outcomes for muscle hypertrophy and strength in older adults (Layman *et al.*, 2015; Bauer *et al.*, 2013).

Concurrent exercise training has been established as an effective strategy to increase strength and function in older adults (Timmons *et al.,* In Press; Holviala *et al.*, 2010; Sillanpää *et al.*, 2009; Wood *et al.*, 2001). Indeed, both training groups (NUTR+EX and EX) experience improvements in strength (handgrip, upper and lower limb strength) and all functional parameters (sit-to-stand, timed-up-and-go test, stair climbing power test and gait speed) after 12 weeks. While there was a positive effect on handgrip strength, upper limb strength and physical function in NUTR alone and EX alone, the combination of both nutrition and training in NUTR+EX was more effective, particularly for lower limb strength. The considerable improvement in lower limb strength in NUTR+EX, compared to EX, which has been reported elsewhere (Daly *et al.*, 2014; Chalé *et al.*, 2013) and

supports the synergistic effect of exercise and nutrition in targeting the age-related decline in muscle strength.

Participants in NUTR produced an increase in total fat mass (FM) (+0.71±1.15kg) with a negligible change in LBM (+0.03±1.30kg) from PRE to POST. These results are in contrast with Norton *et al.*, (2016) who reported minimal change in FM and a positive change in LBM when breakfast and lunch were enriched with whey protein, in the absence of exercise. Similar to the present study, participants increased daily protein intake from 1.2g/kg/d to 1.6g/kg/d and the focus of the additional protein was to enrich breakfast and lunch, in which a protein intake of 0.4g/kg and 0.47g/kg was achieved. An increase of 0.24kg FM and 0.45g in LBM was apparent in the protein group, compared to an isocaloric maltodextrin control group, who produced a 0.06kg loss in FM and 0.16kg loss in LBM over the 24-week period. Participants in the protein supplement group reported a modest 5% increase in calorie intake. Meanwhile, in the present study, there was a 20.7% increase in energy intake in NUTR, with no changes in exercise habits, and therefore energy expenditure. These data suggest that in the absence of exercise, there was a propensity for the excess calories to be deposited as body fat. Excess adipose tissue, particularly in the abdominal area, is a major risk factor for morbidity and mortality during the ageing process (Kopelmam, 2000), therefore the present outcome of the ab libitum approach to energy intake, in the absence of exercise, warrants concern. The positive changes in LBM reported by Norton *et al.,* (2016) may be explained by the age of the participants (50-70 years), which may not be entirely representative of an 'elderly' cohort, who exhibit anabolic resistance to dietary protein (Morton *et al.*, 2018). In the absence of changes in LBM, the improvements in functional capacity apparent in NUTR may appear surprising. However, increases in muscle strength and function with protein supplementation, in the absence of exercise and measurable changes in lean mass is reported elsewhere (Kim and Lee, 2013; Tieland *et al.*, 2012b). Furthermore, a weak correlation exists between changes in muscle size and change in muscle strength following and training intervention is well-reported (Buckner *et al.*, 2016). Nonetheless, these findings suggest that a potentially clinically significant improvement in physical function can be achieved without a need for a sizeable increase in LBM.

The main limitation to the present study is the lack of a true (non-intervention) control group. The positive findings on functional outcomes in relation to NUTR cannot be entirely attributed to the daily consumption of added protein alone. There may be other mitigating factors regarding the physical and cognitive improvements, such as changes in physical activity. Of note, participants were recruited under the premise that they would be involved in a nutrition and/or training study. Due to their interest in participation, one would

assume that these individualised would be eager to introduce a change in both nutrition and exercise for the upcoming 12 weeks. It is possible that, although asked not to introduce any changes in physical activity, that individuals in the NUTR did indeed change their physical activity, thereby rendering improvements in physical and cognitive function.

An even protein intake per meal is reported to increase daily MPS rates (Mamerow *et al.,* 2014; Areta *et al.,* 2013), and has been proposed as a strategy that should be targeted to attenuate the age-related decline of LBM (Arentson-Lantz *et al.*, 2015). This has been demonstrated in older adults with (Daly *et al.*, 2014) and without exercise (Bauer *et al.,* 2015). In NUTR+EX (and NUTR) there was a focus on achieving equal distribution of protein per meal, and reaching the ≥30g and/or ≥0.4g/kg per meal threshold. However, we cannot attribute the positive changes in LBM in NUTR+EX entirely to a more 'even' distribution of protein alone, as the 79% increase in daily protein intake very likely played a role in skeletal muscle accretion. Moreover, the role of equal distribution of protein across meals yields conflicting findings (Kim *et al.*, 2015; Bouillanne *et al.*, 2013; Arnal *et al.,* 1999). It remains to be determined if the distribution of protein plays a large role in skeletal mass accretion and/or retention. Alternatively, it is possible that simply promoting an equal distribution of protein throughout the day inadvertently encourages an increase in protein intake at breakfast and lunch (two meals often low in protein, (Cardon-Thomas *et al.*, 2017; Tieland *et al.*, 2015), thereby increasing overall daily protein intake and resulting in positive changes in LBM. Nonetheless, promoting a greater protein intake at all three meals in the present study resulted in positive changes in LBM compared to either a nutrition or exercise control. While the mechanism (increase in total protein, a more even distribution of protein, or both?) is not fully understood, the results are promising and shed light on an effective nutrition and exercise strategy in promoting gains in skeletal muscle mass and physical function in older adults.

9.5 Conclusion

In summary, concurrent exercise training alone is efficacious in improving physical and cognitive function in older adults over 12 weeks. However, increasing daily protein consumption to 1.5g/kg body mass, and targeting the equivalent of 3g leucine per meal, is necessary to elicit gains in LBM and further augment improvements in lower limb strength. This study is unique in that participants followed a whole food nutrition strategy, as opposed to relying on powdered protein and oral nutrition solutions. Considering the positive outcomes achieved with the addition of the nutrition intervention to exercise, as well as some of the potentially deleterious outcomes when the same nutrition intervention is administered in the absence of exercise, the

combination of exercise and nutrition should be strongly emphasised when targeting the age-related decline in muscle mass and function in older adults.

Chapter 10

10.0 General Discussion

10.1 Introduction

The amino acid leucine is seen as a critical driver of the muscle protein synthetic response to a meal. Therefore, the use of free leucine as a supplement, and in particular, the enrichment of lower leucine foods, has received much interest in recent years. Indeed, leucine-enrichment, in which free leucine is added to a meal or food matrices, is emerging as a promising means of 'rescuing' the inferior anabolic response apparent after the ingestion of low leucine/plant-based protein sources and meals. Therefore, this thesis aimed to address several knowledge gaps around leucine supplementation and protein intakes in exercise, health and ageing contexts. Firstly, the postprandial leucinemia achieved following the ingestion of a free leucine with different dose and timing regimens, or in response to leucine-enriched meal was not previously established. Secondly, amino acid supplementation may play a role in exercise recovery by minimising the negative effects of exercise-induced muscle damage after intense exercise, but the role of leucine in particular in these recovery processes was not well-investigated. Lastly, the prescription to consume leucine-rich foods may be efficacious in preventing or treating the decline in muscle mass and function apparent with advancing age, but a whole food-based dietary strategy, which specifically targets leucine-rich meals, in augmenting exercise training-mediated changes in skeletal muscle mass and function, had not been previously investigated.

Therefore, the aims of the present thesis were as follows;

- To determine the current habitual dietary protein intake and protein distribution in Irish adults across the lifespan
- To determine how different leucine supplementation strategies and leucine-enriched meals affect postprandial plasma leucinemia
- To investigate the application of these supplementation strategies in recovery from intense exercise in young healthy males
- To investigate if a high protein diet, targeting leucine-rich meals, augments exercise-mediated effects on LBM and function over a 12-week period in older adults

10. 2 Plasma Kinetics Following Free Leucine and a Leucine-Enriched Mixed Meal

Despite plasma leucine representing the most important amino acid for stimulating postprandial muscle protein synthesis, the plasma kinetics after the ingestion of leucine alone, and in combination with a meal,

were underexplored. The aim of chapter 5 was to determine the plasma leucine kinetics after the ingestion of free leucine in a 3g bolus, or two 1.5g boluses, separated by 2 hours, on plasma kinetics, compared to a maltodextrin control. In chapter 6, plasma leucine kinetics were determined after these same doses and timing strategies were co-ingested with a mixed meal. The presence of macronutrients and fibre in the mixed meals in chapter 6 results in a delayed absorption of leucine into the circulation and attenuated the increase in leucinemia, compared to free leucine ingested in chapter 5. It appears that postprandial leucinemia is strongly compromised by the co-ingestion of a mixed meal. This is an important consideration when aiming to increase the anabolic potential of a meal through leucine-enrichment, since the purported 'leucine threshold' is seen as as a critical driver of the magnitude of postprandial MPS. Indeed, a higher dose of leucine may be required when enriching a mixed meal in order to create the desired leucine plasma kinetics associated with maximal postprandial MPS. While it is well supported that plasma leucinemia has an influence on the postprandial MPS response, the optimal plasma concentration and kinetics for maximising magnitude and duration of MPS remain to be determined. Future research that investigates the efficacy of leucine-enrichment on acute changes of MPS, and long-term changes in lean body mass (LBM), should investigate the postprandial plasma kinetics after the ingestion of the leucine-enriched meal, thereby offering more of an insight into the ideal plasma leucine kinetics for maximal stimulation of MPS. Noteworthy, the modulatory effect of plasma leucine kinetics has been contested in recent years. Indeed, plasma leucinemia is not the only factor that influences the anabolic effect of a meal and the potential relevance of factors such as the accompanying milieu of amino acids and the co-ingestion of macronutrients and micronutrients should not be discounted. Furthermore, although beyond the scope of the current thesis, factors such as muscle perfusion, intramuscular amino acid availability and amino acid sensing are important determinants of the muscle protein synthetic response. The relationship between these factors, the plasma leucine kinetics and the stimulation of MPS are all worthy of further study.

10.3 The Role of Leucine in Ameliorating the Deleterious Effects of Intense Resistance Exercise

The use of protein and amino acids in the post-exercise window is emerging as a potential strategy for ameliorating the deleterious effects of intense exercise in producing exercise-induced muscle damage and prolonged impairment of physical function. The aim of chapter 7 was to investigate the efficacy of 12g leucine, either bolus- or pulse-fed, in the 14 h post-exercise period, on recovery of muscle function, markers of muscle damage and perceived soreness following an intense resistance exercise bout. Our findings suggest that

leucine supplementation offered no benefit on recovery of muscle function, markers of muscle damage and perceived soreness following an intense resistance exercise bout. However, the study design and outcome measures used had several limitations, which may have increased the likelihood of a type 2 error i.e. false negative. In particular, the cross-over design used may have resulted in interference from the repeated bout effect, in which the first exercise trial created the most amount of muscle damage, thereby resulting in a substantial adaptation and rendering a dampened response to subsequent exercise trials. Furthermore, participants were perhaps not given adequate time to rest from their own training prior to each exercise trial, which meant that true 'rested' measures were not being established, particularly for blood markers of muscle damage. Another plausible explanation for the lack of benefit for leucine on exercise recovery is that that the meal plan provided sufficient protein to recover from intense exercise, and the additional leucine offered no further advantage on recovery. Future research should combine leucine supplement strategy with suboptimal protein intake from food, or less than that prescribed in the present study (1.2g/kg of protein). This may shed light on the true effect of leucine on ameliorating the negative effect of intense exercise.

10.4 Protein and Healthy Ageing

The age-related decline in skeletal muscle mass and function is a fundamental threat to ageing with independence and a good quality of life. Appropriate exercise and nutrition play a key role in the growth and maintenance of skeletal muscle. However, there remains debate about the efficacy of exercise and/or nutrition in ameliorating the age-related decline in muscle mass and function.

Firstly, chapter 4 sought to determine the age and gender patterns for protein intake, protein distribution and protein source in Irish adults between 2008-2010, with the aim of identifying areas where nutrition may improve the outcome of those at risk of declining muscle mass with age. Through secondary analysis of the National Adult Nutrition Survey, a survey of 1500 adults aged 18 and upwards, we demonstrated that protein intake is greatest in young adults (aged 18-35 y), and is lower with increasing age, with the lowest protein intake occurring in adults aged ≥65 y. Furthermore, the purported 'meal thresholds' of protein required to elicit maximal postprandial MPS tends to be lower with increasing age, and lowest in adults aged ≥65 y. Of interest, breakfast was the lowest total protein- and animal protein-containing main meal across all age categories, and may represent an opportunity for improving overall protein intake, and protein distribution, particularly in older adults. Indeed, a high protein nutrition intervention targeting 0.4g/kg protein at breakfast and lunch has previously been shown to be effective in increasing LBM in middle aged adults over 24 weeks

(Norton *et al.*, 2016). Therefore, targeting 'per meal' protein intake, particularly breakfast and lunch, which are often lower in protein intake compared to dinner, may be a promising strategy in combatting the age-related decline in muscle mass and function.

Since dietary protein, and particularly leucine, play a key role in postprandial anabolism and these effects are enhanced by acute exercise, it follows that providing the equivalent of 3g leucine from whole food sources at three meals per day would positively influence exercise training-mediated changes in LBM. Chapter 8 sought to determine if a leucine-rich, whole food-based nutrition intervention was effective in increasing protein intake, and creating a daily protein distribution pattern that is considered most optimal for maximising daily rates of MPS. Our findings in chapter 8 suggest that a nutrition intervention, targeting 3g leucine per meal from whole foods, resulted in substantial increases in protein intake, energy intake, and a more even distribution of protein intake (≥0.4g/kg protein per meal).

The aim of chapter 9 was to investigate if this same nutrition intervention, in the presence of exercise, could augment exercise training-mediated effects on body composition and physical function in older adults. In the presence of concurrent aerobic and resistance exercise training (three times per week), the nutrition co-intervention resulted in positive changes in LBM and muscle function compared to exercise or nutrition alone. Of interest, in the absence of exercise, the nutrition intervention resulted in an increase in body fat mass, with no change in LBM, and an increase in some assessments of muscle function over 12 weeks. These results suggest that a high protein nutrition intervention, targeting leucine-rich meals, in combination with exercise training, is efficacious in increasing LBM and improving muscle function. However, in the absence of exercise, excess energy in the same nutrition intervention had a propensity to be deposited as body fat. The main limitation in the present study is the lack of a true (non-intervention) control group, which makes it difficult to attribute improvements in functional outcomes solely to the daily consumption of added protein alone. While the mechanism which underpins these improvements (increase in total protein, a more even distribution of protein, or a combination of both) is not fully understood, the results are promising and shed light on an effective nutrition and exercise strategy in promoting positive change in LBM and muscle function in older adults. Future research in this area should focus on intervention studies which determine the true impact of protein distribution on muscle accretion, possibly over longer time frames, in older adults. Furthermore, much of the research in older adults has focused on the use of powdered protein and oral nutrition solutions for additional protein, making chapter 8 and 9 novel, since the supplementary protein was derived solely from

whole foods. However, while this nutrition intervention was successful in increasing protein intake and improvement protein distribution, the deteriorating appetite in older adults is a factor which may impede the ability of older adults to stick to an entirely whole food-based high-protein diet, particularly those who are unwell, have swallowing difficulties, are malnourished, or confined to nursing homes and so on. This factor needs consideration when designing a long-term, sustainable nutrition strategy that targets preservation of LBM in older adults. For these reasons, there may be benefits to supplementation with powdered protein and EAAs, or leucine-enrichment of small meals in older adults. Future research should focus on the role of leucine-enrichment of main meals in supporting exercise training-mediated changes in muscle mass and function, as this may offer a more pragmatic strategy for enhancing the anabolic potential of meals in older adults.

10.5 Future Research

In summary, there is a wealth of research to support the efficacy of leucine, administered within an amino acid mixtures and protein powders, in increasing postprandial leucinemia and muscle protein synthesis. However, the plasma leucinemia and the resultant muscle protein synthetic response, following leucine ingestion with a mixed meal (which is representative of leucine-enrichment in a real-world setting), remains underexplored and warrants further investigation. Furthermore, while the application of amino acids supplemented in the post-exercise period shows promise in accelerating recovery from exercise-induced muscle damage, the role of solely supplementary leucine and leucine-enrichment is a potential focus for future research. Finally, with the challenges faced by older adults in consuming adequate dietary protein and energy to maintain muscle mass and function, the investigation of leucine-enrichment of smaller meals in ameliorating the age-related decline in muscle mass represents an area for future research.

References

Abdulla, H., Smith, K., Atherton, P. J., and Idris, I. (2016). Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis. *Diabetologia*, *59*(1), 44–55. https://doi.org/10.1007/s00125-015-3751-0

Achour, L., Méance, S., and Briend, A. (2001). Comparison of gastric emptying of a solid and a liquid nutritional rehabilitation food. *European Journal of Clinical Nutrition*, *55*(9), 769–772. https://doi.org/10.1038/sj.ejcn.1601221

Adibi, S. A., and Gray, S. J. (1967). Intestinal Absorption of Essential Amino Acids in Man. *Gastroenterology*, *52*(5), 837–845. https://doi.org/10.1016/S0016-5085(67)80071-4

Almoosawi, S., Prynne, C. J., Hardy, R., and Stephen, A. M. (2013). Time-of-day and nutrient composition of eating occasions: Prospective association with the metabolic syndrome in the 1946 British birth cohort. *International Journal of Obesity*, *37*(5), 725–731. https://doi.org/10.1038/ijo.2012.103

Arentson-Lantz, E., Clairmont, S., Paddon-Jones, D., Tremblay, A., and Elango, R. (2015). Protein: A nutrient in focus [1]. *Applied Physiology, Nutrition, and Metabolism*, *40*(8), 755–761. https://doi.org/10.1139/apnm-2014-0530

Areta, J.L., Burke, L.M., Ross, M.L., Camera, D.M., West, D.W., Broad, E.M., Jeacocke, N.A., Moore, D.R., Stellingwerff, T., Phillips, S.M. and Hawley, J.A. (2013). Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. *Journal of Physiology*, *591*(9), 2319–2331. https://doi.org/10.1113/jphysiol.2012.244897

Arnal, M.A., Mosoni, L., Boirie, Y., Houlier, M.L., Morin, L., Verdier, E., Ritz, P., Antoine, J.M., Prugnaud, J., Beaufrère, B. and Mirand, P.P (1999). Protein pulse feeding improves protein retention in elderly women. *American Journal of Clinical Nutrition*, *69*(6), 1202–1208. https://doi.org/10.1093/ajcn/69.6.1202

Arnarson, A., Gudny Geirsdottir, O., Ramel, A., Briem, K., Jonsson, P. V., and Thorsdottir, I. (2013). Effects of whey proteins and carbohydrates on the efficacy of resistance training in elderly people: Double blind, randomised controlled trial. *European Journal of Clinical Nutrition*, *67*(8), 821–826. https://doi.org/10.1038/ejcn.2013.40

Astrup, A. (2005). The satiating power of protein - A key to obesity prevention? *American Journal of Clinical Nutrition*, *82*(1), 1–2. https://doi.org/82/1/1 [pii]

Atherton, P. J., and Smith, K. (2012). Muscle protein synthesis in response to nutrition and exercise. *Journal of Physiology*, *590*(5), 1049–1057. https://doi.org/10.1113/jphysiol.2011.225003

Atherton, P. J., Smith, K., Etheridge, T., Rankin, D., and Rennie, M. J. (2010a). Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. *Amino Acids*, *38*(5), 1533–1539. https://doi.org/10.1007/s00726-009-0377-x

Atherton, P.J., Etheridge, T., Watt, P.W., Wilkinson, D., Selby, A., Rankin, D., Smith, K. and Rennie, M.J. (2010b). Muscle full effect after oral protein: Time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *American Journal of Clinical Nutrition*, *92*(5), 1080–1088. https://doi.org/10.3945/ajcn.2010.29819

Babault, N., Païzis, C., Deley, G., Guérin-Deremaux, L., Saniez, M. H., Lefranc-Millot, C., and Allaert, F. A. (2015). Pea proteins oral supplementation promotes muscle thickness gains during resistance training: a double-blind, randomized, Placebo-controlled clinical trial vs. Whey protein. *Journal of the International Society of Sports Nutrition*, *12*(1), 3.

Bano, G., Trevisan, C., Carraro, S., Solmi, M., Luchini, C., Stubbs, B., Manzato, E., Sergi, G. and Veronese, N. (2017). Inflammation and sarcopenia: A systematic review and meta-analysis. *Maturitas*, *96*, 10-15

Bauer, J.M., Verlaan, S., Bautmans, I., Brandt, K., Donini, L.M., Maggio, M., McMurdo, M.E., Mets, T., Seal, C., Wijers, S.L. and Ceda, G.P. (2015). Effects of a vitamin D and leucine-enriched whey protein nutritional supplement on measures of

sarcopenia in older adults, the PROVIDE study: a randomized, double-blind, placebo-controlled trial. *Journal of the American Medical Directors Association*, *16*(9), 740-747.

Bauer, J., Biolo, G., Cederholm, T., Cesari, M., Cruz-Jentoft, A.J., Morley, J.E., Phillips, S., Sieber, C., Stehle, P., Teta, D. and Visvanathan, R., (2013). Evidence-based recommendations for optimal dietary protein intake in older people: A position paper from the prot-age study group. *Journal of the American Medical Directors Association*, *14*(8), 542–559. https://doi.org/10.1016/j.jamda.2013.05.021

Bautmans, I., Gorus, E., Njemini, R., and Mets, T. (2007). Handgrip performance in relation to self-perceived fatigue, physical functioning and circulating IL-6 in elderly persons without inflammation. *BMC Geriatrics*, *7*(8), 755–63. https://doi.org/10.1186/1471-2318-7-5

Beasley, J. M., Shikany, J. M., and Thomson, C. A. (2013). The role of dietary protein intake in the prevention of sarcopenia of aging. *Nutrition in Clinical Practice*. https://doi.org/10.1177/0884533613507607

Beelen, J., de Roos, N. M., and de Groot, L. C. (2017). Protein enrichment of familiar foods as an innovative strategy to increase protein intake in institutionalized elderly. *Journal of Nutrition, Health and Aging*, *21*(2), 173–179. https://doi.org/10.1007/s12603-016-0733-y

Betts, J. A., Toone, R. J., Stokes, K. A., and Thompson, D. (2009). Systemic indices of skeletal muscle damage and recovery of muscle function after exercise: effect of combined carbohydrate–protein ingestion. *Applied Physiology, Nutrition, and Metabolism*, *34*(4), 773–784. https://doi.org/10.1139/H09-070

Biolo, G., Tipton, K. D., Klein, S., and Wolfe, R. R. (1997). An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *The American Journal of Physiology*, *273*(1 Pt 1), E122–E129. https://doi.org/10.1152/ajpendo.1997.273.1.E122

Bohé, J., Low, J. F., Wolfe, R. R., and Rennie, M. J. (2001). Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. *The Journal of Physiology*, *532*(2), 575–579. https://doi.org/PHY_12181 [pii]

Boirie, Y., Gachon, P., and Beaufrère, B. (1997). Splanchnic and whole-body leucine kinetics in young and elderly men. *American Journal of Clinical Nutrition*, *65*(2), 489–495. https://doi.org/10.1093/ajcn/65.2.489

Bollwein, J., Diekmann, R., Kaiser, M. J., Bauer, J. M., Uter, W., Sieber, C. C., and Volkert, D. (2013). Distribution but not amount of protein intake is associated with frailty: A cross-sectional investigation in the region of Nürnberg. *Nutrition Journal*, *12*(1), 1–7. https://doi.org/10.1186/1475-2891-12-109

Bos, C., Juillet, B., Fouillet, H., Turlan, L., Daré, S., Luengo, C., N'tounda, R., Benamouzig, R., Gausserès, N., Tomé, D. and Gaudichon, C. (2005). Postprandial metabolic utilization of wheat protein in humans. *American Journal of Clinical Nutrition*, *81*(1), 87–94.

Bos, C., Metges, C.C., Gaudichon, C., Petzke, K.J., Pueyo, M.E., Morens, C., Everwand, J., Benamouzig, R. and Tome, D. (2003). Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *The Journal of Nutrition*, *133*(5), 1308–1315. https://doi.org/10.1093/jn/133.5.1308

Bouillanne, O., Curis, E., Hamon-Vilcot, B., Nicolis, I., Chrétien, P., Schauer, N., Vincent, J.P., Cynober, L. and Aussel, C. (2013). Impact of protein pulse feeding on lean mass in malnourished and at-risk hospitalized elderly patients: A randomized controlled trial. *Clinical Nutrition*, *32*(2), 186–192. https://doi.org/10.1016/j.clnu.2012.08.015

Breen, L., and Phillips, S. M. (2011). Skeletal muscle protein metabolism in the elderly: Interventions to counteract the "anabolic resistance" of ageing. *Nutrition and Metabolism*, *8*(1), 68. https://doi.org/10.1186/1743-7075-8-68

Brodkorb, A., and Doherty, S. (2015). *U.S. Patent No. 9,089,152*. Washington, DC: U.S. Patent and Trademark Office.

Mobley, C.B., Haun, C.T., Roberson, P.A., Mumford, P.W., Romero, M.A., Kephart, W.C., Anderson, R.G., Vann, C.G., Osburn, S.C., Pledge, C.D. and Martin, J.S. (2017). Effects of whey, soy or leucine supplementation with 12 weeks of resistance training on strength, body composition, and skeletal muscle and adipose tissue histological attributes in college-aged males. *Nutrients*, *9*(9), 1–22. https://doi.org/10.3390/nu9090972

Brown, E. C., DiSilvestro, R. A., Babaknia, A., and Devor, S. T. (2004). Soy versus whey protein bars: Effects on exercise training impact on lean body mass and antioxidant status. *Nutrition Journal*, *3*. https://doi.org/10.1186/1475-2891-3-22

Buckner, S. L., Dankel, S. J., Mattocks, K. T., Jessee, M. B., Mouser, J. G., Counts, B. R., and Loenneke, J. P. (2016). The problem of muscle hypertrophy: Revisited. *Muscle and Nerve*. https://doi.org/10.1002/mus.25420

Bukhari, S.S., Phillips, B.E., Wilkinson, D.J., Limb, M.C., Rankin, D., Mitchell, W.K., Kobayashi, H., Greenhaff, P.L., Smith, K. and Atherton, P.J. (2015). Intake of low-dose leucine-rich essential amino acids stimulates muscle anabolism equivalently to bolus whey protein in older women at rest and after exercise. *American Journal of Physiology - Endocrinology And Metabolism*, *308*(12), E1056–E1065. https://doi.org/10.1152/ajpendo.00481.2014

Burd, N. A., Gorissen, S. H., Van Vliet, S., Snijders, T., & Van Loon, L. J. (2015). Differences in postprandial protein handling after beef compared with milk ingestion during postexercise recovery: a randomized controlled trial, 2. *The American journal of clinical nutrition*, *102*(4), 828-836.

Burd, N. A., Gorissen, S. H., and Van Loon, L. J. C. (2013). Anabolic resistance of muscle protein synthesis with aging. *Exercise and Sport Sciences Reviews*, *41*(3), 169–173. https://doi.org/10.1097/JES.0b013e318292f3d5

Burd, N. A., Yang, Y., Moore, D. R., Tang, J. E., Tarnopolsky, M. A., and Phillips, S. M. (2012). Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men. *British Journal of Nutrition*, *108*(6), 958–962. https://doi.org/10.1017/S0007114511006271

Burke, L. M., Winter, J. a, Cameron-Smith, D., Enslen, M., Farnfield, M., and Decombaz, J. (2012). Effect of intake of different dietary protein sources on plasma amino acid profiles at rest and after exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, *22*, 452–462. https://doi.org/10.1123/jab.2017-0008

Buttery, R. G., Teranishi, R., Flath, R. A., and Ling, L. C. (1999). Flavor Chemistry. *Flavor Chemistry: Trends and Developments*, *388*, 211–222. https://doi.org/10.1007/978-1-4615-4693-1

Cardon-Thomas, D. K., Riviere, T., Tieges, Z., and Greig, C. A. (2017). Dietary protein in older adults: Adequate daily intake but potential for improved distribution. *Nutrients*, *9*(3). https://doi.org/10.3390/nu9030184

Cashman, K.D., Muldowney, S., McNulty, B., Nugent, A., FitzGerald, A.P., Kiely, M., Walton, J., Gibney, M.J. and Flynn, A. (2013). Vitamin D status of Irish adults: Findings from the National Adult Nutrition Survey. *British Journal of Nutrition*. https://doi.org/10.1017/S0007114512003212

Casperson, S. L., Sheffield-Moore, M., Hewlings, S. J., and Paddon-Jones, D. (2012). Leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein. *Clinical Nutrition*, *31*(4), 512–519. https://doi.org/10.1016/j.clnu.2012.01.005

Chalé, A., Cloutier, G. J., Hau, C., Phillips, E. M., Dallal, G. E., and Fielding, R. A. (2013). Efficacy of whey protein supplementation on resistance exercise-induced changes in lean mass, muscle strength, and physical function in mobility-limited older adults. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *68*(6), 682–690. https://doi.org/10.1093/gerona/gls221

Champagne, C. P., and Fustier, P. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2007.03.001

Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., and Norat, T. (2011). Red and processed meat and colorectal cancer incidence: Meta-analysis of prospective studies. *PLoS ONE*, *6*(6). https://doi.org/10.1371/journal.pone.0020456

Chernoff, R. (2004). Protein and Older Adults. *Journal of the American College of Nutrition*, *23*, 627S–630S. https://doi.org/10.1080/07315724.2004.10719434

Cheung, K., Hume, P. A., and Maxwell, L. (2003). Delayed onset muscle soreness: Treatment strategies and performance factors. *Sports Medicine*, *33*(2), 145–164. https://doi.org/10.2165/00007256-200333020-00005

Churchward-Venne, T.A., Breen, L., Di Donato, D.M., Hector, A.J., Mitchell, C.J., Moore, D.R., Stellingwerff, T., Breuille, D., Offord, E.A., Baker, S.K. and Phillips, S.M. (2014). Leucine supplementation of a low-protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: A double-blind, randomized trial1-3. *American Journal of Clinical Nutrition*, *99*(2), 276–286. https://doi.org/10.3945/ajcn.113.068775

Churchward-Venne, T.A., Burd, N.A., Mitchell, C.J., West, D.W., Philp, A., Marcotte, G.R., Baker, S.K., Baar, K. and Phillips, S.M. (2012). Supplementation of a suboptimal protein dose with leucine or essential amino acids: Effects on myofibrillar protein synthesis at rest and following resistance exercise in men. *Journal of Physiology*, *590*(11), 2751–2765. https://doi.org/10.1113/jphysiol.2012.228833

Clarkson, P. M., Nosaka, K., and Braun, B. (1992). Muscle function after exercise-induced muscle damage and rapid adaptation. *Medicine and Science in Sports and Exercise.* https://doi.org/0195-9131/92/2405-0512$3.00/0

Cockburn, E., Hayes, P. R., French, D. N., Stevenson, E., and St Clair Gibson, A. (2008). Acute milk-based protein–CHO supplementation attenuates exercise-induced muscle damage. *Applied Physiology, Nutrition, and Metabolism*, *33*(4), 775–783. https://doi.org/10.1139/H08-057

Cockburn, E., Robson-Ansley, P., Hayes, P. R., and Stevenson, E. (2012). Effect of volume of milk consumed on the attenuation of exercise-induced muscle damage. *European Journal of Applied Physiology*, *112*(9), 3187–3194. https://doi.org/10.1007/s00421-011-2288-2

Cockburn, E., Stevenson, E., Hayes, P. R., Robson-Ansley, P., and Howatson, G. (2010). Effect of milk-based carbohydrate-protein supplement timing on the attenuation of exercise-induced muscle damage. *Applied Physiology, Nutrition, and Metabolism*, *35*(3), 270–277. https://doi.org/10.1139/H10-017

Colbert, L.H., Visser, M., Simonsick, E.M., Tracy, R.P., Newman, A.B., Kritchevsky, S.B., Pahor, M., Taaffe, D.R., Brach, J., Rubin, S. and Harris, T.B. (2004). Physical activity, exercise, and inflammatory markers in older adults: Findings from the health, aging and body composition study. *Journal of the American Geriatrics Society*, *52*(7), 1098–1104. https://doi.org/10.1111/j.1532-5415.2004.52307.x

Connolly, D. A. J., Sayers, S. E., and McHugh, M. P. (2003). Treatment and Prevention of Delayed Onset Muscle Soreness. *Journal of Strength and Conditioning Research*, *17*(1), 197–208. https://doi.org/10.1519/00124278-200302000-00030

Cuthbertson, D. (2004). Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *The Federation of American Societies for Experimental Biology Journal*. https://doi.org/10.1096/fj.04-2640fje

Dalle, S., Rossmeislova, L., and Koppo, K. (2017). The role of inflammation in age-related sarcopenia. *Frontiers in Physiology*. https://doi.org/10.3389/fphys.2017.01045

Daly, R. M., O'Connell, S. L., Mundell, N. L., Grimes, C. A., Dunstan, D. W., and Nowson, C. A. (2014). Protein-enriched diet, with the use of lean red meat, combined with progressive resistance training enhances lean tissue mass and muscle strength and reduces circulating IL-6 concentrations in elderly women: A cluster randomized controlled trial. *American Journal of Clinical Nutrition*, *99*(4), 899–910. https://doi.org/10.3945/ajcn.113.064154

Damas, F., Phillips, S., Vechin, F. C., and Ugrinowitsch, C. (2015). A Review of Resistance Training-Induced Changes in Skeletal Muscle Protein Synthesis and Their Contribution to Hypertrophy. *Sports Medicine*. https://doi.org/10.1007/s40279-015-0320-0

Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., Callier, P., Ballèvre, O. and Beaufrère, B.(2001). The digestion rate of protein is an independent regulating factor of postprandial protein retention. *American Journal of Physiology-Endocrinology and Metabolism*, *280*(2), E340–E348. https://doi.org/10.1152/ajpendo.2001.280.2.E340

de Vries, M., and de Boer, I. J. M. (2010). Comparing environmental impacts for livestock products: A review of life cycle assessments. *Livestock Science*. https://doi.org/10.1016/j.livsci.2009.11.007

Devries, M.C., McGlory, C., Bolster, D.R., Kamil, A., Rahn, M., Harkness, L., Baker, S.K. and Phillips, S.M. (2018). Leucine, Not Total Protein, Content of a Supplement Is the Primary Determinant of Muscle Protein Anabolic Responses in Healthy Older Women. *The Journal of Nutrition*. https://doi.org/10.1093/jn/nxy091

Dias, M. I., Ferreira, I. C. F. R., and Barreiro, M. F. (2015). Microencapsulation of bioactives for food applications. *Food and Function*. https://doi.org/10.1039/c4fo01175a

Dillon, E.L., Sheffield-Moore, M., Paddon-Jones, D., Gilkison, C., Sanford, A.P., Casperson, S.L., Jiang, J., Chinkes, D.L. and Urban, R.J. (2009). Amino acid supplementation increases lean body mass, basal muscle protein synthesis, and insulin-like growth factor-I expression in older women. *Journal of Clinical Endocrinology and Metabolism*, *94*(5), 1630–1637. https://doi.org/10.1210/jc.2008-1564

Doran, S., Jones, K. L., Andrews, J. M., and Horowitz, M. (1998). Effects of meal volume and posture on gastric emptying of solids and appetite. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *275*(5), R1712–R1718.

Dreyer, H.C., Drummond, M.J., Pennings, B., Fujita, S., Glynn, E.L., Chinkes, D.L., Dhanani, S., Volpi, E. and Rasmussen, B.B. (2008). Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *The American Journal of Physiology Endocrinology and Metabolism*, *1144*, 392–400. https://doi.org/10.1152/ajpendo.00582.2007

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia*. https://doi.org/10.1016/j.fitote.2005.11.008

Egan, B. (2016). Protein intake for athletes and active adults: Current concepts and controversies. *Nutrition Bulletin*. https://doi.org/10.1111/nbu.12215

Elliot, T. A., Cree, M. G., Sanford, A. P., Wolfe, R. R., and Tipton, K. D. (2006). Milk ingestion stimulates net muscle protein synthesis following resistance exercise. *Medicine and Science in Sports and Exercise*, *38*(4), 667–674. https://doi.org/10.1249/01.mss.0000210190.64458.25

Engelen, M.P., Rutten, E.P., De Castro, C.L., Wouters, E.F., Schols, A.M. and Deutz, N.E. (2007). Supplementation of soy protein with branched-chain amino acids alters protein metabolism in healthy elderly and even more in patients with chronic obstructive pulmonary disease. *The American Journal of Clinical Nutrition*, *85*, 431–9

Etheridge, T., Philp, A., and Watt, P. W. (2008). A single protein meal increases recovery of muscle function following an

acute eccentric exercise bout. *Applied Physiology, Nutrition, and Metabolism*, *33*(3), 483–488. https://doi.org/10.1139/H08-028

European Food Safety Authority. (2012). Guidance on Dermal Absorption. *EFSA Journal*, *10*(4), 2665. https://doi.org/10.2903/j.efsa.2012

FAO. (2013). Dietary protein quality evaluation in human nutrition. *Food and Agriculrure Organization of the United Nations*, 1–79.

Fielding, R.A., Vellas, B., Evans, W.J., Bhasin, S., Morley, J.E., Newman, A.B., van Kan, G.A., Andrieu, S., Bauer, J., Breuille, D. and Cederholm, T. (2011). Sarcopenia: An Undiagnosed Condition in Older Adults. Current Consensus Definition: Prevalence, Etiology, and Consequences. International Working Group on Sarcopenia. *Journal of the American Medical Directors Association*, *12*(4), 249–256. https://doi.org/10.1016/j.jamda.2011.01.003

Finger, D., Goltz, F. R., Umpierre, D., Meyer, E., Rosa, L. H. T., and Schneider, C. D. (2015). Effects of Protein Supplementation in Older Adults Undergoing Resistance Training: A Systematic Review and Meta-Analysis. *Sports Medicine*, *45*(2), 245–255. https://doi.org/10.1007/s40279-014-0269-4

Food and Nutriton Board. (2015). Panel on Macronutrients, Panel on the Definition of Dietary Fiber, Subcommittee on Upper Reference Levels of Nutrients, Subcommittee on Interpretation and Uses of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes Library of Congress Cataloguing-in-Publication Data

Fouillet, H., Mariotti, F., Gaudichon, C., Bos, C., and Tomé, D. (2002). Peripheral and splanchnic metabolism of dietary nitrogen are differently affected by the protein source in humans as assessed by compartmental modeling. *The Journal of Nutrition*, *132*(1), 125–133. https://doi.org/10.1093/JN/132.1.125

Fouré, A., Nosaka, K., Gastaldi, M., Mattei, J.P., Boudinet, H., Guye, M., Vilmen, C., Le Fur, Y., Bendahan, D. and Gondin, J. (2016). Effects of branched-chain amino acids supplementation on both plasma amino acids concentration and muscle energetics changes resulting from muscle damage: A randomized placebo controlled trial. *Clinical Nutrition*, *35*(1), 83–94. https://doi.org/10.1016/j.clnu.2015.03.014

Fraga, S., Serrao, M. P., and Soares-da-Silva, P. (2002). L-type amino acid transporters in two intestinal epithelial cell lines function as exchangers with neutral amino acids. *The Journal of nutrition*, *132*(4), 733-738

Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M.P., Invidia, L., Celani, L., Scurti, M. and Cevenini, E (2007). Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans. *Mechanisms of Ageing and Development*, *128*(1), 92–105. https://doi.org/10.1016/j.mad.2006.11.016

Fujita, S., Dreyer, H.C., Drummond, M.J., Glynn, E.L., Cadenas, J.G., Yoshizawa, F., Volpi, E. and Rasmussen, B.B. (2007). Nutrient signalling in the regulation of human muscle protein synthesis. *Journal of Physiology*, *582*(2), 813–823. https://doi.org/10.1113/jphysiol.2007.134593

Fujita, S., Rasmussen, B. B., Cadenas, J. C., Grady, J. J., and Volpi, E. (2006). Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability. *The American Journal of Physiology Endocrinology and Metabolism*, *291*(4), E745–E754. https://doi.org/10.1152/ajpendo.00271.2005

Fulgoni, V. L. (2008). Current protein intake in America: analysis of the National Healthand Nutrition Examination Survey, 2003–2004. *The American Journal of Clinical Nutrition*

Gardner, C.D., Trepanowski, J.F., Del Gobbo, L.C., Hauser, M.E., Rigdon, J., Ioannidis, J.P., Desai, M. and King, A.C. (2018). Effect of low-fat VS low-carbohydrate diet on 12-month weight loss in overweight adults and the association with genotype pattern or insulin secretion the DIETFITS randomized clinical trial. *Journal of the American Medical Association*, *319*(7), 667–679. https://doi.org/10.1001/jama.2018.0245

Gillen, J.B., Trommelen, J., Wardenaar, F.C., Brinkmans, N.Y., Versteegen, J.J., Jonvik, K.L., Kapp, C., de Vries, J., van den Borne, J.J., Gibala, M.J. and van Loon, L.J. (2017). Dietary protein intake and distribution patterns of well-trained Dutch athletes. *International Journal of Sport Nutrition and Exercise Metabolism*, *27*(2), 105–114. https://doi.org/10.1123/ijsnem.2016-0154

Glynn, E. L., Fry, C. S., Drummond, M. J., Timmerman, K. L., Dhanani, S., Volpi, E., and Rasmussen, B. B. (2010). Excess Leucine Intake Enhances Muscle Anabolic Signaling but Not Net Protein Anabolism in Young Men and Women. *Journal of Nutrition*, *140*(11), 1970–1976. https://doi.org/10.3945/jn.110.127647

Godard, M. P., Williamson, D. L., and Trappe, S. W. (2002). Oral amino-acid provision does not affect muscle strength or size gains in older men. *Medicine and Science in Sports and Exercise*, *34*(7), 1126–1131

Gorissen, S. H. M., and Witard, O. C. (2018). Characterising the muscle anabolic potential of dairy, meat and plant-based protein sources in older adults. In *Proceedings of the Nutrition Society* (Vol. 77, pp. 20–31). https://doi.org/10.1017/S002966511700194X

Gorissen, S.H., Horstman, A.M., Franssen, R., Crombag, J.J., Langer, H., Bierau, J., Respondek, F. and Van Loon, L.J. (2016). Ingestion of Wheat Protein Increases In Vivo Muscle Protein Synthesis Rates in Healthy Older Men in a Randomized Trial. *The Journal of Nutrition*, *146*(9), 1651–1659. https://doi.org/10.3945/jn.116.231340

Greer, B. K., Woodard, J. L., White, J. P., Arguello, E. M., and Haymes, E. M. (2007). Branched-chain amino acid supplementation and indicators of muscle damage after endurance exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, *17*(6), 595–607. https://doi.org/10.1123/ijsnem.17.6.595

Gropper, S. S., and Smith, J. L. (2012). *Advanced nutrition and human metabolism*. Cengage Learning.

Hall, W. L., Millward, D. J., Long, S. J., and Morgan, L. M. (2003). Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *British Journal of Nutrition*, *89*(2), 239. https://doi.org/10.1079/BJN2002760

Haran, P. H., Rivas, D. A., and Fielding, R. A. (2012). Role and potential mechanisms of anabolic resistance in sarcopenia. *Journal of cachexia, sarcopenia and muscle*, *3*(3), 157-162

Hartman, J. W., Tang, J. E., Wilkinson, S. B., Tarnopolsky, M. A., Lawrence, R. L., Fullerton, A. V., and Phillips, S. M. (2007). Consumption of fat-free fluid milk after resistance exercise promotes greater lean mass accretion than does consumption of soy or carbohydrate in young, novice, male weightlifters. *American Journal of Clinical Nutrition*, *86*(2), 373–381. https://doi.org/10.1016/S0162-0908(08)79232-8

Have, G.A.T., Engelen, M.P., Luiking, Y.C. and Deutz, N.E. (2007). Absorption Kinetics of Amino Acids, Peptides, and intact Proteins. *International Journal of Sport Nutrition and Exercise Metabolism*, *17*, 23–36

Henderson, J. W., and Brooks, A. (2010). Improved amino acid methods using Agilent ZORBAX Eclipse Plus C18 columns for a variety of Agilent LC instrumentation and separation goals. *Santa Clara: Agilent*

Hind, K., Slater, G., Oldroyd, B., Lees, M., Thurlow, S., Barlow, M., & Shepherd, J. (2018). Interpretation of Dual Energy X-Ray Absorptiometry-Derived Body Composition Change in Athletes: a Review and Recommendations for Best Practice. *Journal of Clinical Densitometry*.

Holviala, J., Häkkinen, A., Karavirta, L., Nyman, K., Izquierdo, M., Gorostiaga, E.M., Avela, J., Korhonen, J., Knuutila, V.P., Kraemer, W.J. and Häkkinen, K. (2010). Effects of combined strength and endurance training on treadmill load carrying walking performance in aging men. *Journal of Strength and Conditioning Research*, *24*(6), 1584–1595. https://doi.org/10.1519/JSC.0b013e3181dba178

Holwerda, A. M., Lenaerts, K., Bierau, J., Wodzig, W. K. W. H., and van Loon, L. J. C. (2017). Food ingestion in an upright sitting position increases post-prandial amino acid availability when compared to food ingestion in a lying down position. *Applied Physiology, Nutrition, and Metabolism*, *5027*, apnm-2016-0522. https://doi.org/10.1139/apnm-2016-0522

Hone, M., Kent, R. M., Scotto di Palumbo, A., Bleiel, S. B., De Vito, G., and Egan, B. (2017). Plasma Creatine Kinetics After Ingestion of Microencapsulated Creatine Monohydrate with Enhanced Stability in Aqueous Solutions. *Journal of Dietary Supplements*, *14*(4), 433–445. https://doi.org/10.1080/19390211.2016.1267060

Hopkins, S.M., Gibney, M.J., Nugent, A.P., McNulty, H., Molloy, A.M., Scott, J.M., Flynn, A., Strain, J.J., Ward, M., Walton, J. and McNulty, B.A. (2015). Impact of voluntary fortification and supplement use on dietary intakes and biomarker status of folate and vitamin B-12 in Irish adults. *American Journal of Clinical Nutrition*, *101*(6), 1163–1172. https://doi.org/10.3945/ajcn.115.107151

Howatson, G., and Someren, K. A. (2008). The prevention and treatment of exercise-induced muscle damage. *Sports Medicine*, *36*(6), 483–503

Howatson, G., Hoad, M., Goodall, S., Tallent, J., Bell, P. G., and French, D. N. (2012). Exercise-induced muscle damage is reduced in resistance-trained males by branched chain amino acids: A randomized, double-blind, placebo controlled study. *Journal of the International Society of Sports Nutrition*, *9*, 20. https://doi.org/10.1186/1550-2783-9-20

Humayun, M. A., Elango, R., Ball, R. O., and Pencharz, P. B. (2007). Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. *American Journal of Clinical Nutrition*, *86*(4), 995–1002. https://doi.org/86/4/995 [pii]

Hunt, J. N. (1980). A possible relation between the regulation of gastric emptying and food intake. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *239*(1), G1-G4

Iannoli, R., Miller, J. H., Wang, H. T., Bode, B., Souba, W. W., Avissar, N. E., and Sax, H. C. (1999). Characterization of L-leucine transport system in brush border membranes from human and rabbit small intestine. *Metabolism: Clinical and Experimental*, *48*(11), 1432–1436. https://doi.org/10.1016/S0026-0495(99)90155-5

Iglay, H. B., Apolzan, J. W., Gerrard, D. E., Eash, J. K., Anderson, J. C., and Campbell, W. W. (2009). Moderately increased protein intake predominately from egg sources does not influence whole body, regional, or muscle composition responses to resistance training in older people. *Journal of Nutrition, Health and Aging*, *13*(2), 108–114. https://doi.org/10.1007/s12603-009-0016-y

Iuliano, S., Woods, J., and Robbins, J. (2013). Consuming two additional serves of dairy food a day significantly improves energy and nutrient intakes in ambulatory aged care residents: A feasibility study. *Journal of Nutrition, Health and Aging*, *17*(6), 509–513. https://doi.org/10.1007/s12603-013-0025-8

Jackman, S. R., Witard, O. C., Jeukendrup, A. E., and Tipton, K. D. (2010). Branched-chain amino acid ingestion can ameliorate soreness from eccentric exercise. *Medicine and Science in Sports and Exercise*, *42*(5), 962–970. https://doi.org/10.1249/MSS.0b013e3181c1b798

Janssen, I. (2010). Evolution of sarcopenia research. *Applied Physiology, Nutrition, and Metabolism*, *35*(5), 707–712. https://doi.org/10.1139/H10-067

Janssen, I., Heymsfield, S. B., and Ross, R. (2002). Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. *Journal of the American Geriatrics Society*, *50*(5), 889–896. https://doi.org/10.1046/j.1532-5415.2002.50216.x

Jensen, G. L. (2008). Inflammation: Roles in aging and sarcopenia. In *Journal of Parenteral and Enteral Nutrition* (Vol. 32, pp. 656–659). https://doi.org/10.1177/0148607108324585

Joy, J.M., Lowery, R.P., Wilson, J.M., Purpura, M., De Souza, E.O., Wilson, S.M., Kalman, D.S., Dudeck, J.E. and Jäger, R. (2013). The effects of 8 weeks of whey or rice protein supplementation on body composition and exercise performance. *Nutrition Journal*, *12*(1). https://doi.org/10.1186/1475-2891-12-86

Kato, H., Suzuki, H., Mimura, M., Inoue, Y., Sugita, M., Suzuki, K., and Kobayashi, H. (2015). Leucine-enriched essential amino acids attenuate muscle soreness and improve muscle protein synthesis after eccentric contractions in rats. *Amino Acids*, *47*(6), 1193–1201. https://doi.org/10.1007/s00726-015-1946-9

Kato, H., Suzuki, K., Bannai, M., and Moore, D. R. (2016). Protein requirements are elevated in endurance athletes after exercise as determined by the indicator amino acid oxidation method. *PLoS ONE*, *11*(6). https://doi.org/10.1371/journal.pone.0157406

Katsanos, C. S. (2006). A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *AJP: Endocrinology and Metabolism*, *291*(2), E381–E387. https://doi.org/10.1152/ajpendo.00488.2005

Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., and Wolfe, R. R. (2005). Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. *American Journal of Clinical Nutrition*, *82*(5), 1065–1073. https://doi.org/82/5/1065 [pii]

Kephart, W.C., Mumford, P.W., McCloskey, A.E., Holland, A.M., Shake, J.J., Mobley, C.B., Jagodinsky, A.E., Weimar, W.H., Oliver, G.D., Young, K.C. and Moon, J.R. (2016). Post-exercise branched chain amino acid supplementation does not affect recovery markers following three consecutive high intensity resistance training bouts compared to carbohydrate supplementation. *Journal of the International Society of Sports Nutrition*, *13*(1). https://doi.org/10.1186/s12970-016-0142-y

Kim, C. O., and Lee, K. R. (2013). Preventive effect of protein-energy supplementation on the functional decline of frail older adults with low socioeconomic status: A community-based randomized controlled study. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *68*(3), 309–316. https://doi.org/10.1093/gerona/gls167

Kim, I.Y., Schutzler, S., Schrader, A., Spencer, H., Kortebein, P., Deutz, N.E., Wolfe, R.R. and Ferrando, A.A. (2015). Quantity of dietary protein intake, but not pattern of intake, affects net protein balance primarily through differences in protein synthesis in older adults. *AJP: Endocrinology and Metabolism*, *308*(1), E21–E28. https://doi.org/10.1152/ajpendo.00382.2014

Kim, J., and Lee, J. (2014). A review of nutritional intervention on delayed onset muscle soreness. Part I. *Journal of Exercise Rehabilitation*, *10*(6), 349–356. https://doi.org/10.12965/jer.140179

Kirby, T. J., Triplett, N. T., Haines, T. L., Skinner, J. W., Fairbrother, K. R., and McBride, J. M. (2012). Effect of leucine supplementation on indices of muscle damage following drop jumps and resistance exercise. *Amino Acids*, *42*(5), 1987–1996. https://doi.org/10.1007/s00726-011-0928-9

Koopman, R., Walrand, S., Beelen, M., Gijsen, A.P., Kies, A.K., Boirie, Y., Saris, W.H. and van Loon, L.J., (2009). Dietary protein digestion and absorption rates and the subsequent postprandial muscle protein synthetic response do not differ between young and elderly men. *The Journal of nutrition*, *139*(9),1707-1713

Koopman, R., Saris, W. H. M., Wagenmakers, A. J. M., and van Loon, L. J. C. (2007). Nutritional interventions to promote post-exercise muscle protein synthesis. *Sports Medicine (Auckland, N.Z.)*, *37*(10), 895–906. https://doi.org/37105 [pii]

Koopman, R., Verdijk, L.B., Beelen, M., Gorselink, M., Kruseman, A.N., Wagenmakers, A.J., Kuipers, H. and van Loon, L.J. (2008). Co-ingestion of leucine with protein does not further augment post-exercise muscle protein synthesis rates in elderly men. *British Journal of Nutrition*, *99*(3), 571–580. https://doi.org/10.1017/S0007114507812013

Koopman, R., Verdijk, L., Manders, R.J., Gijsen, A.P., Gorselink, M., Pijpers, E., Wagenmakers, A.J. and van Loon, L.J. (2006). Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly lean men. *American Journal of Clinical Nutrition*, *84*(3), 623–632. https://doi.org/84/3/623 [pii]

Kopelman, P. G. (2000). Obesity as a medical problem. *Nature*, *404*(6778), 635

Kraemer, W.J., Ratamess, N.A., Volek, J.S., Häkkinen, K., Rubin, M.R., French, D.N., Gómez, A.L., McGuigan, M.R., Scheett, T.P., Newton, R.U. and Spiering, B.A. (2006). The effects of amino acid supplementation on hormonal responses to resistance training overreaching. *Metabolism: Clinical and Experimental*, *55*(3), 282–291. https://doi.org/10.1016/j.metabol.2005.08.023

Kramer, I.F., Verdijk, L.B., Hamer, H.M., Verlaan, S., Luiking, Y.C., Kouw, I.W., Senden, J.M., van Kranenburg, J., Gijsen, A.P., Bierau, J. and Poeze, M. (2017). Both basal and post-prandial muscle protein synthesis rates, following the ingestion of a leucine-enriched whey protein supplement, are not impaired in sarcopenic older males. *Clinical Nutrition*, *36*(5), 1440–1449. https://doi.org/10.1016/j.clnu.2016.09.023

Kreider, R. B., and Campbell, B. (2009). Protein for exercise and recovery. *Physician and Sportsmedicine*, *37*(2), 13–21. https://doi.org/10.3810/psm.2009.06.1705

Laplante, M., and Sabatini, D. M. (2012). MTOR signaling in growth control and disease. *Cell*. https://doi.org/10.1016/j.cell.2012.03.017

Latham, N. K., Bennett, D. A., Stretton, C. M., and Anderson, C. S. (2004). Systematic Review of Progressive Resistance Strength Training in Older Adults. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *59*(1), M48–M61. https://doi.org/10.1093/gerona/59.1.M48

Layman, D. K., Anthony, T. G., Rasmussen, B. B., Adams, S. H., Lynch, C. J., Brinkworth, G. D., and Davis, T. A. (2015). Defining meal requirements for protein to optimize metabolic roles of amino acids. *American Journal of Clinical Nutrition*, *101*(6), 1330S–1338S. https://doi.org/10.3945/ajcn.114.084053

Leenders, M., and van Loon, L. J. (2011). Leucine as a pharmaconutrient to prevent and treat sarcopenia and type 2 diabetes. *Nutrition Reviews*, *69*(11), 675–689. https://doi.org/10.1111/j.1753-4887.2011.00443.x

Leenders, M., Verdijk, L.B., van der Hoeven, L., van Kranenburg, J., Hartgens, F., Wodzig, W.K., Saris, W.H. and van Loon, L.J.(2011). Prolonged Leucine Supplementation Does Not Augment Muscle Mass or Affect Glycemic Control in Elderly Type 2 Diabetic Men. *Journal of Nutrition*, *141*(6), 1070–1076. https://doi.org/10.3945/jn.111.138495

Leenders, Marika, Lex B. Verdijk, Letty Van der Hoeven, Janneau Van Kranenburg, Rachel Nilwik, W. K. Wodzig, Joan M. Senden, Hans A. Keizer, and L. J. Van Loon. "Protein supplementation during resistance-type exercise training in the elderly." *Medicine and Science in Sports and Exercise*, 45, no. 3 (2013): 542-552

Leitzmann, C. (2014). Vegetarian nutrition: past, present, future–. *The American journal of clinical nutrition*, *100*(suppl_1), 496S-502

Lewis, P. B., Ruby, D., and Bush-Joseph, C. A. (2012). Muscle Soreness and Delayed-Onset Muscle Soreness. *Clinics in Sports Medicine*. https://doi.org/10.1016/j.csm.2011.09.009

Lexell, J., Taylor, C. C., and Sjöström, M. (1988). What is the cause of the ageing atrophy?: Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15-to 83-year-old men. *Journal of the neurological sciences*, *84*(2-3), 275-294

Liao, C.D., Tsauo, J.Y., Wu, Y.T., Cheng, C.P., Chen, H.C., Huang, Y.C., Chen, H.C. and Liou, T.H. (2017). Effects of protein supplementation combined with resistance exercise on body composition and physical function in older adults: a systematic review and meta-analysis. *The American journal of clinical nutrition*, *106*(4), 1078-1091

Lieber, R. L., and Friden, J. (2002). Morphologic and mechanical basis of delayed-onset muscle soreness. *The Journal of the American Academy of Orthopaedic Surgeons*, *10*(1), 67–73. https://doi.org/10.5435/00124635-200201000-00009

Liu, C., and Latham, N. (2010). Progressive resistance strength training for improving physical function in older adults. *Australasian Journal of Ageing*, *29*(2), 98. https://doi.org/10.1111/j.1741-6612.2010.00435.x

Loenneke, J. P., Loprinzi, P. D., Murphy, C. H., and Phillips, S. M. (2016). Per meal dose and frequency of protein consumption is associated with lean mass and muscle performance. *Clinical Nutrition*, *35*(6), 1506–1511. https://doi.org/10.1016/j.clnu.2016.04.002

Macdonald, G. Z., Button, D. C., Drinkwater, E. J., and Behm, D. G. (2014). Foam rolling as a recovery tool after an intense bout of physical activity. *Medicine and Science in Sports and Exercise*, *46*(1), 131–142. https://doi.org/10.1249/MSS.0b013e3182a123db

MacKenzie, K., Slater, G., King, N., and Byrne, N. (2015). The measurement and interpretation of dietary protein distribution during a rugby preseason. *International Journal of Sport Nutrition and Exercise Metabolism*, *25*(4), 353–358. https://doi.org/10.1123/ijsnem.2014-0168

Malafarina, V., Uriz-Otano, F., Iniesta, R., and Gil-Guerrero, L. (2013). Effectiveness of Nutritional Supplementation on Muscle Mass in Treatment of Sarcopenia in Old Age: A Systematic Review. *Journal of the American Medical Directors Association*. https://doi.org/10.1016/j.jamda.2012.08.001

Malm, C., Nyberg, P., Engstrom, M., Sjodin, B., Lenkei, R., Ekblom, B., and Lundberg, I. (2000). Muscular adaptation to physical stress is of significant importance for normal muscular development and function. Without stimulation from physical activity.

Mamerow, M.M., Mettler, J.A., English, K.L., Casperson, S.L., Arentson-Lantz, E., Sheffield-Moore, M., Layman, D.K. and Paddon-Jones, D. (2014). Dietary Protein Distribution Positively Influences 24-h Muscle Protein Synthesis in Healthy Adults. *Journal of Nutrition*, *144*(6), 876–880. https://doi.org/10.3945/jn.113.185280

Matsumoto, K., Mizuno, M., Mizuno, T., Dilling-Hansen, B., Lahoz, A., Bertelsen, V., Münster, H., Jordening, H., Hamada, K. and Doi, T. (2007). Branched-chain amino acids and arginine supplementation attenuates skeletal muscle proteolysis induced by moderate exercise in young individuals. *International Journal of Sports Medicine*, *28*(6), 531–538. https://doi.org/10.1055/s-2007-964940

Matsumoto, T., Nakamura, K., Matsumoto, H., Sakai, R., Kuwahara, T., Kadota, Y., Kitaura, Y., Sato, J. and Shimomura, Y. (2014). Bolus ingestion of individual branched-chain amino acids alters plasma amino acid profiles in young healthy men. *SpringerPlus*, *3*(1), 1–13. https://doi.org/10.1186/2193-1801-3-35

Matsumoto, K., Koba, T., Hamada, K., Sakurai, M., Higuchi, T., and Miyata, H. (2009). Branched-chain amino acid supplementation attenuates muscle soreness, muscle damage and inflammation during an intensive training program. *Journal of Sports Medicine and Physical Fitness*, *49*, 424-31

McGlory, C., and Phillips, S. M. (2014). Assessing the regulation of skeletal muscle plasticity in response to protein ingestion and resistance exercise: Recent developments. *Current Opinion in Clinical Nutrition and Metabolic Care*, *17*(5), 412–417. https://doi.org/10.1097/MCO.0000000000000083

McGowan, M., Harrington, K., Kiely, M., Robson, P., Livingstone, M., and Gibney, M. (2001). An evaluation of energy intakes and the ratio of energy intake to estimated basal metabolic rate (EI/BMRest) in the North/South Ireland Food Consumption Survey. *Public Health Nutrition*, *4*(5a). https://doi.org/10.1079/PHN2001185

McHugh, M. P. (2003). Recent advances in the understanding of the repeated bout effect: The protective effect against muscle damage from a single bout of eccentric exercise. *Scandinavian Journal of Medicine and Science in Sports*, *13*(2), 88–97. https://doi.org/10.1034/j.1600-0838.2003.02477.x

Micha, R., Wallace, S. K., and Mozaffarian, D. (2010). Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus: A systematic review and meta-analysis. *Circulation*, *121*(21), 2271–2283. https://doi.org/10.1161/CIRCULATIONAHA.109.924977

Millward, D. J. (1995). A protein-stat mechanism for regulation of growth and maintenance of the lean body mass. *Nutrition Research Reviews*, *8*, 93–120. https://doi.org/10.1079/NRR19950008

Mitchell, W.K., Phillips, B.E., Williams, J.P., Rankin, D., Lund, J.N., Wilkinson, D.J., Smith, K. and Atherton, P.J. (2015a). The impact of delivery profile of essential amino acids upon skeletal muscle protein synthesis in older men: clinical efficacy of pulse vs. bolus supply. *American Journal of Physiology-Endocrinology and Metabolism*, *309*(5), E450–E457. https://doi.org/10.1152/ajpendo.00112.2015

Mitchell, W. K., Phillips, B. E., Williams, J. P., Rankin, D., Lund, J. N., Smith, K., and Atherton, P. J. (2015b). A Dose- rather than Delivery Profile-Dependent Mechanism Regulates the "Muscle-Full" Effect in Response to Oral Essential Amino Acid Intake in Young Men. *Journal of Nutrition*, *145*(2), 207–14. https://doi.org/10.3945/jn.114.199604.1

Moore, D.R., Areta, J., Coffey, V.G., Stellingwerff, T., Phillips, S.M., Burke, L.M., Cléroux, M., Godin, J.P. and Hawley, J.A. (2012). Daytime pattern of post-exercise protein intake affects whole-body protein turnover in resistance-trained males. *Nutrition and Metabolism*, *9*. https://doi.org/10.1186/1743-7075-9-91

Moore, D. R., Atherton, P. J., Rennie, M. J., Tarnopolsky, M. A., and Phillips, S. M. (2011). Resistance exercise enhances mTOR and MAPK signalling in human muscle over that seen at rest after bolus protein ingestion. *Acta Physiologica*, *201*(3), 365–372. https://doi.org/10.1111/j.1748-1716.2010.02187.x

Moore, D. R., Churchward-Venne, T. A., Witard, O., Breen, L., Burd, N. A., Tipton, K. D., and Phillips, S. M. (2015). Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *70*(1), 57–62. https://doi.org/10.1093/gerona/glu103

Moore, D.R., Robinson, M.J., Fry, J.L., Tang, J.E., Glover, E.I., Wilkinson, S.B., Prior, T., Tarnopolsky, M.A. and Phillips, S.M. (2009). Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *American Journal of Clinical Nutrition*, *89*(1), 161–168. https://doi.org/10.3945/ajcn.2008.26401

Morley, J. E. (2001). Decreased food intake with aging. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *56 Spec No*, 81–8. https://doi.org/10.1093/gerona/56.suppl_2.81

Morton, R. W., and Phillips, S. M. (2018). Does protein supplementation really augment hypertrophy in older persons with resistance exercise training?. *The American journal of clinical nutrition*, *107*(6), 1054-1056

Morton, R. W., McGlory, C., and Phillips, S. M. (2015). Nutritional interventions to augment resistance training-induced skeletal muscle hypertrophy. *Frontiers in Physiology*. https://doi.org/10.3389/fphys.2015.00245

Morton, R. W., Traylor, D. A., Weijs, P. J. M., and Phillips, S. M. (2018). Defining anabolic resistance: Implications for delivery of clinical care nutrition. *Current Opinion in Critical Care*, *24*(2), 124–130. https://doi.org/10.1097/MCC.0000000000000488

Munger, R. G., Cerhan, J. R., & Chiu, B. C. (1999). Prospective study of dietary protein intake and risk of hip fracture in postmenopausal women. *The American journal of clinical nutrition*, *69*(1), 147-152.

Murphy, C. H., Saddler, N. I., Devries, M. C., McGlory, C., Baker, S. K., and Phillips, S. M. (2016). Leucine supplementation enhances integrative myofibrillar protein synthesis in free-living older men consuming lower-and higher-protein diets: A parallel-group crossover study1. *American Journal of Clinical Nutrition*, *104*(6), 1594–1606. https://doi.org/10.3945/ajcn.116.136424

Norton, C., Toomey, C., McCormack, W. G., Francis, P., Saunders, J., Kerin, E., and Jakeman, P. (2016). Protein Supplementation at Breakfast and Lunch for 24 Weeks beyond Habitual Intakes Increases Whole-Body Lean Tissue Mass in Healthy Older Adults. *Journal of Nutrition*, *146*(1), 65–69. https://doi.org/10.3945/jn.115.219022

Norton, L. E., and Layman, D. K. (2006). Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. *Journal of Nutrition*, *136*(2), 533S–537S

Norton, L. E., Layman, D. K., Bunpo, P., Anthony, T. G., Brana, D. V., and Garlick, P. J. (2009). 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. *Journal of Nutrition*, *139*(6), 1103–1109. https://doi.org/10.3945/jn.108.103853

Norton, L. E., Wilson, G. J., Room, A., and Gregory, W. (2009). Optimal Protein Intake To Maximize Muscle Protein Synthesis and Frequency for Athletes. *AgroFOOD Industry Hi-Tech*, *20*(2), 54–57.

Nosaka, K., Newton, M., and Sacco, P. (2002). Delayed-onset muscle soreness does not reflect the magnitude of eccentric exercise-induced muscle damage. *Scandinavian Journal of Medicine and Science in Sports*, *12*(6), 337–346. https://doi.org/10.1034/j.1600-0838.2002.10178.x

Nosaka, K., Sacco, P., and Mawatari, K. (2006). Effects of amino acid supplementation on muscle soreness and damage. *International Journal of Sport Nutrition and Exercise Metabolism*, *16*(6), 620–635. https://doi.org/10.1123/ijsnem.16.6.620

Nowson, C., and O'Connell, S. (2015). Protein requirements and recommendations for older people: A review. *Nutrients*. https://doi.org/10.3390/nu7085311

O'Donovan, C.B., Devlin, N.F., Buffini, M., Walton, J., Flynn, A., Gibney, M.J., Nugent, A.P. and McNulty, B.A. (2018). Whole grain intakes in Irish adults: findings from the National Adults Nutrition Survey (NANS). *European Journal of Nutrition*, *0*, 1–10. https://doi.org/10.1007/s00394-018-1615-3

Onambélé-Pearson, G. L., Breen, L., and Stewart, C. E. (2010). Influence of exercise intensity in older persons with unchanged habitual nutritional intake: Skeletal muscle and endocrine adaptations. *Age*, *32*(2), 139–153. https://doi.org/10.1007/s11357-010-9141-0

Paddon-Jones, D. (2004). Exogenous amino acids stimulate human muscle anabolism without interfering with the

response to mixed meal ingestion. *AJP: Endocrinology and Metabolism*, *288*(4), E761–E767. https://doi.org/10.1152/ajpendo.00291.2004

Paddon-Jones, D., and Leidy, H. (2014). Dietary protein and muscle in older persons. *Current Opinion in Clinical Nutrition and Metabolic Care*. https://doi.org/10.1097/MCO.0000000000000011

Paddon-Jones, D., and Rasmussen, B. B. (2009). Dietary protein recommendations and the prevention of sarcopenia. *Current Opinion in Clinical Nutrition and Metabolic Care*. https://doi.org/10.1097/MCO.0b013e32831cef8b

Paddon-jones, D., Campbell, W. W., Jacques, P. F., Kritchevsky, S. B., Moore, L. L., and Rodriguez, N. R. (2015). Protein and healthy aging 1 – 5. *American Journal of Clinical Nutrition*, *101*(Suppl), 1339S–1345S. https://doi.org/10.3945/ajcn.114.084061.1

Paddon-Jones, D., Sheffield-Moore, M., Creson, D. L., Sanford, A. P., Wolf, S. E., Wolfe, R. R., and Ferrando, A. A. (2003). Hypercortisolemia alters muscle protein anabolism following ingestion of essential amino acids. *American Journal of Physiology - Endocrinology And Metabolism*, *284*(5), E946–E953. https://doi.org/10.1152/ajpendo.00397.2002

Pasiakos, S. M. (2012). Exercise and amino acid anabolic cell signaling and the regulation of skeletal muscle mass. *Nutrients*. https://doi.org/10.3390/nu4070740

Pearcey, G. E. P., Bradbury-Squires, D. J., Kawamoto, J.-E., Drinkwater, E. J., Behm, D. G., and Button, D. C. (2015). Foam Rolling for Delayed-Onset Muscle Soreness and Recovery of Dynamic Performance Measures. *Journal of Athletic Training*, *50*(1), 5–13. https://doi.org/10.4085/1062-6050-50.1.01

Pennings, B., Boirie, Y., Senden, J. M. G., Gijsen, A. P., Kuipers, H., and Van Loon, L. J. C. (2011a). Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *American Journal of Clinical Nutrition*, *93*(5), 997–1005. https://doi.org/10.3945/ajcn.110.008102

Pennings, B., Groen, B., de Lange, A., Gijsen, A. P., Zorenc, A. H., Senden, J. M. G., and van Loon, L. J. C. (2012). Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *AJP: Endocrinology and Metabolism*, *302*(8), E992–E999. https://doi.org/10.1152/ajpendo.00517.2011

Pennings, B., Koopman, R., Beelen, M., Senden, J. M. G., Saris, W. H. M., and Van Loon, L. J. C. (2011b). Exercising before protein intake allows for greater use of dietary protein-derived amino acids for de novo muscle protein synthesis in both young and elderly men. *American Journal of Clinical Nutrition*, *93*(2), 322–331. https://doi.org/10.3945/ajcn.2010.29649

Petersen, K.F., Dufour, S., Savage, D.B., Bilz, S., Solomon, G., Yonemitsu, S., Cline, G.W., Befroy, D., Zemany, L., Kahn, B.B. and Papademetris, X. (2007). The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proceedings of the National Academy of Sciences*, *104*(31), 12587–12594. https://doi.org/10.1073/pnas.0705408104

Phillips, S. M., Tipton, K. D., Aarsland, A. S. L. E., Wolf, S. E., & Wolfe, R. R. (1997). Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *American journal of physiology-endocrinology and metabolism*, *273*(1), 99-E107

Phillips, S. M. (2012a). Dietary protein requirements and adaptive advantages in athletes. *British Journal of Nutrition*, *108*(SUPPL. 2). https://doi.org/10.1017/S0007114512002516

Phillips, S. M. (2012b). Nutrient-rich meat proteins in offsetting age-related muscle loss. *Meat Science*. https://doi.org/10.1016/j.meatsci.2012.04.027

Phillips, S. M. (2015). Nutritional Supplements in Support of Resistance Exercise to Counter Age-Related Sarcopenia.

Advances in Nutrition: An International Review Journal, *6*(4), 452–460. https://doi.org/10.3945/an.115.008367

Phillips, S. M., and van Loon, L. J. C. (2011). Dietary protein for athletes: From requirements to optimum adaptation. *Journal of Sports Sciences*, *29*(SUPPL. 1). https://doi.org/10.1080/02640414.2011.619204

Phillips, S. M., Fulgoni, V. L., Heaney, R. P., Nicklas, T. A., Slavin, J. L., and Weaver, C. M. (2015). Commonly consumed protein foods contribute to nutrient intake, diet quality, and nutrient adequacy. *American Journal of Clinical Nutrition*, *101*(6), 1346S–1352S. https://doi.org/10.3945/ajcn.114.084079

Philpott, J. D., Donnelly, C., Walshe, I. H., MacKinley, E. E., Dick, J., Galloway, S.D., Tipton, K.D., and Witard, O. C. (2018). Adding fish oil to whey protein, leucine, and carbohydrate over a six-week supplementation period attenuates muscle soreness following eccentric exercise in competitive soccer players. *International Journal of Sport Nutrition and Exercise Metabolism*, *28*(1), 26–36. https://doi.org/10.1123/ijsnem.2017-0161

Pitkänen, H. T., Oja, S. S., Rusko, H., Nummela, A., Komi, P. V., Saransaari, P., and Mero, A. A. (2003). Leucine supplementation does not enhance acute strength or running performance but affects serum amino acid concentration. *Amino Acids*, *25*(1), 85–94. https://doi.org/10.1007/s00726-002-0343-3

Power-Grant, O., McCormack, W. G., Ramia De Cap, M., Amigo-Benavent, M., Fitzgerald, R. J., and Jakeman, P. (2016). Evaluation of the antioxidant capacity of a milk protein matrix in vitro and in vivo in women aged 50-70 years. *International Journal of Food Sciences and Nutrition*, *67*(3), 325–334. https://doi.org/10.3109/09637486.2016.1153607

Proske, U., and Morgan, D. L. (2001). Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. *The Journal of physiology*, *537*(2), 333-345

Ra, S.G., Miyazaki, T., Ishikura, K., Nagayama, H., Komine, S., Nakata, Y., Maeda, S., Matsuzaki, Y. and Ohmori, H. (2013). Combined effect of branched-chain amino acids and taurine supplementation on delayed onset muscle soreness and muscle damage in high-intensity eccentric exercise. *Journal of the International Society of Sports Nutrition*, *10*. https://doi.org/10.1186/1550-2783-10-51

Rafii, M., Chapman, K., Elango, R., Campbell, W. W., Ball, R. O., Pencharz, P. B., and Courtney-Martin, G. (2016). Dietary Protein Requirement of Men andgt;65 Years Old Determined by the Indicator Amino Acid Oxidation Technique Is Higher than the Current Estimated Average Requirement. *Journal of Nutrition*, *146*(4), 681–687. https://doi.org/10.3945/jn.115.225631

Rafii, M., Chapman, K., Owens, J., Elango, R., Campbell, W.W., Ball, R.O., Pencharz, P.B. and Courtney-Martin, G. (2015). Dietary Protein Requirement of Female Adults andgt;65 Years Determined by the Indicator Amino Acid Oxidation Technique Is Higher Than Current Recommendations. *Journal of Nutrition*, *145*(1), 18–24. https://doi.org/10.3945/jn.114.197517

Rand, W. M., Pellett, P. L., and Young, V. R. (2003). Meta-analysis of nitrogen balance studies for estimating protein requirements in healthy adults. *American Journal of Clinical Nutrition*, *77*(1), 109–127. https://doi.org/10.1093/ajcn/77.1.109

Reiser, R., Rocheford, E., and Armstrong, C. J. (2006). Basic Mechanical Principles Through Analysis of the Vertical Jump. *Strength and Conditioning Journal*, *28*(4), 70–80. https://doi.org/10.1519/1533-4295(2006)028[0070:BABUOB]2.0.CO;2

Rondanelli, M., Aquilani, R., Verri, M., Boschi, F., Pasini, E., Perna, S., Faliva, A. and Condino, A.M. (2017). Plasma kinetics of essential amino acids following their ingestion as free formula or as dietary protein components. *Aging Clinical and Experimental Research*, *29*(4), 801–805. https://doi.org/10.1007/s40520-016-0605-7

Roubenoff, R. (2003). Catabolism of aging: Is it an inflammatory process? *Current Opinion in Clinical Nutrition and Metabolic Care*, *6*(3), 295–299. https://doi.org/10.1097/00075197-200305000-00005

Ruiz Valenzuela, R. E., Ponce, J. A., Morales-Figueroa, G. G., Aguilar Muro, K., Ramírez Carreón, V., and Alemán-Mateo, H. (2013). Insufficient amounts and inadequate distribution of dietary protein intake in apparently healthy older adults in a developing country: Implications for dietary strategies to prevent sarcopenia. *Clinical Interventions in Aging*, *8*, 1143–1148. https://doi.org/10.2147/CIA.S49810

Ruiz, J. R., Sui, X., Lobelo, F., Morrow, J. R., Jackson, A. W., Sjöström, M., and Blair, S. N. (2008). Association between muscular strength and mortality in men: Prospective cohort study. *British Medical Journal*, *337*(7661), 92–95. https://doi.org/10.1136/bmj.a439

Saxton, R. A., & Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell*, *168*(6), 960-976.

Sette, S., Le Donne, C., Piccinelli, R., Arcella, D., Turrini, A., and Leclercq, C. (2011). The third Italian National Food Consumption Survey, INRAN-SCAI 2005-06 - Part 1: Nutrient intakes in Italy. *Nutrition, Metabolism and Cardiovascular Diseases*, *21*(12), 922–932. https://doi.org/10.1016/j.numecd.2010.03.001

Shimomura, Y., Inaguma, A., Watanabe, S., Yamamoto, Y., Muramatsu, Y., Bajotto, G., Sato, J., Shimomura, N., Kobayashi, H. and Mawatari, K. (2010). Branched-chain amino acid supplementation before squat exercise and delayed-onset muscle soreness. *International Journal of Sport Nutrition and Exercise Metabolism*, *20*(3), 236–244

Shimomura, Y., Yamamoto, Y., Bajotto, G., Sato, J., Murakami, T., Shimomura, N., Kobayashi, H. and Mawatari, K. (2006). Branched-Chain Amino Acids in Exercise Nutraceutical Effects of Branched-Chain Amino Acids on Skeletal Muscle. *Journal of Nutrition*, *136*, 529–532

Sillanpää, E., Laaksonen, D.E., Häkkinen, A., Karavirta, L., Jensen, B., Kraemer, W.J., Nyman, K. and Häkkinen, K. (2009). Body composition, fitness, and metabolic health during strength and endurance training and their combination in middle-aged and older women. *European Journal of Applied Physiology*, *106*(2), 285–296. https://doi.org/10.1007/s00421-009-1013-x

Singh, M. N., Hemant, K. S. Y., Ram, M., and Shivakumar, H. G. (2010). Microencapsulation: A promising technique for controlled drug delivery. *Research in pharmaceutical sciences*, *5*(2), 65

Smith, K., Reynolds, N., Downie, S., Patel, A., and Rennie, M. J. (1998). Effects of flooding amino acids on incorporation of labelled amino acids into human muscle protein. *The American Physiological Society*

Snijders, T., and Parise, G. (2017). Role of muscle stem cells in sarcopenia. *Current Opinion in Clinical Nutrition and Metabolic Care*, *20*(3), 186-190

Snijders, T., Nederveen, J. P., McKay, B. R., Joanisse, S., Verdijk, L. B., van Loon, L. J. C., and Parise, G. (2015). Satellite cells in human skeletal muscle plasticity. *Frontiers in Physiology*. https://doi.org/10.3389/fphys.2015.00283

Snijders, T., Nederveen, J. P., Verdijk, L. B., Houben, A. J. H. M., Goossens, G. H., Parise, G., and van Loon, L. J. C. (2017). Muscle fiber capillarization as determining factor on indices of insulin sensitivity in humans. *Physiological Reports*, *5*(10). https://doi.org/10.14814/phy2.13278

Solerte, S.B., Gazzaruso, C., Bonacasa, R., Rondanelli, M., Zamboni, M., Basso, C., Locatelli, E., Schifino, N., Giustina, A. and Fioravanti, M. (2008). Nutritional Supplements with Oral Amino Acid Mixtures Increases Whole-Body Lean Mass and Insulin Sensitivity in Elderly Subjects with Sarcopenia. *American Journal of Cardiology*, *101*(11 SUPPL.). https://doi.org/10.1016/j.amjcard.2008.03.004

Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. (1997). Dietary Reference Intakes. *Nutrition Reviews*, *55*(9), 319–326. https://doi.org/10.1016/S0002-8223(02)90346-9

Steffl, M., Bohannon, R. W., Sontakova, L., Tufano, J. J., Shiells, K., and Holmerova, I. (2017). Relationship between sarcopenia and physical activity in older people: A systematic review and meta-analysis. *Clinical Interventions in Aging*. https://doi.org/10.2147/CIA.S132940

Stock, M. S., Young, J. C., Golding, L. A., Kruskall, L. J., Tandy, R. D., Conway-Klaassen, J. M., and Beck, T. W. (2010). The effects of adding leucine to pre and postexercise carbohydrate beverages on acute muscle recovery from resistance training. *Journal of Strength and Conditioning Research*, *24*(8), 2211–2219. https://doi.org/10.1519/JSC.0b013e3181dc3a10

Symons, T. B., Sheffield-Moore, M., Wolfe, R. R., and Paddon-Jones, D. (2009). A Moderate Serving of High-Quality Protein Maximally Stimulates Skeletal Muscle Protein Synthesis in Young and Elderly Subjects. *Journal of the American Dietetic Association*, *109*(9), 1582–1586. https://doi.org/10.1016/j.jada.2009.06.369

Tang, J. E., Moore, D. R., Kujbida, G. W., Tarnopolsky, M. A., and Phillips, S. M. (2009). Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *Journal of Applied Physiology*, *107*(3), 987–992. https://doi.org/10.1152/japplphysiol.00076.2009

Tartibian, B., Maleki, B. H., and Abbasi, A. (2009). The Effects of Ingestion of Omega-3 Fatty Acids on Perceived Pain and External Symptoms of Delayed Onset Muscle Soreness in Untrained Men. *Clinical Journal of Sport Medicine*, *19*(2), 115–119. https://doi.org/10.1097/JSM.0b013e31819b51b3

Thomas, D. K., Quinn, M. A., Saunders, D. H., and Greig, C. A. (2016). Protein Supplementation Does Not Significantly Augment the Effects of Resistance Exercise Training in Older Adults: A Systematic Review. *Journal of the American Medical Directors Association*. https://doi.org/10.1016/j.jamda.2016.07.002

Thomas, D. T., Erdman, K. A., and Burke, L. M. (2016). Position of the Academy of Nutrition and Dietetics, Dietitians of Canada, and the American College of Sports Medicine: Nutrition and Athletic Performance. *Journal of the Academy of Nutrition and Dietetics*, *116*(3), 501–528. https://doi.org/10.1016/j.jand.2015.12.006

Thompson, D. D. (2007). Aging and sarcopenia. *Journal of Musculoskeletal and Neuronal Interactions*, *7*(4), 344–345. https://doi.org/10.1152/japplphysiol.00347.2003

Thomson, J. S., Ali, A., and Rowlands, D. S. (2011). Leucine-protein supplemented recovery feeding enhances subsequent cycling performance in well-trained men. *Applied Physiology, Nutrition, and Metabolism*, *36*(2), 242–253. https://doi.org/10.1139/h10-10

Tieland, M., Borgonjen-Van den Berg, K.J., Van Loon, L.J. and de Groot, L.C. (2015). Dietary protein intake in dutch elderly people: A focus on protein sources. *Nutrients*, *7*(12), 9697–9706. https://doi.org/10.3390/nu7125496

Tieland, M., Dirks, M.L., van der Zwaluw, N., Verdijk, L.B., Van De Rest, O., de Groot, L.C. and Van Loon, L.J. (2012a). Protein Supplementation Increases Muscle Mass Gain During Prolonged Resistance-Type Exercise Training in Frail Elderly People: A Randomized, Double-Blind, Placebo-Controlled Trial. *Journal of the American Medical Directors Association*, *13*(8), 713–719. https://doi.org/10.1016/j.jamda.2012.05.020

Tieland, M., van de Rest, O., Dirks, M.L., van der Zwaluw, N., Mensink, M., van Loon, L.J. and de Groot, L.C. (2012b). Protein Supplementation Improves Physical Performance in Frail Elderly People: A Randomized, Double-Blind, Placebo-Controlled Trial. *Journal of the American Medical Directors Association*, *13*(8), 720–726. https://doi.org/10.1016/j.jamda.2012.07.005

Timmerman, K.L., Lee, J.L., Fujita, S., Dhanani, S., Dreyer, H.C., Fry, C.S., Drummond, M.J., Sheffield-Moore, M., Rasmussen, B.B. and Volpi, E. (2010). Pharmacological vasodilation improves insulin-stimulated muscle protein anabolism but not glucose utilization in older adults. *Diabetes*

Timmons, J., Minnock, D., Hone, M., Cogan, K., E., Murphy, J., C., Egan, B. (In Press). Combined exercise training is more effective than time-matched aerobic or resistance exercise training for improving physical function in older adults. Manuscript submitted for publication, *Journal of American Medical Directors Association*, ID JAMDA-D-17-00541

Tipton, K. D., and Wolfe, R. R. (2004). Protein and amino acids for athletes. In *Journal of Sports Sciences* (Vol. 22, pp. 65–79). https://doi.org/10.1080/02640410310001405554

Tipton, K. D., Elliott, T. A., Ferrando, A. A., Aarsland, A. A., and Wolfe, R. R. (2009). Stimulation of muscle anabolism by resistance exercise and ingestion of leucine plus protein. *Applied Physiology, Nutrition, and Metabolism*, *34*(2), 151–161. https://doi.org/10.1139/H09-006

Trabal, J., Forga, M., Leyes, P., Torres, F., Rubio, J., Prieto, E., and Farran-Codina, A. (2015). Effects of free leucine supplementation and resistance training on muscle strength and functional status in older adults: A randomized controlled trial. *Clinical Interventions in Aging*, *10*, 713–723. https://doi.org/10.2147/CIA.S75271

Traylor, D. A., Gorissen, S. H. M., and Phillips, S. M. (2018). Perspective: Protein requirements and optimal intakes in aging: Arewe ready to recommend more than the recommended daily allowance? *Advances in Nutrition*, *9*(3), 171–182. https://doi.org/10.1093/advances/nmy003

Trumbo, P., Schlicker, S., Yates, A. A., and Poos, M. (2002). Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. *Journal of the American Dietetic Association*, *102*(11), 1621–1630. https://doi.org/10.1016/S0002-8223(02)90346-9

USDA National Nutrient Database for Standard Reference, Release 22. U.S. Department of Agriculture, Agricultural Research Service; 20

van Vliet, S., Burd, N. A., and van Loon, L. J. (2015). The Skeletal Muscle Anabolic Response to Plant- versus Animal-Based Protein Consumption. *Journal of Nutrition*, *145*(9), 1981–1991. https://doi.org/10.3945/jn.114.204305

Van Vliet, S., Shy, E. L., Sawan, S. A., Beals, J. W., West, D. W. D., Skinner, S.K., Ulanov, A.V., Li, Z., Paluska, S.A., Parsons, C.M. and Moore, D.R. and Burd, N. A. (2017). Consumption of whole eggs promotes greater stimulation of postexercise muscle protein synthesis than consumption of isonitrogenous amounts of egg whites in young men. *American Journal of Clinical Nutrition*, *106*(6), 1401–1412. https://doi.org/10.3945/ajcn.117.159855

Veldhorst, M.A., Nieuwenhuizen, A.G., Hochstenbach-Waelen, A., Westerterp, K.R., Engelen, M.P., Brummer, R.J.M., Deutz, N.E. and Westerterp-Plantenga, M.S. (2009). Comparison of the effects of a high- and normal-casein breakfast on satiety, "satiety" hormones, plasma amino acids and subsequent energy intake. *British Journal of Nutrition*, *101*(2), 295–303. https://doi.org/10.1017/S0007114508003061

Verdijk, L.B., Jonkers, R.A., Gleeson, B.G., Beelen, M., Meijer, K., Savelberg, H.H., Wodzig, W.K., Dendale, P. and van Loon, L.J. (2009). Protein supplementation before and after exercise does not further augment skeletal muscle hypertrophy after resistance training in elderly men. *American Journal of Clinical Nutrition*, *89*(2), 608–616. https://doi.org/10.3945/ajcn.2008.26626

Verhoeven, S., Vanschoonbeek, K., Verdijk, L. B., Koopman, R., Wodzig, W. K., Dendale, P., and van Loon, L. J. (2009). Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. *American Journal of Clinical Nutrition*, *89*(5), 1468–1475. https://doi.org/10.3945/ajcn.2008.26668

Visser, M., Pahor, M., Taaffe, D.R., Goodpaster, B.H., Simonsick, E.M., Newman, A.B., Nevitt, M. and Harris, T.B. (2002). Relationship of Interleukin-6 and Tumor Necrosis Factor- With Muscle Mass and Muscle Strength in Elderly Men and Women: The Health ABC Study. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *57*(5), M326–M332. https://doi.org/10.1093/gerona/57.5.M326

Volpi, E., Mittendorfer, B., Rasmussen, B. B., and Wolfe, R. R. (2000). The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *Journal of Clinical Endocrinology and Metabolism*, *85*(12), 4481–4490. https://doi.org/10.1210/jc.85.12.4481

Volpi, E., Mittendorfer, B., Wolf, S. E., and Wolfe, R. R. (1999). Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. *American Journal of Physiology-Endocrinology and Metabolism*, *277*(3), E513–E520. https://doi.org/10.1152/ajpendo.1999.277.3.E513

Waldron, M., Whelan, K., Jeffries, O., Burt, D., Howe, L., and Patterson, S. D. (2017). The effects of acute branched-chain amino acid supplementation on recovery from a single bout of hypertrophy exercise in resistance-trained athletes. *Applied Physiology, Nutrition, and Metabolism*, *42*(6), 630–636. https://doi.org/10.1139/apnm-2016-0569

Wall, B. T., Gorissen, S. H., Pennings, B., Koopman, R., Groen, B. B. L., Verdijk, L. B., and Van Loon, L. J. C. (2015). Aging is accompanied by a blunted muscle protein synthetic response to protein ingestion. *PLoS ONE*, *10*(11). https://doi.org/10.1371/journal.pone.0140903

Webb, K. E. (1990). Intestinal absorption of protein hydrolysis products: a review. *Journal of Animal Science*, *68*(9), 3011–3022

Weigle, D.S., Breen, P.A., Matthys, C.C., Callahan, H.S., Meeuws, K.E., Burden, V.R. and Purnell, J.Q. (2005). A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body we. *American Journal of Clinical Nutrition*, *82*(1), 41–48. https://doi.org/10.1093/ajcn.82.1.41

Weinert, B. T., Timiras, P. S., Brian, T., and Timiras, P. S. (2003). Physiology of Aging Invited Review: Theories of aging. *Journal of Applied Physiology*, 1706–1716.

Welle, S., Thornton, C., Jozefowicz, R., and Statt, M., (1993). Myofibrillar protein synthesis in young and old men. *American Journal of Physiology-Endocrinology And Metabolism*, *264*(5), E693-E698.

West, D.W., Burd, N.A., Coffey, V.G., Baker, S.K., Burke, L.M., Hawley, J.A., Moore, D.R., Stellingwerff, T. and Phillips, S.M. (2011). Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. *American Journal of Clinical Nutrition*, *94*(3), 795–803. https://doi.org/10.3945/ajcn.111.013722

Wilkinson, D.J., Bukhari, S.S., Phillips, B.E., Limb, M.C., Cegielski, J., Brook, M.S., Rankin, D., Mitchell, W.K., Kobayashi, H., Williams, J.P. and Lund, J. (2017). Effects of leucine-enriched essential amino acid and whey protein bolus dosing upon skeletal muscle protein synthesis at rest and after exercise in older women. *Clinical Nutrition*. https://doi.org/10.1016/j.clnu.2017.09.008

Wilkinson, D.J., Hossain, T., Hill, D.S., Phillips, B.E., Crossland, H., Williams, J., Loughna, P., Churchward-Venne, T.A., Breen, L., Phillips, S.M. and Etheridge, T. and Atherton, P. J. (2013). Effects of leucine and its metabolite β-hydroxy-β-methylbutyrate on human skeletal muscle protein metabolism. *Journal of Physiology*, *591*(11), 2911–2923. https://doi.org/10.1113/jphysiol.2013.253203

Wilkinson, S. B., Tarnopolsky, M. A., MacDonald, M. J., MacDonald, J. R., Armstrong, D., and Phillips, S. M. (2007). Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *American Journal of Clinical Nutrition*, *85*(4),

1031–1040. https://doi.org/10.3945/ajcn.113.067389

Witard, O. C., Jackman, S. R., Breen, L., Smith, K., Selby, A., and Tipton, K. D. (2014). Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *American Journal of Clinical Nutrition*, *99*(1), 86–95. https://doi.org/10.3945/ajcn.112.055517

Witard, O. C., McGlory, C., Hamilton, D. L., and Phillips, S. M. (2016). Growing older with health and vitality: a nexus of physical activity, exercise and nutrition. *Biogerontology*. https://doi.org/10.1007/s10522-016-9637-9

Wolfson, R. L., & Sabatini, D. M. (2017). The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell metabolism*, *26*(2), 301-309.

Wood, R.H., Reyes, R., Welsch, M.A., Favaloro-Sabatier, J., Sabatier, M., Lee, C.M., Johnson, L.G. and Hooper, P.F. (2001). Concurrent cardiovascular and resistance training in healthy older adults. *Medicine and Science in Sports and Exercise*, *33*(10), 1751–1758. https://doi.org/10.1097/00005768-200110000-00021

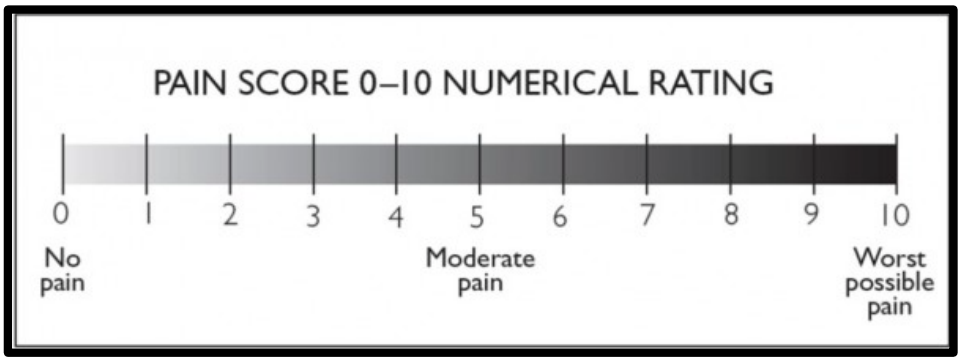
Yang, Y., Breen, L., Burd, N.A., Hector, A.J., Churchward-Venne, T.A., Josse, A.R., Tarnopolsky, M.A. and Phillips, S.M. (2012a). Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. *British Journal of Nutrition*, *108*(10), 1780–1788. https://doi.org/10.1017/S0007114511007422

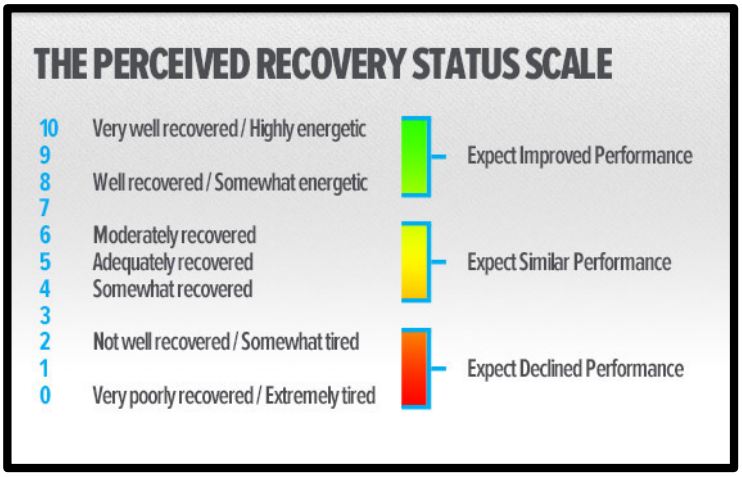
Yang, Y., Churchward-Venne, T. A., Burd, N. A., Breen, L., Tarnopolsky, M. A., and Phillips, S. M. (2012b). Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. *Nutrition and Metabolism*, *9*(1), 57. https://doi.org/10.1186/1743-7075-9-57

Young, V. R., and Pellett, P. L. (1994). Plant proteins in relation to human and amino acid nutrition. *The American Journal of Clinical Nutrition*, *59*(suppl), 1203S–12S

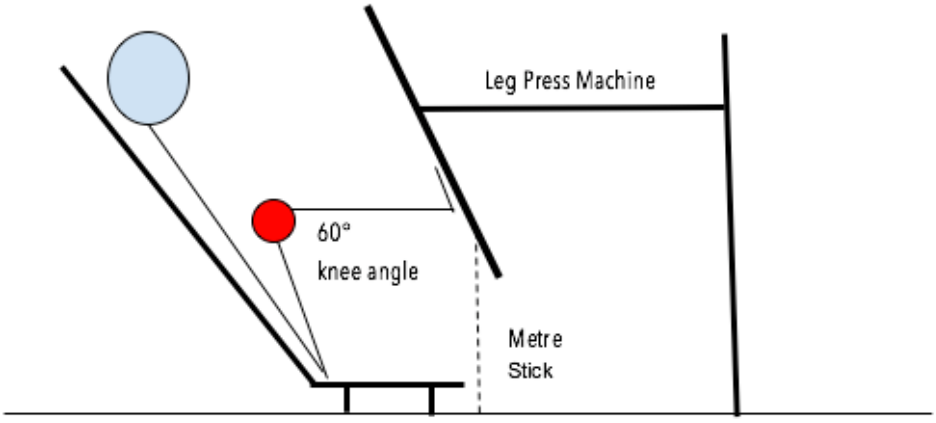
Appendices

Appendix 1 – Chapter 7 –Pain Scale and Perceived Recovery Scale





Appendix 2 – Chapter 7 –Position of participant on leg press, to control for movement range-of-motion



Appendix 3 – Chapter 7 – Nutrition and supplement protocol instruction following the 14-hour post-exercise period

The effect of leucine supplementation on muscle soreness and the recovery of muscle function after intense exercise

Instruction on following the Nutrition & Supplement Protocol

It is of utmost importance for the validity of this research study that you follow the meal plan and supplementation protocol very closely.

We have provided you with 14 bottles to go home with. Every 2 hours, 2 bottles are to be taken at the time indicated on the bottle.

You have also been instructed to eat 4 meals at specific times throughout the day. We ask you:

- Not to eat anything outside of the foods we have outlined on the meal plan
- To be very precise when you are measuring out your food (we have provided you with measurement utensils)
- Not to have snacks between your 4 meals

For example, your protocol will look like this:

9:00am - Meal provided in the lab + 2 bottles
11:00am - 2 bottles only
1:00pm - Meal specified on meal plan + 2 bottles
3:00pm - 2 bottles only
5:00pm - Meal specified on meal plan + 2 bottles
7:00pm - 2 bottles only
9:00pm - Meal specified on meal plan + 2 bottles
11:00pm - 2 bottles only

"Please take the instructed bottles **at the same time as** your meal"

Please fill out the following form and return it to us tomorrow morning:

Participant Code and Trial (for lab use): _____

Scheduled Time:		Time taken:	Comments:
9:00am	Meal & 2 bottles		
11:00am	x 2 bottles		
1:00pm	Meal & 2 bottles		
3:00pm	x 2 bottles		
5:00pm	Meal & 2 bottles		
7:00pm	x 2 bottles		
9:00pm	Meal & 2 bottles		
11:00pm	x 2 bottles		

Note: It is very important that your **do not foam roll, or do any mobility** between the time that you complete the 10x10 and the 48 hour post testing visit.

Appendix 4– Chapter 7 –Sample meal plan for male of body mass 75-80kg

Meal Plan for 75kg - 80kg

Male, 46, 80kg, 180cm, 24.7 BMI

Testing Day

FOOD NAME:	QUANTITY:	DESCRIPTION:	CALORIES	CARBS	PROTEIN	FAT
Meal 1 - Provided in Lab (9:00am/10:00am)						
Manitoba Hemp Protein	25	(Provided)	115	7.3g	12.5g	2.9g
Almonds, with skin	35	(Provided)	209	2.6g	7.3g	18.4g
Tesco Active Isotonic	700	(Provided)	168	45g	0g	0g
Nutri-Grain Chocolate Chip Bake	90	(Provided)	362	63g	4.3g	11.7g
		Mea l Total:	854	117g	24g	33g
Meal 2 - Vegetable Stirfry with Avocado (1:00pm/2:00pm)						
Basmati rice, dry	100	1/2 Dry Cup	363	88g	7.4g	0.5g
Onions, raw	15	1/4 onion	5.3	1.2g	0.2g	0.02g
Avocado, Hass, flesh only	95	x 2/3 avocado	181	1.8g	1.5g	18.7g
Manitoba Hemp Protein	25	(Provided)	115	7.3g	12.5g	2.9g
Peppers, bell, raw, mixed	160	1 full pepper	39	7.7g	1.6g	0.5g
Garlic, raw	3	1 clove	2.9	0.5g	0.2g	0.02g
Oil, coconut	9	x1 Tsp (solids)	81	0g	trace	9g
Soy sauce, light and dark varieties	18	x 1 tbsp	14.2	3.2g	0.5g	0g
		Mea l Total:	802	109g	24g	32g
Meal 3 - Oats Banana and Peanut Butter (5:00pm/6:00pm)						
Manitoba Hemp Protein	25	(Provided)	115	7.3g	12.5g	2.9g
Oat flakes, rolled	40	x 1/2 Dry Cup (Made on wate	152	28g	4.4g	3.2g
Bananas, flesh only	120	x Large banana or 1/2 large	97	24g	1.4g	0.1g
Peanut butter, smooth	25	x 1 tbsp	152	3.3g	5.7g	13g
		Mea l Total:	517	63g	24g	19.2g
Meal 4 - Vegetable Stirfry with Avocado (9:00pm/10:00pm)						
Soy sauce, light and dark varieties	18	x 1 tbsp	14.2	3.2g	0.5g	0g
Onions, raw	15	1/4 onion	5.3	1.2g	0.2g	0.02g
Garlic, raw	3	1 clove	2.9	0.5g	0.2g	0.02g
Manitoba Hemp Protein	25	(Provided)	115	7.3g	12.5g	2.9g
Avocado, Hass, flesh only	95	x 2/3 avocado	181	1.8g	1.5g	18.7g
Oil, coconut	9	x1 Tsp (solids)	81	0g	trace	9g

Appendix 5 – Chapter 8&9 –Nutrition intervention instructions for participants in nutrition and exercise group

Nutrition to Support Your New Exercise Regime

Firstly, thank you for signing up for this research study. For the purpose of this study, you have been assigned to the **Training & Nutrition Intervention Group**, which means we will be bringing in changes to your eating habits over the next 12 weeks.

As you will be introducing exercises that are new to your body, it is important to compliment these exercise sessions with good nutrition. If we support the body with the right foods, your muscles will repair, grow and get stronger from each exercise session, and you should truly benefit from the entire study.

The main change we want you to introduce during this study is **increasing your protein intake**. The reason protein is of particular importance is because it provides the building blocks we need to repair and build muscle. We will be honing in on your **breakfast, lunch** and **dinner** everyday, making them each **high protein meals.**

It is also imperative that **one of these meals is eaten directly after (No more than 1 hour after) your training session on a Monday/Wednesday/Friday.** After training your body needs to take on this protein to enable this repair and recovery process to take place.

Here are some tips which will help you apply these changes with ease:

- **Preparation is key:** Make sure that you have your kitchen stocked up with the protein foods on the list overleaf
- **Use left-overs:** Cook a little extra at dinner to have for your lunch the following day
- **Batch cook meals:** Making a couple of meals to last you a few days is a great way of saving time! You can also separate them into portions and freeze them. Examples of these meals are chicken curry, beef mince chilli concarni, lasagne, and chicken stifry
- **Cook a roasted chicken/turkey/beef:** This can be left in the fridge for a few days, and can be used for lunch/dinner with salad, bread, potatoes, etc to make up a high protein meal
- **Prepare some hard-boiled eggs**: Keep these in the fridge and use some to make up a high protein breakfast/lunch

University College Dublin
Nutrition Intervention

Firstly, thank you for signing up for this research study, and we hope you're excited about your involvement over the next 12 weeks. For the purpose of this study, you have been assigned to the **Nutrition Intervention Group**, which means we will be bringing in changes to your eating habits over the next 12 weeks.

The main change we want you to introduce during this study is **increasing your protein intake**. We will be honing in on your **breakfast, lunch** and **dinner** everyday, making them each high protein meals.

It is imperative to the success of this intervention, for you and for the research team that you stick to the guide overleaf for next 12 weeks.

Here are some tips which will help you apply these changes with ease:

- **Preparation is key:** Make sure that you have your kitchen stocked up with the protein foods on the list overleaf
- **Use left-overs:** Cook a little extra at dinner to have for your lunch the following day
- **Batch cook meals:** Making a couple of meals to last you a few days is a great way of saving time! You can also separate them into portions and freeze them. Examples of these meals are chicken curry, beef mince chilli concarni, lasagne, and chicken stifry
- **Cook a roasted chicken/turkey/beef:** This can be left in the fridge for a few days, and can be used for lunch/dinner with salad, bread, potatoes, etc to make up a high protein meal
- **Prepare some hard-boiled eggs**: Keep these in the fridge and use some to make up a high protein breakfast/lunch

Please choose a **combination of TWO of the following** to have at **EACH main meal. It is imperative that you stick to the correct portion size** (You can of course combine two of the same food choices)

Food	How much you need to eat
Chicken/Turkey	**1/2 fillet or steak**
Turkey mince **Beef/Lamb/Pork steak/mince** **Fish (Cod, Hake, Salmon, etc)**	**Half a palm sized** (You can combine two of these palm sized portions if for example you wanted to have a full steak or fish fillet)
Prawns	14 small/6 jumbo prawns
Tinned Tuna/Mackerel	**Small tin** (56-60g drained)
Low fat yoghurt	**150g pot** (0% Glenisk, Liberte, or Fage yoghurt)
Bacon/Rashers	**3**
Pork/Turkey sausages	**2**
Eggs (Poached, boiled, fried, etc)	**2 medium eggs**
Whole/Low fat/Supermilk	**450 ml**
Hard cheeses	**"Deck of cards" size**
Quark cheese/Cottage cheese	**130g**

Here are two examples of how you might combined the above option to create a nutritious, high protein breakfast, lunch and dinner:

Breakfast	Porridge oats, made on **450ml milk, 150g pot of low fat yoghurt** and fresh berries
Lunch	Sandwich with "**deck of cards" size of cheddar cheese** and **2 hard-boiled eggs**
Dinner	**Palm sized portion of sirloin steak**, baby potatoes and mixed vegetables

Breakfast	**2 scrambled eggs, small portion of smoked salmon,** grilled tomato and 1 slice of toast
Lunch	**Palm sized portion of roast chicken** with avocado and ryvita crackers
Dinner	**Palm sized portion of salmon** with roasted vegetables

Appendix 6 – Chapter 8&9 – Dietary adherence sheet

Weekly Protein Intake Checklist - Week Starting 3rd April 2017

Please tick the **TWO** portions of protein that you have had at each meal. Remember, you will need to eat **TWO** of the following examples at three main meals:

½ palm sized portion of chicken, steak, beef mince, turkey breast, turkey mince, fish	150g pot low fat yoghurt
Small tin of tuna/mackerel	'Deck of cards' size of hard cheese
3 piece of bacon/rashers	450ml milk
2 pork/turkey sausages	2 medium eggs

	Portion 1	**Portion 2**
MONDAY		
Breakfast		
Lunch		
Dinner		
TUESDAY		
Breakfast		
Lunch		
Dinner		
WEDNESDAY		
Breakfast		
Lunch		
Dinner		
THURSDAY		
Breakfast		
Lunch		
Dinner		
FRIDAY		
Breakfast		
Lunch		
Dinner		
SATURDAY		
Breakfast		
Lunch		
Dinner		
SUNDAY		
Breakfast		
Lunch		
Dinner		

3-Day Food Diary

Please keep a record of everything you eat and drink over the next 3 days

Instructions:

1. Every time you eat or drink something, write it down in the diary provided.

2. For mixed meals, try to estimate the amount of each component of the meal.

For example, if making a salad:

If the bag of leaves says 70 g, and you use half the bag, then fill in diary as e.g. Rocket leaves 35 g

If the container of tomatoes says 200 g, and you use a quarter of these, then fill in diary as e.g. Cherry tomatoes 50 g
And so on through all components of a meal

3. If this is not practical, then simply give an estimate of portion size e.g. a large handful of salad leaves, 8-10 cherry tomatoes

4. Try to describe the food as accurately as possible, e.g. skimmed milk, multi-seed granary bread, milk chocolate-coated digestive biscuits as opposed to simply writing milk, bread and biscuits

5. In the case of eating out, eating pre-packed meals/foods and similar, make an estimate of portion size. Note the brand and any other comments you want to add

6. Where possible, always give estimates of the portion size: to do this you can use household measurements or write down the weight of the product from the packet where possible.

For example:

- Large, medium or small banana
- Three dessert spoons of peas
- 200 g tin of baked beans
- One large egg
- Tayto, salt & vinegar, 40 g packet
- One mug of instant coffee, black

7. Where appropriate, be sure to note whether the food is cooked or uncooked. Try to describe the cooking method:

For example:

- 1 large egg, boiled, scrambled or fried in oil
- Lamb chop, grilled
- Frozen peas, boiled

8. Don't forget to include any sauces, condiments, or second helpings

9. Remember to include all **foods AND drinks AND supplements consumed** at home and at other places such as restaurants and friend's houses etc.

10. Try to fill in the diary as you eat, instead of leaving it till the end of the day. This ensures that you won't forget what you have eaten

11. At all times, try to be as accurate and descriptive as possible

University College Dublin
School of Public Health, Physiotherapy and Population Sciences
Institute of Sport and Health
Health Science Centre
Belfield, Dublin 4, Ireland

STUDY OF THE PLASMA KINETICS OF AN ORAL LEUCINE SUPPLEMENT DELIVERED BY A NOVEL MICROENCAPSULATION METHOD

PARTICIPANT INFORMATION LEAFLET

01/02/2015

Thank you very much for your interest this research project. We are looking for volunteers to take part in a research study under the direction of Ms. Michelle Hone and Dr. Brendan Egan at the UCD Institute for Sport and Health, School of Public Health, Physiotherapy and Population Science. Please read the following paragraphs which should explain the research in greater detail.

The aim of this study is to investigate the effect of delivering the nutrition supplement, leucine, in a novel form known as microencapsulation on the amount of leucine that is present in blood in the few hours after swallowing. Ms. Hone is funded by a postgraduate award known as the Irish Research Council Enterprise Partnership Scheme, in collaboration with AnaBio Technology. AnaBio are an Irish start-up who have developed this new technique for encapsulation of food ingredients to be used in this study.

1. What is this research about?

Leucine is an amino acid (a building block for protein) that is found naturally in certain foods. Unlike some other amino acids, the body cannot produce leucine itself, and it must be obtained from food sources. These sources include protein-containing foods such as meats and dairy products, as well as supplements such as whey protein and recovery shakes.

Leucine plays an important role in recovery from exercise and in building muscle tissue. The process in which muscle tissue is created is referred to as muscle protein synthesis (MPS). It is possible that if the delivery of leucine is optimised, this process of creating muscle tissue may be maximised. Therefore, a novel encapsulation technique, which may enhance the delivery of leucine, is being tested in this study.

2. Why are we doing this research?

Research suggests that MPS has an upper limit in response to a quantity of leucine, meaning after a certain dose of leucine, the rate of MPS cannot be increased any further. However, when the level of leucine in the blood rises and falls intermittently, similar to what happens when we eat a meal, and then, eat another meal several hours later, the rate of muscle growth may be maximised.

To mimic this meal-to-meal scenario, and recreate this intermittent rising and falling of leucine in the blood, a novel microencapsulation technique has been developed which enables leucine to be released in intervals over several hours. Microencapsulation means that the individual leucine molecules are coated in a special protein that allows them to be put into a drink, and when the drink is consumed, this special protein coating is broken down at a slower rate than if leucine was consumed in its normal form.

In addition, the use of encapsulated leucine may also improve solubility and the taste of leucine-containing protein supplements used both in sport and in supplements that support healthy ageing.
Thus, we are interested in the way that the delivery of this encapsulated leucine can influence the way in which leucine is digested and absorbed into the blood.

3. How will the data be used?

The results we obtain from your tests will be grouped together with those of others in the study. When the study is complete we would like to submit the grouped results to a scientific journal for publication. A report on the study will also be written.

4. What will happen if I decide to take part in this research study?

If you are happy to proceed, this research is due to commence in February 2015 and will run for about 5 months. However, your involvement will last only a few weeks, in which you will make five separate visits to the Human Performance Laboratory in the Institute for Sport and Health in UCD. Because the menstrual cycle affects metabolism of carbohydrate, fat and protein, this means that females would be required to visit once per month to complete visits, whereas males can visit once per week. For that reason and the time-constraints of the project, we are *only recruiting males* on this study.

Before this, however, you will be asked to provide written consent to be a volunteer, and state that you have read this information sheet and understand the study protocol. Next you will come to UCD for your first visit. That visit and all subsequent visits will last approximately 5 hours.

Each visit will be identical except for the varying the drink that you will drink, which will be in random order and you will not know which drink is which until after the study. This is known as “blinding”. For each visit, you will come to the Human Performance Laboratory in the Institute for Sport and Health at UCD (Newstead Block C) at between 8:00 and 9;00 AM after an overnight fast. This means that you will come to the lab without eating your breakfast or consuming any drinks except water that morning. The testing session will consist as follows:

- *Blood collection*: A small plastic needle known as a cannula will be placed in your arm vein to permit blood withdrawal. During the whole session, 11 blood samples will be collected each of about the size of a teaspoon, and this is a minimal quantity not able to alter any of your body’s normal functions.
- *Response to an oral leucine load*: An initial resting blood sample will be taken before you have drunk any beverage. Next, you will be asked to consume one of five beverages (a drink containing encapsulated leucine, normal leucine in one dose, normal leucine in two doses a combination of normal leucine and encapsulated leucine, or a beverage containing no leucine). During the next four hours, while remaining in the lab, 10 blood samples will be collected at 15 minutes, 30 minutes, 45 minutes, a hour, and every half hour for the remaining 3 hours.

After the collection of the last blood sample, you will be provided with a small meal consisting of a meat-based sandwich, a cereal bar and a piece of fruit. This will complete your commitment in the lab on that day.

If you have any specific dietary requirements such as gluten intolerance or prefer a vegetarian option, you will have the opportunity to let us know prior to your first visit and

we can arrange for alternative foods..

Once your first visit is complete, you will visit the lab again on four more occasions. Identical procedures will take place during these visits, except on each occasion, you will consume one of four other beverages.

Prior to your first visit you will be asked to complete a two day food intake diary, a food frequency questionnaire and a habitual physical activity level questionnaire. These are standard research questionnaires and will take no longer than 25 minutes on total. During your first visit, you will also have your height and weight measured, and you will have your body composition (%fat and muscle) assessed using a dual energy x-ray body absorptiometry body scan also known as DXA. Wearing light, loose clothing, you will lay on a flat bed over which the scanner moves. A trained operator will position you correctly based on your size and stature, and you will lay still during the scan, which takes about 10 minutes to complete.

5. How will my privacy be protected?

All study data will be stored securely in UCD. All study participants will be given a study code upon entering the study. Your name will appear beside this code on a master sheet that will be held in confidence by the lead investigator. This master sheet is the only link between your name and your study code. Your study code number will appear on all study documentation from there onwards. There will be no way in which you may be identified in the reported study findings.

In addition, the master sheet containing your name and your assigned code will be destroyed after data collection is complete. After this point, it will no longer be possible to identify your data, in which case it will no longer be possible for you to withdraw the data from the study. However, this now anonymous data will be stored indefinitely and used in future research and scientific publications.

6. What are the benefits of taking part in this research study?

You will receive a comprehensive report of your current muscle and bone function, and physical activity level. You will receive a comprehensive report on the composition of your normal diet, and how to improve this, if necessary. These reports will be in the form of a Word document that will be emailed to you by Ms. Hone on the completion of analysis of your DXA scan and the questionnaires attached to the study. Within this report, we will provide a comparison of your data with other participants on the study as well as in comparison to international norms and health guidelines as appropriate.

7. What are the risks of taking part in this research study?

The risks associated with participation in the study are minimal. However, you should consider the following prior to giving consent to participate:

There is a potential for a small amount of bruising to occur when a blood sample is drawn. To ensure this risk is kept to a minimum, a person trained specifically for this purpose will be employed to undertake this procedure. You may be assured that the procedures to be employed have been used extensively by the researchers conducting this study and are generally well-tolerated by participants.

With respect to blood sampling, we will take all possible precautions to avoid infection during these procedures. These samples will be taken with sterile disposable needles, drapes and gauze; in fact, sterile (aseptic) techniques are used during all sampling procedures.

You will undergo iDXA scanning once during the study. The iDXA scan for body composition works in a similar manner to an X-ray, but the radiation dose that you are subjected to is very much less than a standard X-ray (about 1/40th). At this level, the risk

to you, as described by the international authorities regulating the using of X-rays, is 'trivial'.

After an overnight fast, there is a small change that your blood sugar levels may drop below normal levels, a situation known as fasting hypoglycemia. Symptoms include nausea, extreme hunger, feeling nervous or jittery, clammy, wet skin and/or excessive sweating not caused by exercise, rapid heartbeat, trembling. This is rare in young healthy men but if we observe, or you experience, any of these symptoms, we will measure blood sugar levels directly and provide a small snack consisting of a cereal bar and a sports drink as these provide a small amount of sugar to return you to normal levels.

8. Can I change my mind at any stage and withdraw from the study?

If at any time during the study you are uncomfortable with any of the testing or protocols, or if you can no longer commit to the study for whatever reason, you have the option to withdraw from the study up until the date of the final data collection. If you are a UCD student, withdrawal will not result in any penalty or affect your rights as a student of UCD, or your participant in recreational or competitive sport at UCD.

9. How will I find out what happens with this project?

You are free to contact any of the researchers involved in this study (contact details below) and ask additional information regarding your results, data etc.

10. Contact details and further information:

- Dr. Brendan Egan, PhD
  Email: brendan.egan@ucd.ie
  Phone: 01 716 3419
  Role: Principal investigator for this research and responsible for study design
  Responsible for medical assessment and support on the study

- Michelle Hone, Postgraduate Research Student
  Email: michelle.hone@ucdconnect.ie
  Phone: 087 9342326
  Role: Responsible for coordination of all testing procedures

We will be happy to answer any questions you may have.

Dublin City University
School of Health and Human Performance

STUDY OF THE PLASMA KINETICS OF LEUCINE WHEN COMBINED WITH A MIXED MEAL

PARTICIPANT INFORMATION LEAFLET

01/02/17

Thank you very much for your interest this research project. We are looking for volunteers to take part in a research study under the direction of Ms. Michelle Hone and Dr. Brendan Egan at the DCU School of Health and Human Performance. Please read the following paragraphs, which should explain the research in greater detail.

The aim of this study is to investigate the effect of delivering the nutrition supplement, leucine, on the amount of leucine that is present in blood in the few hours after swallowing.

11. What is this research about?

Leucine is an amino acid (a building block for protein) that is found naturally in certain foods. Unlike some other amino acids, the body cannot produce leucine itself, and it must be obtained from food sources. These sources include protein-containing foods such as meats and dairy products, as well as supplements such as whey protein and recovery shakes.

Leucine plays an important role in recovery from exercise and in building muscle tissue. The process in which muscle tissue is created is referred to as muscle protein synthesis (MPS). It is possible that if the delivery of leucine is optimised, this process of creating muscle tissue may be maximised.

12. Why are we doing this research?

Research has shown that MPS is dictated by the concentration of leucine in the blood. A previous study in our labs investigated the concentrations of leucine in the blood after a leucine drink (leucine powder and water) was drank on its own. To mimic a real-life scenario, we want to investigate the concentrations of leucine in the blood after the same leucine drink has been drank, while a balanced meal of carbohydrates, fats and carbohydrates has been eaten with this drink.

13. How will the data be used?

The results we obtain from your tests will be grouped together with those of others in the study. When the study is complete we would like to submit the grouped results to a

scientific journal for publication. A report on the study will also be written.

14. What will happen if I decide to take part in this research study?

If you are happy to proceed, this research is due to commence in February 2017 and will run for about 2 months. However, your involvement will last only a few weeks, in which you will make three separate visits to the Human Performance Laboratory in the School of Health and Human Performance in DCU. Because the menstrual cycle affects metabolism of carbohydrate, fat and protein, this means that females would be required to visit once per month to complete visits, whereas males can visit once per week. For that reason and the time-constraints of the project, we are *only recruiting males* on this study.

Before this, however, you will be asked to provide written consent to be a volunteer, and state that you have read this information sheet and understand the study protocol. Next you will come to DCU for your first visit. That visit and all subsequent visits will last approximately 4.5 hours.

Each visit will be identical except we will be varying the drink that you will drink, which will be in random order and you will not know which drink is which until after the study. This is known as “blinding”. For each visit, you will come to the Human Performance Laboratory in the School of Health and Human Performance in DCU at between 7:00 and 9;00 AM after an overnight fast. This means that you will come to the lab without eating your breakfast or consuming any drinks except water that morning. The testing session will consist as follows:

- *Blood collection*: A small plastic needle known as a cannula will be placed in your arm vein to permit blood withdrawal. During the whole session, 11 blood samples will be collected each of about the size of a teaspoon, and this is a minimal quantity not able to alter any of your body’s normal functions.
- *Response to an oral leucine load and meal*: An initial resting blood sample will be taken before you have eaten or drunk anything. Next, you will be asked to consume one of three beverages (a drink containing leucine in one dose, a drink containing leucine in two doses, or a beverage containing no leucine). During the next four hours, while remaining in the lab, 10 blood samples will be collected at 15 minutes, 30 minutes, 45 minutes, a hour, and every half hour for the remaining 3 hours.

Once your first visit is complete, you will visit the lab again on two more occasions. Identical procedures will take place during these visits, except on each occasion, you will consume one of the other two beverages.

15. How will my privacy be protected?

All study data will be stored securely in DCU. All study participants will be given a study code upon entering the study. Your name will appear beside this code on a master sheet that will be held in confidence by the lead investigator. This master sheet is the only link between your name and your study code. Your study code number will appear on all study documentation from there onwards. There will be no way in which you may be identified in the reported study findings.

In addition, the master sheet containing your name and your assigned code will be destroyed after data collection is complete. After this point, it will no longer be possible to identify your data, in which case it will no longer be possible for you to withdraw the data from the study. However, this now anonymous data will be stored indefinitely and used in future research and scientific publications.

16. What are the risks of taking part in this research study?

The risks associated with participation in the study are minimal. However, you should consider the following prior to giving consent to participate:

There is a potential for a small amount of bruising to occur when a blood sample is drawn. To ensure this risk is kept to a minimum, a person trained specifically for this purpose will be employed to undertake this procedure. You may be assured that the procedures to be employed have been used extensively by the researchers conducting this study and are generally well-tolerated by participants.

With respect to blood sampling, we will take all possible precautions to avoid infection during these procedures. These samples will be taken with sterile disposable needles, drapes and gauze; in fact, sterile (aseptic) techniques are used during all sampling procedures.

After an overnight fast, there is a small change that your blood sugar levels may drop below normal levels, a situation known as fasting hypoglycemia. Symptoms include nausea, extreme hunger, feeling nervous or jittery, clammy, wet skin and/or excessive sweating not caused by exercise, rapid heartbeat, trembling. This is rare in young healthy men but if we observe, or you experience, any of these symptoms, we will measure blood sugar levels directly and provide a small snack consisting of a cereal bar and a sports drink as these provide a small amount of sugar to return you to normal levels.

17. Can I change my mind at any stage and withdraw from the study?

If at any time during the study you are uncomfortable with any of the testing or protocols, or if you can no longer commit to the study for whatever reason, you have the option to withdraw from the study up until the date of the final data collection. If you are a DCU student, withdrawal will not result in any penalty or affect your rights as a student of UCD, or your participant in recreational or competitive sport at DCU.

18. How will I find out what happens with this project?

You are free to contact any of the researchers involved in this study (contact details below) and ask additional information regarding your results, data etc.

19. Contact details and further information:

- Dr. Brendan Egan, PhD
  Email: brendan.egan@dcu.ie
  Role: Principal investigator for this research and responsible for study design
  Responsible for medical assessment and support on the study

- Michelle Hone, Postgraduate Research Student
  Email: michelle.hone2@mail.dcu.ie
  Phone: 087 9342326
  Role: Responsible for coordination of all testing procedures

We will be happy to answer any questions you may have.

University College Dublin
School of Public Health, Physiotherapy and Sports Science
Institute of Sport and Health
Health Science Centre
Belfield, Dublin 4, Ireland

EFFECT OF LEUCINE SUPPLEMENTATION ON MUSCLE SORENESS AND THE RECOVERY OF MUSCLE FUNCTION AFTER INTENSE EXERCISE

PARTICIPANT INFORMATION LEAFLET
01/02/2016

Thank you very much for your interest in participating in this research project. We are looking for volunteers to take part in a research study for both a PhD study and final year research dissertation as part of the BSc in Health and Performance Science. This research study will be carried out by Ms. Michelle Hone and colleagues under the supervision of Dr. Brendan Egan at the School of Public Health, Physiotherapy and Sports Science. Please read the following paragraphs, which will explain the research study in greater detail.

The aim of this study is to investigate the delivery strategies of an amino acids, known as leucine, to optimise recovery in the 24 hours after intense weight lifting exercise.

What is this research about?

Most athletes are aware of a "post-exercise window of opportunity" existing during which it is recommended to consume nutrients as soon as possible during recovery from exercise. However, many previous studies have focused primarily on markers of muscle growth shortly after exercise, whereas we are interested in this phenomenon in team sport and strength/power athletes and their recovery over 24 hours in terms of strength and muscle soreness after a gym-type session.

Why are we doing this research?

This research aims to investigate if leucine supplementation can have a positive effect on recovery and muscle soreness in team sports athletes who have done an intense gym session. This research is important as it is still unknown whether leucine can impact recovery in team sports athletes, such as rugby, GAA, soccer, hockey, as well as strength/power athletes, despite the fact that leucine continue to soar in popularity amongst athletes.

The post-exercise window of opportunity describes a period of time where repair and recovery processes are faster such as recovery of carbohydrates stores and repair of muscle. It is widely accepted that eating as soon as possible after exercise is best practice but many of the studies on which this advice is based were performed in 3 to 6 hour recovery windows, while complete recovery takes longer than this, likely up to 24 or 48 hours.

Leucine is an amino acid that has strong effects on muscle growth but less is known about

how the method of leucine delivery throughout a given day effects muscle soreness and recovery.

We will investigate the influence of leucine supplementation during 24 hours of recovery on physical tests and blood-based markers of recovery as this has important implications for the type of nutrition advice given by nutritionists in team sport settings.

How will the data be used?

The results we obtain from your tests will be grouped together with those of others in the study. The data gathered from the study will be used to write up a final year dissertation for the BSc in Health and Performance Science at UCD, as well as Ms. Hone's doctoral thesis. When the study is complete, we would like to submit the grouped results to a peer-reviewed scientific journal for publication.

What will happen if I decide to take part in this research study?

If you are happy to proceed, this research is due to commence in late February 2016 and will run for about three months. However, your involvement will last only one month during which you will be required to report to the High Performance Unit at UCD Institute for Sport and Health on four separate occasions (on the same day of the week at the same time of day) over those four weeks. Before this however you will be asked to provide written consent to be a volunteer, and state that you have read this information sheet and understand the study protocol.

During the first visit, your leg muscle strength will be assessed by means of three repetition maximum test also known as a 3RM test using a leg press machine. You must have at least one year worth of experience in gym training with this exercise before you can be allowed to participate in the study. Following a warm-up, you will begin to lift progressively heavier weights with two minutes rest between sets until you cannot lift any heavier. This will be your 3RM off which the rest of the study's protocols will be based. We will also measure your height and weight and determine your quantities of muscle and fat using a DXA scan. This is a scan that measures your body composition (%fat and muscle). Wearing light, loose clothing, you will lay on a flat bed over which the scanner moves. A trained operator will position you correctly based on your size and stature, and you will lay still during the scan, which takes approximately 10 minutes to complete. Your total visit time will be approximately one hour.

Over the next several weeks, you will then have three main "trials". Each trial will be identical except for the different conditions for the recovery supplement explained below. Each main trial will proceed as follows:

- Having not eaten anything since the night previous, and having not trained for 24 hours previous, you will come to lab and undergo a battery of tests to measure your jumping ability and your leg and arm muscle strength. You will also provide a small blood sample from a vein in your arm.
- Next, after a brief warm-up, you will perform an intense weightlifting session that involves 10 sets of 10 repetitions in the leg press exercise at 60% of the 1RM measured in the first visit, with two minutes rest between each set. Your form and timing of each repetition will be monitored throughout the session.
- Fifteen minutes after the test, you will undergo the same battery of tests as earlier, to measure your jumping ability and your leg and arm muscle strength. You will also provide another small blood sample. Your total visit time will be approximately two hours.
- Between the end of the exercise test and bedtime that night, you will be asked to drink 8 "shots" (about two mouthfuls) of the test nutrient dissolved in sweetened water. These will be taken at 2 hour intervals. You will also be not to do any training for the next 24 hours. You will be asked to follow a meal plan for the duration of the day, which consists of 4 main meals. Examples of what will be included on the meal plan are hemp protein (which will be provided by us), oats, banana, rice, avocado, nuts, etc.

- The next day, 24 hours after the end of the weightlifting session, you will come back again and undergo the same battery of tests to measure your jumping ability and your leg and arm muscle strength. You will also provide another small blood sample. This visit will last 30 minutes.
- The following day, 48 hours after the end of the weightlifting session, you will come back again and undergo the same battery of tests to measure your jumping ability and your leg and arm muscle strength. You will also provide another small blood sample. This visit will last 30 minutes.

You will repeat this whole pattern of a main trial three times over three weeks on the same day of the week, and this will complete your participation.

The three trials reflect the fact that we are investigating three different drinks that will be provided to you in random order depending on the trial. These drinks are glucose (sugar), leucine delivered at four time points throughout and the day, and leucine delivered at eight time points throughout and the day.

Why have I been invited to take part in this research?

You have been asked to take part in this research because you are physically active and have a good history of training. Because we are interested in the response to individual exercise sessions and the effect of drinking different nutrients, having people who already are well-trained will provide with the best insight into the responses that would occur if these sessions were undertaken as training.

How will my privacy be protected?

All study data will be stored securely in UCD. All study participants will be given a study code upon entering the study. Your name will appear beside this code on a master sheet that will be held in confidence by the project supervisor, Dr. Egan. This master sheet is the only link between your name and your study code. Your study code number will appear on all study documentation from there onwards. There will be no way in which you may be identified in the reported study findings.

In addition, the master sheet containing your name and your assigned code will be destroyed after data collection is complete. After this point, it will no longer be possible to identify your data, in which case it will no longer be possible for you to withdraw the data from the study. However, this now anonymous data will be stored indefinitely and used in future research and scientific publications.

What are the benefits of taking part in this research study?

As each testing day is similar to an intense training session, by the end of this study, you may see an improvement in your overall strength and performance, which is likely to benefit you in your sport. As we also measure strength and body composition, we will be able to assess your fitness status relative to your peers and international norms. You will receive a comprehensive report of your current muscle and bone health (DXA scan) and information on the composition of your normal diet.

What are the risks of taking part in this research study?

The risks associated with participation in the study are minimal. However, you should consider the
following prior to giving consent to participate:

As with any exercise of high intensity and heavy lifting, there is a chance of muscle strain, but as these sessions are similar to those performed as part of your own sport and/or training, the risk is similar to that present in training. You will be taken through a gradual warm-up to reduce this risk. In general, there is little risk associated with these tests but the most likely event to occur immediately after or within the next few hours after the test is

local muscle soreness in the legs, similar to the feeling of a hard training session in your respective sport. This will subside with after 36 hours. Should any emergency arise during the testing, the investigators are trained and certified in emergency first aid and CPR.

When a blood sample is drawn, there may be a small amount of discomfort when the needle breaks the skin and potential for a small amount of bruising to occur. To ensure this risk is kept to a minimum, you may be assured that the procedure to be employed has been used extensively by the researchers conducting this study and is generally well-tolerated by participants. The blood samples will be stored for up to 6 months at -20°C in a secure freezer at UCD Institute of Sport and Health to which only the project supervisor has access. Samples will be coded, so your name will not be associated with the samples, once the master coding sheet has been destroyed. The purpose of these samples are to analyze markers of inflammation and recovery in the blood.

You will undergo DXA scanning on during the study. The iDXA scan for body composition works in a similar manner to an X-ray, but the radiation dose that you are subjected to is very much less than a standard X-ray (about 1/40th). At this level, the risk to you, as described by the international authorities regulating the using of X-rays, is 'trivial'.

All of the procedures described in section 4 above are standard procedures for the evaluation of strength, and nutrient supplementation effects. These procedures are currently the best methods for the questions being addressed. The project supervisor is experienced in the implementation of these techniques and the students are trained in the same.

Can I change my mind at any stage and withdraw from the study?

If at any time during the study you are uncomfortable with any of the testing or protocols, or if you can no longer commit to the study for whatever reason, you have the option to withdraw from the study up until the date of the final data collection. If you are a UCD student, withdrawal will not result in any penalty or affect your rights as a student of UCD, or your participant in recreational or competitive sport at UCD.

How will I find out what happens with this project?

You are free to contact any of the researchers involved in this study (contact details below) and ask additional information regarding your results, data etc. You will be provided written feedback on your results once the whole study is completed and the data are analyzed.

Contact details and further information:

Dr. Brendan Egan, PhD (Project supervisor for this research and responsible for study design)

- brendan.egan@ucd.ie
- 01 716 3419

Michelle Hone (PhD student responsible for coordination of all testing procedures)

- michelle.hone@ucdconnect.ie
- 00353 87 934 2326

We will be happy to answer any questions that you may have.

University College Dublin
School of Public Health, Physiotherapy and Sports Sciences
Institute for Sport and Health
Health Science Centre
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EFFICACY OF NUTRITION GUIDELINES TO AUGMENT EXERCISE TRAINING EFFECTS IN SKELETAL MUSCLE MASS AND FUNCTION IN OLDER ADULTS

PARTICIPANT INFORMATION LEAFLET

27/03/17

Thank you very much for taking time to consider participating in this research project. We are looking for volunteers to take part in a research study conducted by Mr. James Timmons and Ms. Michele Hone under the direction of Dr. Brendan Egan. Mr. Timmons is a post-graduate researcher at the UCD Institute for Sport and Health, School of Public Health, Physiotherapy and Sports Science. Ms. Hone is a post-graduate researcher in the School of Health and Human Performance, Dublin City University. Dr. Egan is a visiting associate professor in the School of Public Health, Physiotherapy and Sports Science, but whose primary role is as Senior Lecturer in Sports and Exercise Physiology at Dublin City University. Please read the following paragraphs, which should explain the research in greater detail. Mr. Timmons is funded by the Irish Research Council (IRC), an industry-academic collaboration supported by Medfit Proactive Healthcare, and Ms. Hone is also funded by the IRC.

1. What is this research about?

From approximately 50 years of age people begin to gradually lose muscle and strength. This is concerning because losses in muscle are associated with an increased risk of falls, fractures and physical disability. Therefore, it is important to identify nutrition strategies along with exercise interventions to slow muscle loss in older individuals.

This study will examine how the body and mind adapt to 12 weeks of either exercise alone or exercise combined with food-based advice and substitutions to support exercise training in people over the age of 65 years. We think that providing a series of nutrition tips involving whole food substitutions will increase the benefits of exercise training and support healthy ageing.

2. Why are we doing this research?

Having low muscle and strength is associated with increased risk of negative health conditions and reduced quality of life. This study will assess your muscle and strength. This study will test whether a series of nutrition tips, along with a supervised exercise training programme, will assist in the adaptation of the training programme in helping to preserve muscle and strength in older adults. This study will inform the development of an evidence-based lifestyle intervention for Irish older adults at risk for physical and mental decline, which may enable healthcare specialists to support healthy ageing.

3. How will the data be used?

The data gathered from the study will be used to write up part of a PhD thesis at University College Dublin (UCD) and Dublin City University (DCU), and may be used in the future for publication in a peer-reviewed scientific journal.

4. What will happen if I decide to take part in this research study?

If you decide to take part in this research and fulfill the inclusion criteria, you will be required to participate in three assessment sessions: at baseline (0 weeks), mid-point (6 weeks) and after completion of training (12 weeks) at the Human Performance Laboratory at the UCD Institute for Sport and Health and in Medfit Proactive Healthcare, consisting of body composition, functional capacity, and cognitive performance assessment.

The total expected time per visit/session is 1 hour. They will consist as follows:

Test battery 1: @UCD Institute for Sport and Health

You will arrive to the lab at UCD ISH after an overnight fast, i.e. you will not eat your breakfast that day, for the following assessments:
Body Mass: Height and weight will be measured with a standard clinical scale and stadiometer

DEXA (dual energy x-ray body absorptiometry) body scan: this is a scan that measures body composition (percentage of fat and muscle). Wearing light, loose clothing, you will lie on a flat bed over which the scanner moves. A trained operator will position the participant correctly based on your size and stature, and you will lie still during the scan, which takes about 10 minutes to complete.

Blood Pressure: Immediately after your DXA while still lying down, blood pressure will be measured at the elbow using a standard blood pressure assessment

Blood analysis: You will have a blood sample (about a tablespoon) taken by a trained technician from a vein in your arm, and this is a minimal quantity not able to alter any of your body's normal functions.

At this point, you will be provided with a small snack of a piece of fruit and cereal bar. You may drink water but you will still not be allowed to have tea or coffee

Handgrip strength: will next be measured with a device that will measure your ability ability to exert force in the handgrip.

Timed Up and Go Test (TUGT): In this test, you will stand up from a standard chair, walk a distance of 3m as fast as possible, turn, walk back to the chair, and sit again.

Short Physical Performance Battery (SPPB): The SPPB is based on a timed short-distance walk, repeated chair stands, and balance test. Each of the performance measures is assigned a score ranging from 0 to 4, with 4 indicating the highest level of performance and 0 the inability to complete the test. Lower body function will be evaluated using tests of walking speed (3 m), standing balance, and the time which you need to rise from a chair five consecutive times as quickly as possible with the arms folded across their chest.

Your mental function will be assessed using the **Montreal Cognitive Assessment (MoCA),** a brief measure of function that includes aspects of attention, language, verbal memory, and so on. The MoCA will be administered by trained personnel.

The Chester Step Test will be used to estimate your aerobic fitness. During the test you will be asked to step on to and off a 15-cm step at a rate set by a beat on a computer. The initial step rate is 15 steps per minute and every 2 minutes the tempo will be increased by 5 steps per minute. You will continue stepping until you reach 80% of your maximum predicted maximum heart rate, or rate the test as too hard, or reach the end of the 10-minutes 5-stage test.

Test battery 2: @ Medfit Proactive Healthcare

Stair Climbing Power Test (SCPT): This test requires you to climb a flight of stairs as quickly as possible but you are allowed to using a handrail for support if you wish.

Upper/Lower Limb Strength Tests: Your upper and lower body strength will be measured using what is known as one repetition maximum (1RM), which means the most amount of weight that you can lift once. We will assess this on the chest press, a rowing exercise and a leg press.

Exercise training programme

This will take in Medfit Proactive Healthcare (Blackrock Business Park, Carysfort Avenue, Blackrock, Co. Dublin). All training sessions will be supervised and performed in a group setting, three days per week (Mon, Wed, Fri) for twelve weeks (36 training sessions in total). A gradual progression will be built into the training programme and the programme will consist of aerobic- and strength-based activities. We have recently completed a study using an identical exercise training protocol that was very well received by the participants who were also over 65 years of age. You will be randomised into one of two groups as follows:

Group 1: A group who trains as described above and will also be provided with nutrition guidelines to assist in the adaptation of the training programme. These guidelines will suggest food-based substitutions to consume both at breakfast and at lunch.
Group 2: A group who trains as described above and does not change their habitual daily eating habits.

5. How will we protect your privacy?

Your privacy will be protected in a number of ways. Your data will be stored on a password-protected computer, which only the project supervisor will have access to. This data will be coded, so that you will be only potentially identifiable. Upon completion of the study, identifiable data will be destroyed, and only anonymous data will remain.

6. What are the benefits of taking part in this research study?

Regular exercise has many positive effects on health and mood, and the proposed study will provide you with the recommended amount of weekly exercise for the twelve weeks of the training study The assessment of body composition by DXA, and the assessment of aerobic fitness, strength and cognitive ability are common measures of fitness, so we will be able to assess you relative to your peers and international norms. These results can be used to help plan your future exercise programme relative to current health and fitness. Additionally, based on your food diary, we will provide feedback on your dietary habits relative to your health status. During the testing period or at any time during the study, if we discover any medical issue that would warrant further investigation, we will inform you directly. The questionnaires completed and assessments performed in the study do not constitute a diagnosis and therefore will not be reported to your GP. However, when you receive your feedback pack after completion of the study, you are welcome to take your results to your GP for discussion if desired.

It should be noted that there is a random allocation procedure within this study whereby you will randomly assigned to an intervention (exercise training & nutrition) or control group (exercise training only). If assigned to the control group, you will be asked to maintain your normal diet for the duration of the 12 week intervention period. You will still receive all of the same benefits of feedback on your current health and nutrition status.

7. What are the risks of taking part in this research study?

In general, there is little risk associated with exercise training but the most likely event to occur immediately after or within the next few hours after training is, as with any exercise of moderate-to-high intensity, a chance of muscle strain and local muscle soreness. Any local muscle soreness will subside with after 24 hours. You will be taken through a gradual warm-up to reduce this risk. Should any emergency arise during the testing, the investigators are trained and certified in emergency first aid and CPR.

When a blood sample is drawn, there may be a small amount of discomfort when the needle breaks the skin and potential for a small amount of bruising to occur. To ensure this risk is kept to a minimum, you may be assured that the procedure to be employed has been used extensively by the researchers conducting this study and is generally well tolerated by participants. We will take all

possible precautions to avoid infection during these procedures. These samples will be taken with sterile disposable needles, drapes and gauze; in fact, sterile (aseptic) techniques are used during all sampling procedures. These techniques will be performed by trained staff, and support staff within the Institute for Sport and Health are trained in first aid, and will be available throughout each trial if you require attention.

You will undergo iDXA scanning once during the study. The iDXA scan for body composition works in a similar manner to an X-ray, but the radiation dose that you are subjected to is very much less than a standard X-ray (about 1/40th). At this level, the risk to you, as described by the international authorities regulating the using of X-rays, is 'trivial'.

8. Can I change my mind at any stage and withdraw from the study?

If at any time during the study you are uncomfortable with any of the testing or protocols, or if you can no longer commit to the study for whatever reason, you have the option to withdraw from the study up until the date of the final data collection.

9. How will I find out what happens with this project?

We will inform you of all outcomes and results when all data is collected and finalised. In the interim, we will provide feedback on your relative scores throughout the testing period

10. Contact details and further information:

If you have any further questions or queries please forward them to:

- Dr. Brendan Egan, PhD
  Email: brendan.egan@ucd.ie / brendan.egan@dcu.ie
  Phone: 01 700 8803
  Role: Principal investigator for this research and responsible for study design

- Mr. James Timmons, Postgraduate Research Student
  Email: james.timmons@ucdconnect.ie
  Role: Responsible for coordination of all testing procedures

- Ms. Michele Hone, Postgraduate Research Student
  Email: michelle.hone2@mail.dcu.ie
  Role: Responsible for coordination of all testing procedures

We will be happy to answer any questions you may have.

Appendix 12 – Chapter 5&6 – Ethical Approval Letter

UCD Office of Research Ethics

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hrec@ucd.ie
www.ucd.ie/researchethics

31st March 2015

Ms Michelle Hone
c/o Dr Brendan Egan
UCD School of School of Public Health, Physiotherapy and Population Science
Woodview House
Belfield
Dublin 4

RE: LS-15-01-Hone-Egan: *Study of the plasma kinetics of an oral leucine supplement delivered by a novel microencapsulation method*

Dear Ms Hone

Thank you for your response to the Human Research Ethics Committee – Sciences (30/03/15). The Decision of the Committee is that **approval is granted** for this application which is subject to the conditions set out below.

Please note that **public liability insurance for this study has been confirmed** in accordance with our guidelines.[i]

Your request to access UCD students was also reviewed and granted. Please ensure that any additional permissions to access participants, whether internal (heads of Schools) or external are obtained before the recruitment of the participants is commenced.

Please note that approval is for the work and the time period specified in the above protocol and is subject to the following:

- Any amendments or requests to extend the original approved study will need to be approved by the Committee. Therefore you will need to submit by email the *Request to Amend/Extend Form* (HREC Doc 10);
- Any unexpected adverse events that occur during the conduct of your research should be notified to the Committee. Therefore you will need to Submit, by email, an *Unexpected Adverse Events Report* (HREC Doc 11);
- You or your supervisor (if applicable) are required to submit a signed *End of Study Report Form* (HREC Doc 12) to the Committee upon the completion of your study;

.../.

- This approval is granted on condition that you ensure that, in compliance with the Data Protection Acts 1988 and 2003. If applicable, all data will be destroyed in accordance with your application and that you will confirm this in your *End of Study Report* (HREC Doc 12), or indicate when this will occur and how this will be communicated to the Human Research Ethics Committee;
- Please note that further **new** submissions from you may not be reviewed until any **End of Study Reports due** have been submitted to the Office of Research Ethics. That is, any earlier study that you received ethical approval for from the UCD HRECs;
- You may require copies of submitted documentation relating to this approved application and therefore we advise that you retain copies for your own records;
- Please note that the granting of this ethical approval is premised on the assumption that the research will be carried out within the limits of the law;
- Please also note that approved applications and any subsequent amendments are subject to a Research Ethics Compliance Review.

The Committee wishes you well with your research and look forward to receiving your End of Study Report. All forms are available on the website www.ucd.ie/researchethics please ensure that you submit the latest version of the relevant form. If you have any queries regarding the above please contact the Office of Research Ethics and please quote your reference in all correspondence.

Yours sincerely,

Mr T. John O'Dowd
Chairman, Human Research Ethics Committee - Sciences

[i] http://www.ucd.ie/researchethics/information_for_researchers/insurance/

Appendix 13 – Chapter 7 – Ethical Approval Letter

UCD Office of Research Ethics

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hrec@ucd.ie
www.ucd.ie/researchethics

24th January 2016

Dr Brendan Egan
UCD School of Public Health, Physiotherapy and Sports Science
Woodview House
Belfield
Dublin 4

RE: LS-16-08-Egan: *Effect of leucine supplementation on muscle soreness and the recovery of muscle function after intense exercise*

Dear Dr Egan,

Thank you for your response to the Human Research Ethics Committee – Sciences (24/03/16). The Decision of the Committee is that **approval is granted** for this application which is subject to the conditions set out below.

Your request to access UCD students was also reviewed and granted. Please ensure that any additional permissions to access participants, whether internal (heads of Schools) or external are obtained before the recruitment of the participants is commenced.

Please note that **public liability insurance for this study has been confirmed** in accordance with our guidelines.[i]

Please note that approval is for the work and the time period specified in the above protocol and is subject to the following:

- Any amendments or requests to extend the original approved study will need to be approved by the Committee. Therefore you will need to submit by email the *Request to Amend/Extend Form* (HREC Doc 10);
- Any unexpected adverse events that occur during the conduct of your research should be notified to the Committee. Therefore you will need to Submit, by email, an *Unexpected Adverse Events Report* (HREC Doc 11);
- You or your supervisor (if applicable) are required to submit a signed *End of Study Report Form* (HREC Doc 12) to the Committee upon the completion of your study;

.../.

- This approval is granted on condition that you ensure that, in compliance with the Data Protection Acts 1988 and 2003. If applicable, all data will be destroyed in accordance with your application and that you will confirm this in your *End of Study Report* (HREC Doc 12), or indicate when this will occur and how this will be communicated to the Human Research Ethics Committee;
- Please note that further **new** submissions from you may not be reviewed until any **End of Study Reports due** have been submitted to the Office of Research Ethics. That is, any earlier study that you received ethical approval for from the UCD HRECs;
- You may require copies of submitted documentation relating to this approved application and therefore we advise that you retain copies for your own records;
- Please note that the granting of this ethical approval is premised on the assumption that the research will be carried out within the limits of the law;
- Please also note that approved applications and any subsequent amendments are subject to a Research Ethics Compliance Review.

The Committee wishes you well with your research and look forward to receiving your End of Study Report. All forms are available on the website www.ucd.ie/researchethics please ensure that you submit the latest version of the relevant form. If you have any queries regarding the above please contact the Office of Research Ethics and please quote your reference in all correspondence.

Yours sincerely,

Mr T. John O'Dowd
Chairman, Human Research Ethics Committee - Sciences

[i] http://www.ucd.ie/researchethics/information_for_researchers/insurance/

Appendix 13 – Chapter 8&9 – Ethical Approval Letter

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24th February 2017

Dr Brendan Egan
UCD School of Public Health, Physiotherapy and Sports Science
Belfield
Dublin 4

RE: LS-17-22-Timmons-Egan: *Efficacy of nutrition supplementation to augment exercise training effects on skeletal muscle mass and function in older adults*

Dear Dr Egan

Thank you for your response to the Human Research Ethics Committee – Sciences (23/02/17). The Decision of the Committee is that **approval is granted** for this application which is subject to the conditions set out below.

Please note that **public liability insurance for this study has been confirmed** in accordance with our guidelines.[i]

Please note that approval is for the work and the time period specified in the above protocol and is subject to the following:

- Any amendments or requests to extend the original approved study will need to be approved by the Committee. Therefore you will need to submit by email the *Request to Amend/Extend Form* (HR4);
- Any unexpected adverse events that occur during the conduct of your research should be notified to the Committee. Therefore you will need to Submit, by email, an *Unexpected Adverse Events Report* (HR5);
- You or your supervisor (if applicable) are required to submit a signed *End of Study Report Form* (HR6) to the Committee upon the completion of your study;
- This approval is granted on condition that you ensure that, in compliance with the Data Protection Acts 1988 and 2003, all data will be managed in accordance with your application and that you will confirm this in your *End of Study Report* (HR6);
- Please note that further **new** submissions from you may not be reviewed until any **End of Study Reports due** have been submitted to the Office of Research Ethics. That is, any earlier study that you received ethical approval for from the UCD HRECs;
- You may require copies of submitted documentation relating to this approved application and therefore we advise that you retain copies for your own records;

.../.

- Please note that the granting of this ethical approval is premised on the assumption that the research will be carried out within the limits of the law;
- Please also note that approved applications and any subsequent amendments are subject to a Research Ethics Compliance Review.

The Committee wishes you well with your research and look forward to receiving your End of Study Report. All forms are available on the website www.ucd.ie/researchethics please ensure that you submit the latest version of the relevant form. If you have any queries regarding the above please contact the Office of Research Ethics and please quote your reference in all correspondence.

Yours sincerely,

Mr T. John O'Dowd
Chairman, Human Research Ethics Committee - Sciences

[i] http://www.ucd.ie/researchethics/information_for_researchers/insurance/