



The impact of leucine-enriched whey protein on skeletal muscle anabolism

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MRes, BSc Hons

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

28th March 2024

I would like to dedicate this thesis to my Grandma and Grandad.

Both of whom passed away whilst I was studying for my PhD.

Your support and love never left me, and I hope I have made you proud.

I finally finished my homework, Grandad.

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Acknowledgements

I would firstly like to thank Arla Foods Ingredients and the Medical Research Council who supported and funded the research conducted for my PhD.

Thank you to Professor Phil Atherton, Professor Beth Phillips, Dr Mads Larsen, and Professor Leigh Breen for their supervision over the past 3.5 years. My thanks also go to Professor Kenny Smith and Dr Dan Wilkinson for their guidance and laboratory support.

A special thank you goes to Dr Paula Scaife and Mrs Amanda Gates. Thank you so much for all your support in the lab, during study days and being someone to turn to throughout my entire time with the research group.

To my family and friends. Thank you for sticking with me throughout the highs and the lows. Without your constant support, belief, and love, none of this would have been possible.

Finally, to Cameron. You have been with me from the very beginning and supported me all the way. Thank you for everything you have done and continue to do for me. I love you.

Statement of Circumstances

The COVID-19 pandemic dramatically impacted the undertaking of this project in several ways. Even though this project started in the latter stages of 2020 (October), the UK was impacted by several government mandated national lockdowns throughout the first year of the project. Resultingly, access to the laboratory was restricted for a prolonged period. Furthermore, we as a research group were unable to conduct human research until the latter stages of 2021. Even after human research was allowed again, several precautions were in place and there was an understandable lack of willingness to participate in research projects from the public, due to the impact of the pandemic, hence, restricting the progression of the project.

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Abbreviations

1RM	One repetition maximum
4E-BP1	4E-binding protein 1
A-band	Anisotropic band
AA	Amino acids
AV	Arterio-venous
BCAA	Branched chain amino acids
BLG	β -lactoglobulin
BLG_10	β -lactoglobulin with 10 g protein
BLG_5	β -lactoglobulin with 5 g protein
BMI	Body mass index
BSA	Bovine serum albumin
CD98	Cluster of differentiation 98 transporter
CSA	Cross-sectional area
D ₂ O	Deuterium oxide
DIAAS	Digestible indispensable amino acid score
DNA	Deoxyribonucleic acid
DXA	Dual-energy X-ray absorptiometry
EAA	Essential amino acids
eEF2	Eukaryotic Translation Elongation Factor 2
eIF-#	Eukaryotic initiation factor-
ELISA	Enzyme-linked immunosorbent assay
EWGSOP	European Working Group on Sarcopenia in Older People
FAO	Food and Agriculture Organisation of the United Nations

FBR	Fractional breakdown rate
FED	Effects of feeding
FED-EX	Effects of feeding plus exercise
FSR	Fractional synthetic rate
GATOR-#	GTPase-activating protein toward Rags-
GC/MS	Gas chromatography/mass spectrometry
GIP	Glucose-dependent insulintropic polypeptide
GTP	Guanosine-5'-triphosphate
HCL	Hydrochloric acid
HMB	β -Hydroxymethylbutyrate
I-band	Isotropic band
IAAO	Indicator amino acid oxidation
iAUC	Integrated area under the curve
IV	Intravenous
KA	Keto acids
KIC	α -ketoisocaproate
LAT1	L-type amino acid transporter 1
LEAA	Leucine-enriched essential amino acids
LEU	Leucine
LRS	Leucyl-tRNA synthetase
MAFbx	Muscle Atrophy F-box
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid

mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
muRF1	Muscle RING-finger protein-1
Na ₃ VO ₄	Sodium orthovanadate
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids
NPB	Net protein balance
p70S6K1	Ribosomal protein s6 kinase 1
PA	Physical activity
PAT1	Proton-coupled AA transporter 1
PCA	Perchloric acid
PDCAAS	Protein digestibility-corrected amino acid score
PP2A	Protein phosphatase 2A
Ra	Rate of appearance
Rag	Recombination-activating genes
Raptor	Regulatory-associated protein of mTOR
RC-#	Research chapter-#
Rd	Rate of disappearance
RDA	Recommended daily allowance
RE	Resistance exercise
RET	Resistance exercise training
RPS6	Ribosomal protein s6
SKM	Skeletal muscle
SNAT2	Small neutral amino acid transporter 2

SPPBT	Short physical performance batter test
tBDMS	t-butyldimethylsilyl
TBST	Tris buffered saline/Tween 20
TIF-IA	Transcription initiation factor-IA
tRNA	Transfer ribonucleic acid
TUG	Timed-up and go test
VL	Vastus lateralis
WHO	World Health Organisation
WP	Whey protein
WPI	Whey protein isolate

Abstract

The preservation of skeletal muscle (SKM) throughout the lifespan is vital for maintaining health and well-being. SKM is tightly regulated and maintained by the dynamic relationship between muscle protein synthesis (MPS) and muscle protein breakdown (MPB), with SKM protein turnover occurring at a rate of ~1–2% per day in healthy younger adults. Protein feeding and exercise are the most potent stimulators of MPS with synergistic action occurring to promote SKM mass through increased MPS and suppressed MPB. However, rates of MPS and MPB are also influenced by physical inactivity and ageing, contributing to SKM atrophy and functional declines.

Sarcopenia, the age-related decline in muscle mass and function, is a significant predictor of age-related disability and mortality, and impacts every individual across the globe. Although inevitable and inescapable, performing physical activity/exercise and maintaining a balanced healthy diet throughout life significantly alters the rate at which this functional decline progresses and the presence of disease. As sarcopenia is a multifaceted condition, we specifically focus on the role of SKM anabolic resistance in older age and how dietary protein and exercise interventions can maximise healthy ageing throughout this project.

Following consumption of dietary protein and/or amino acids (AA), MPS is increased temporally and transiently. Regardless of protein source, SKM is receptive to the essential amino acid (EAA) component of a protein meal, in particular leucine (LEU), which is thought to be the most potent AA for

stimulating MPS. Interestingly, LEU can stimulate MPS in isolation and through the consumption of low-dose LEU-enriched protein. In some instances, suboptimal doses of LEU-enriched protein have matched the anabolic response of larger doses of protein. β -lactoglobulin (BLG) is the most abundant whey protein (WP) in bovine milk and is rich in EAA and LEU. There has yet to be a thorough investigation of the effects of BLG supplementation on SKM anabolism in both younger and older individuals. Therefore, this thesis is divided into a narrative review and three research chapters aiming to determine the impact of LEU-enriched WP on SKM anabolism.

We initially focus on the importance of LEU supplementation for SKM anabolism in differing physiological states, through our narrative review (Chapter 3). We then aimed to investigate the efficacy of BLG supplementation on SKM anabolism at rest and following acute resistance exercise (RE), using stable isotope tracers. Chapters 4 and 5 focus on the anabolic effects of BLG compared to an isonitrogenous whey protein isolate (WPI) in young and older individuals, respectively. Chapter 6 explores different doses of BLG on SKM anabolism in older adults only. We demonstrate throughout this thesis that BLG is an effective nutritional supplement to stimulate SKM anabolism in differing populations and may be an effective intervention, alongside RE, in offsetting sarcopenia in advancing age.

Chapter 1

1. Introduction

1.1. The human musculoskeletal system

The human musculoskeletal system is a complex network between bone, muscle, and connective tissues, which allows for movement, provides support, structure, and function of the human body (Hayes et al., 2016). The musculoskeletal system also offers protection and provides housing for vital organs and the central nervous system (Elrod, 2014). Due to its location and function, the musculoskeletal system commonly sustains traumatic injuries and degenerative changes (Elrod, 2014), but also alters the ability to perform daily functional tasks throughout life's varying developmental stages. For older adults, this often leads to impaired functional capacity, increased frailty, risk of injury, and decreases quality of life and independence. Muscle is a tissue composed of contractile cells or fibres that effects movement of an organ or part of the body (Hayes et al., 2016). The human body consists of three different types of muscle: skeletal (striated), cardiac and smooth muscle. The function of the muscle can vary considerably depending on type, with skeletal muscle (SKM) being the only type of muscle that can be controlled voluntarily. The focus of this thesis going forward will be specifically on SKM in differing physiological states, including in response to protein nutrition, exercise, ageing and inactivity.

1.1.1. Skeletal muscle structure and function

SKM accounts for approximately 40% of total body mass in healthy adults (Janssen et al., 2000; McLeod et al., 2016). The preservation of SKM across the human lifespan not only allows for the maintenance of physical independence, but also allows for the maintenance of whole-body metabolic health (Wilkinson et al., 2018b) and protection from metabolic morbidities such as insulin resistance and the subsequent development of diabetes (Brook et al., 2016b). SKM is known for its importance in posture, breathing and locomotion (McLeod et al., 2016), with a remarkable ability to adapt to (e.g.) endurance and resistance exercise (RE) (Brook et al., 2016b), both of which result in distinct changes in an individual's phenotype. Importantly, the role of SKM is not solely limited to its force-generating capacities. For example, SKM is responsible for the majority of post-prandial glucose disposal and plays a central role in whole-body protein metabolism (DeFronzo et al., 1985; Shulman et al., 1990; Wolfe, 2006), with the additional ability to liberate large pools of amino acids (AA) in conditions of fasting or stress (Pozefsky et al., 1976; Brook et al., 2016b).

SKM muscle is ultimately constructed from thousands of layers of proteins and consists of type I (slow twitch) and type II (fast twitch) muscle fibres. The most superficial layer of SKM is the muscle “body” created from muscle fascicles, with the deepest being the myofibrils which are subsequently made from thousands of myofilaments (see Figure 1.1 for illustration). It is these myofilaments which house the contractile elements of the muscle, sarcomeres, which are comprised of actin, myosin, nebulin and titin and are uniform in appearance (Marieb, 2005, p. 248). Concerning the structure of sarcomeres, actin (thin) filaments, are anchored to the Z discs at each end of a sarcomere by

nebulin and contain regulatory proteins, troponin, and tropomyosin, which are essential to allow for muscle contraction (Marieb, 2005, p. 250). Tropomyosin provides rigidity to actin and blocks actin's active sites when the muscle is relaxed to prevent a state of tetanus. Whereas troponin, a three-polypeptide complex, allows for the binding of actin, tropomyosin, and calcium ions (Marieb, 2005, p. 250). Myosin (thick) filaments contain a tail and two globular heads which bind to actin to form a “cross-bridge” during contraction (Huxley, 1969). Titin is vitally important in the process of muscle relaxation, with its two main roles being: i) holding myosin filaments in place, thus maintaining the organisation of the anisotropic band (A-band) and, ii) assisting the muscle cell to spring back into shape after contraction (Marieb, 2005, p. 251).

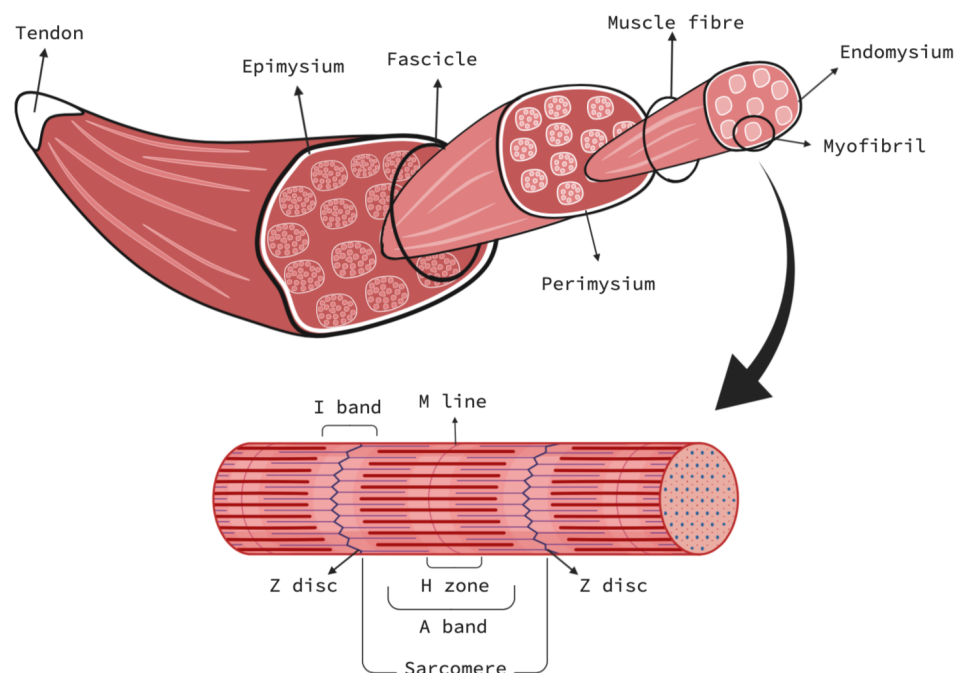


Figure 1.1 Illustration of skeletal muscle and sarcomere structure. A-band, anisotropic band. I-band, isotropic band. Created in Biorender.com.

1.2. Skeletal muscle protein metabolism

Protein regulation accounts from anywhere between 10–35% of daily energy needs in a healthy young individual. In a young healthy male, when normalised to body weight, SKM contains 30% of all body proteins with this value being lower (16%) in females (Cohn et al., 1980). Protein is the only macronutrient (the others being carbohydrate and fat) which cannot be stored by the body, although muscle can provide AA during fasting and times of starvation. Newly synthesised proteins can function as enzymes, transporters, hormones, and antibodies, and can assist in maintaining fluid/electrolyte balance, acid-base balance, energy production, immune defence, and cellular growth, maintenance, and repair (Carbone et al., 2016).

There are 20 different AA which are the most abundant in the human body and diet due to being used for protein synthesis (Table 1.1). All AA have specific metabolism properties, with it long established that not all AA can be synthesised *de novo* in quantities commensurable with the metabolic demands of protein synthesis. Of the 20 AA, nine are classed as essential amino acids (EAA – or indispensable AA) meaning they do not present in the body and must be obtained through the diet. The remaining 11 are classed as non-essential amino acids (NEAA – or dispensable AA) and can be synthesised endogenously. Within the group of EAA, there are three branched chain amino acids (BCAA): leucine (LEU), isoleucine, and valine. Despite these distinct categories, some AA (e.g., arginine and glutamine) may be classed as “conditionally essential” in certain circumstances such as deficiency during infancy or in the presence of disease (World Health Organization, 2007). Subsequently, it is the sequence of

the AA which ultimately determines the structure and function of the protein of interest.

Table 1.1. Amino Acid Classifications.

Essential Amino Acids	Non-essential Amino Acids
Histidine	Alanine
<i>Isoleucine</i>	Arginine
<i>Leucine</i>	Asparagine
Lysine	Aspartate
Methionine	Cysteine
Phenylalanine	Glutamate
Threonine	Glutamine
Tryptophan	Glycine
<i>Valine</i>	Proline
	Serine
	Tyrosine

Branched chain amino acids indicated in italics.

SKM is regulated and maintained by the dynamic relationship between muscle protein synthesis (MPS) and muscle protein breakdown (MPB), with SKM proteins typically turning over at a rate of ~1–2% per day in healthy younger adults (Atherton et al., 2010a; Atherton et al., 2016; Breen et al., 2013). Net protein balance (NPB) is achieved through counterbalanced fluctuations in MPS and MPB via periods of net influx and efflux of AA during fasted and fed periods across the day (Burd et al., 2009; Atherton & Smith, 2012). Indeed, MPS and

MPB are responsive to dietary protein feeding, both alone and when combined with various modalities of exercise (i.e., resistance, endurance, or concurrent exercise) (Burd et al., 2009; Atherton & Smith, 2012), but also to periods of inactivity (de Boer et al., 2007; Glover et al., 2008; Wall et al., 2013b; Wall et al., 2016). Subsequently, muscle protein anabolism varies in differing physiological conditions, which in turn may modulate the capacity of SKM to incorporate AA into protein; ultimately driving rates of SKM hypertrophy or atrophy.

The dynamic balance between MPS and MPB is closely regulated. In the fasted state, MPB exceeds MPS, whereas in the fed state MPS exceeds MPB; thereby creating NPB across the day (see Figure 1.2). Protein feeding and exercise are the most potent stimulators of MPS with synergistic action occurring to promote SKM mass over time, through increased MPS and suppressed MPB. It has been well established that following feeding, MPS increases in response to heightened availability of AA, although despite continued AA availability, this response is limited to ~two hours due to the effects of “muscle full” (Atherton et al., 2010a) (detailed in section 1.3). Whilst MPS is heightened due to feeding, MPB becomes suppressed predominantly due to the reflex actions of insulin (Greenhaff et al., 2008), although this action is blunted in older age (Wilkes et al., 2009). In situations of ageing and/or inactivity, rates of MPS become blunted to key anabolic stimuli, namely exercise and protein nutrition. This has subsequently been termed “anabolic resistance” and significantly limits muscle anabolism. Conversely, in situations of starvation and fasting or advanced stages of disease, MPB increases to meet the demands of protein turnover, although

there is a limit as to how much protein can be catabolised. Additionally, rates of MPS seemingly increase in severe disease states, thus often experiencing greater rates of protein turnover (Phillips et al., 2020). Resultingly, understanding the mechanisms of MPS and MPB and patterns for loss/gain of SKM protein is essential to attempt to combat muscle wasting and restoring/maintaining muscle mass through effective therapies, specifically exercise and nutritional plans.

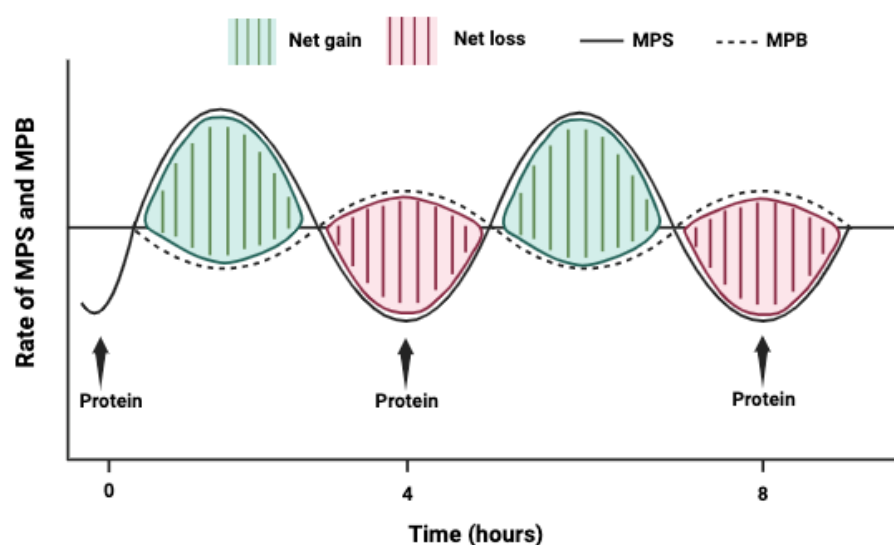


Figure 1.2 Diurnal fluctuations in muscle protein synthesis (MPS) and muscle protein breakdown (MPB) in relation to protein feeding. Adapted from Phillips et al., 2009. Created in Biorender.com.

1.2.1. Muscle protein synthesis

Protein synthesis is a core component of cellular regulation and has profound effects at tissue, organ, and whole-body levels (Carbone et al., 2016). Protein synthesis is the process of translating messenger ribonucleic acid (mRNA) to functional peptides and occurs through three major steps:

- i) **transcription:** the transcribing of a deoxyribonucleic acid (DNA) code into mRNA,
- ii) **translation:** the translation of the sequence mRNA bases on a ribosome into a polypeptide chain, and,
- iii) **post-translational modifications:** further modification to synthesised proteins to enable their mature form.

Transcription is the process of copying a segment of DNA to mRNA. This process occurs in the nucleus by RNA polymerase until a stop codon is encountered. The newly created mRNA strand has the anti-sense sequence to the original strand of the DNA; thus, allowing a template to be created for the process of translation.

The translation process occurs in three steps: initiation, elongation, and termination of the new peptide chain. Translation begins with the initiation process resulting in the formation of the 80S initiation complex, composed of mRNA, charged methionyl-transfer RNA (tRNA), and both the 40S and 60S ribosomal subunits (Carbone et al., 2016). The complete ribosome possesses three tRNA binding sites which span the two subunits, known as the P (peptidyl) site, the A (aminoacyl) site and the E (exit) site. The anticodon of methionyl-tRNA (UAC – the “initiator” tRNA) is aligned with its complementary mRNA codon (AUG) and the ribosome then moves along the mRNA, catalysed by aminoacyl-tRNA synthetase, with specific AA-charged tRNA moving into the A-site of the ribosome based on the subsequent mRNA codon sequences (Carbone et al., 2016). The process of elongation involves the binding of

subsequent aminoacyl-tRNA complexes. The ribosome moves one complex down the mRNA and the initiation process is repeated. Termination of protein synthesis occurs when the A-site encounters a stop codon. At this stage, the polypeptide chain is freed from the ribosome, which dissociates into its subunits again.

Almost all proteins are further modified after their synthesis is completed. Post-translation proteins may be modified or spliced, further increasing the number of proteins. A single protein may also have many interactive domains allowing it to have multiple functions simultaneously. Although the AA constituents of a protein do not necessarily indicate the role of that protein in a cell, they can suggest a common function between proteins, with protein synthesis leading to a myriad of products capable of regulating intra and extracellular processes (Carbone et al., 2016).

In 1982, Rennie and colleagues first demonstrated that a mixed macronutrient meal was able to stimulate both whole body and SKM protein synthesis (Rennie et al., 1982). It was later shown that AA alone were likely the major contributor to the stimulation (Bennet et al., 1990), with the EAA of a protein meal specifically identified to stimulate MPS (Smith et al., 1992; Smith et al., 1998). The AA-induced stimulation of MPS is purportedly dose-dependent with maximal anabolic stimulation being achieved with 20–40 g of protein or 10–20 g of EAA (Cuthbertson et al., 2005; Moore et al., 2009a; Moore et al., 2015; Witard et al., 2014) depending on an individual's age, activity, and health/disease status. However, questions have recently arisen regarding

whether there is, in fact, a dose response to AA intake due to the ability of LEU to robustly stimulate MPS in isolation or in a LEU-enriched “suboptimal” protein dose (Atherton et al., 2017; Bukhari et al., 2015; Wilkinson et al., 2018a). It may, therefore, be that the dose-response of MPS to protein/EAA is not driven by protein quantity *per se* but instead by the quality of the protein, and specifically the LEU content (discussed later in detail). It is, however, important to note that MPS would eventually become limited by the availability of other EAA, due to LEU stimulating MPS via the depletion of endogenous intramyocellular pools (Stokes et al., 2018). We discuss the fundamental responses of SKM to dietary protein feeding and the phenomenon of “muscle full” in section 1.3 and Chapter 3 of this thesis.

1.2.2. Muscle protein breakdown

The MPS response to exercise and nutrition is much more understood compared to MPB due to several reasons: i) studying MPB in humans is technically much more difficult than MPS, ii) changes in MPS in response to exercise/nutrition have a much greater influence on protein balance than MPB and iii) the resolution of measuring MPS on a protein level is more readily accomplished than for MPB. However, it is important to delineate the role of MPB with regards to the remodelling and repair of SKM in response to exercise and how nutrition influences these processes.

MPB is an important metabolic component of muscle remodelling, adaption to training and increasing muscle mass. Degradation of muscle proteins occurs via the integration of three main proteolytic systems; autophagy, the calpain system

and the ubiquitin-proteasome pathway (detailed by Tipton et al., 2018). These systems do not operate independently, and the regulation is complex, with complete degradation of a protein requiring some combination of the systems. Of these three degradation pathways, the ubiquitin-proteasome pathway is the best known and plays a fundamental role in normal physiology with it being central to protein degradation across all cell types (Tipton et al., 2018).

Exercise is known to be a powerful mediator of MPB, with it generally believed RE increases rates of MPB (Pasiakos & Carbone, 2014). This was demonstrated using both stable isotope (Biolo et al., 1995) and the arterio-venous (AV) balance techniques (Phillips et al., 1997), highlighting increases in MPB following RE although to a lesser extent than MPS, meaning NPB increased but did not become positive in the fasted state. Interestingly, fractional breakdown rate (FBR) was elevated for 24 hours post-exercise, whereas fractional synthetic rate (FSR) was elevated for 48 hours (Phillips et al., 1997). Training status may also influence MPB rates, with FBR increasing following a bout of RE in untrained but not trained individuals (Phillips et al., 1999). Further, resting FBR and FSR, was greater following eight weeks resistance exercise training (RET) (Phillips et al., 2002), although this is difficult to compare to the previous study (Phillips et al., 1999) as all measures of FBR were taken following the same absolute exercise intensity and during constant feeding (Tipton et al., 2018). Nevertheless, these studies support the notion that training reduces the response of MPB to RE. Molecular markers of MPB (e.g., Muscle RING-finger protein-1 (MuRF1) and Muscle Atrophy F-box gene (MAFbx or Atrogin-1)) have also been shown to increase following endurance exercise (Coffey et al., 2006; Louis

et al., 2007; Stefanetti et al., 2015; Kim et al., 2011; Pasiakos et al., 2010; Jamart et al., 2012). Despite this, MPB only increased after 10 minutes, but not 60 or 180 minutes, following 45 minutes of treadmill walking (Sheffield-Moore et al., 2004). Consequently, it seems that RE consistently increases MPB, with nonconclusive results following endurance exercise.

Unlike assessment of MPS which has been extensively researched (Witard et al., 2016; Morton et al., 2015; Atherton & Smith, 2012; Phillips et al., 2012; Tipton & Wolfe, 2004; Tipton & Wolfe, 2001), methodological difficulties have impacted measurements of the MPB response (Tipton et al., 2018); thus, complicating the picture of MPB with relation to nutrition and exercise. For example, using the three-pool AV balance model, Biolo and colleagues evidenced MPS increased during hyperaminoacidaemia post-exercise, with no increases in MPB compared to resting fasted levels (Biolo et al., 1997). Following the combined ingestion of EAA and carbohydrates there was the absence of exercise-induced increases in MPB (Glynn et al., 2010a). Interestingly, in states of energy deficit, MPB displayed no change in overweight individuals (Hector et al., 2018). Ultimately, the physiological relationship between MPS/MPB may explain the MPB response to nutrition and exercise. As RE increases MPS (Biolo et al., 1995; Phillips et al., 1997; Phillips et al., 1999), there becomes an increased demand for intracellular free AA to act as a substrate for increases in MPS (Tipton et al., 2018). In the absence of exogenous AA, rates of MPS become limited resulting in increased MPB to supply the AA (Tipton et al., 2018). This notion is supported by a high correlation between MPS and MPB following exercise in the postabsorptive state, whereas in the fed-exercised state

there is no need for MPB to increase due to increased exogenous AA supplies (Biolo et al., 1995; Phillips et al., 1997; Phillips et al., 1999); resulting in increases in MPS but not MPB (Tipton et al., 2018).

1.2.3. Intracellular regulation of muscle protein synthesis and muscle protein breakdown

The mammalian target of rapamycin (mTOR) signalling pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation, and survival (Laplane & Sabatini, 2009). mTOR is, perhaps, the most critical and well-characterised intracellular regulator of exercise and nutrient-induced (i.e., protein) MPS (Atherton & Smith, 2012; Drummond et al., 2009b; Dickinson et al., 2011).

mTOR exists in two regulatory complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is the primary regulator in protein synthesis (Carosi et al., 2022). Activation of the mTORC1 signalling pathway leads to a cascade of signals resulting in phosphorylation of 4E-binding protein 1 (4E-BP1), ribosomal protein S6 kinase (p70S6K1) and ribosomal protein S6 (RPS6), stimulating binding of eukaryotic initiation factor (eIF) 4A and eIF4E to eIF4G forming the eIF4E complex (Anthony et al., 2001). Subsequently, eIF4E mediates mRNA binding to the 43S pre-initiation complex, resulting in upregulation in rates of mRNA translation and protein synthesis (Anthony et al., 2001).

The activation of mTORC1 has also been shown to promote ribosome biogenesis by stimulating the transcription of ribosomal RNA through a process involving the protein phosphatase 2A (PP2A) and the transcription initiation factor IA (TIF-IA) (Mayer et al., 2004). Previously, it has been demonstrated that the recombination-activating gene (Rag) proteins, a family of four related small GTPases, interact with mTORC1 in an AA-sensitive manner and are necessary for the activation of the mTORC1 pathway by AA (Kim et al., 2008; Sancak et al., 2008). In the presence of AA, Rag proteins bind to the regulatory-associated protein of mTOR (Raptor) and promote the re-localisation of mTORC1 from discrete locations throughout the cytoplasm to a perinuclear region that contains its activator Rheb (Sancak et al., 2008). The physical dissociation of mTORC1 and Rheb with AA deprivation might explain why activators of Rheb, such as growth factors, cannot stimulate mTORC1 signalling in the absence of AA.

AA represent a strong signal that positively regulates mTORC1 (Figure 1.3). Increasing intracellular AA availability in SKM cells serves two major functions: i) providing substrate for polypeptide biosynthesis, and ii) directing activation (i.e., phosphorylation) of the regulatory mechanisms of mRNA translation. This dual function ensures that in postprandial periods, increased availability of AA acquired from dietary protein stimulates a transient increase in MPS. Of all the EAA, LEU has been shown to stimulate MPS to a similar extent as complete (i.e., whole) protein or complete mixture of AA in rat muscle (Fulks et al., 1975) but also in humans through IV infusion of multiple EAA

(Smith et al., 1992). Unlike other EAA, LEU can independently activate mTORC1 and downstream anabolic signalling (Anthony et al., 2000a), in addition to the more obvious role as a substrate for newly synthesised proteins. Although knowledge of upstream regulation of the mTORC1 pathway is incomplete, it is now known that LEU binds to an intracellular “LEU sensor” of the mTORC1 pathway, Sestrin-2, leading to subsequent dissociation of Sestrin-2 and GTPase-activating protein toward Rags-2 (GATOR2) which activates the mTORC1 pathway (Wolfson et al., 2016) with leucyl-tRNA synthetase (LRS) playing a critical role through sensing intracellular LEU concentrations and initiating molecular signalling events leading to mTORC1 activation (Han et al., 2012). We discuss intracellular MPS activation via LEU in Chapter 3 in more detail.

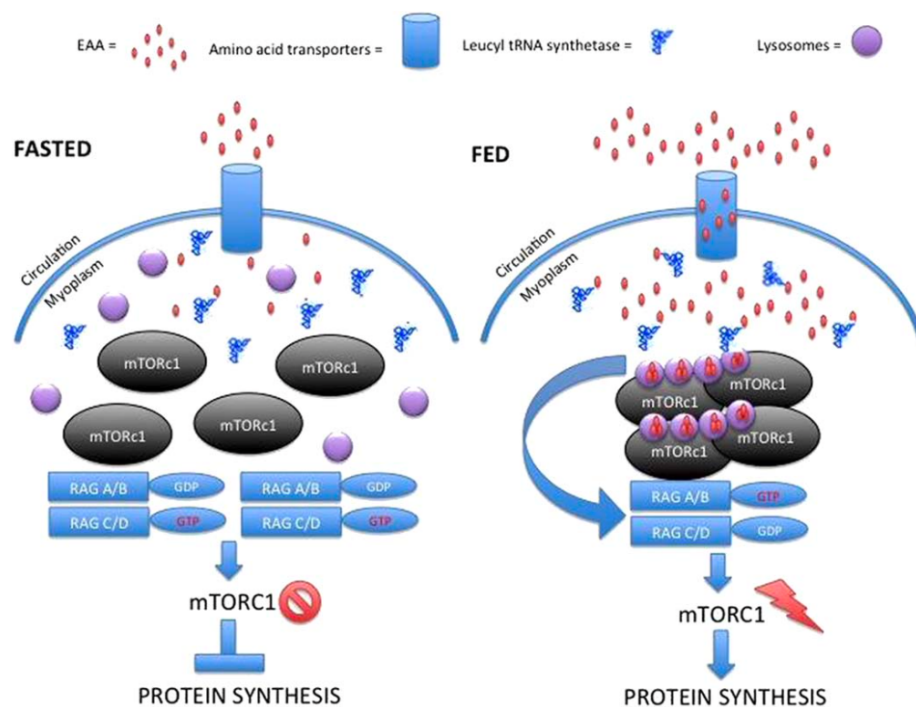


Figure 1.3 Summary diagram of intramuscular molecular signalling resulting from essential amino acid ingestion. Image taken directly from Atherton et al.,

2016 summarising the intramuscular signalling responses driven by amino acids in the fasted and fed states.

With regards to intracellular mechanisms of MPB, data from animal models suggests the ubiquitin proteasome pathway and the calpain system play a larger role than autophagy during MPB (Tipton et al., 2018). Mechanistic data from humans is lacking to confirm this. Autophagy still, however, plays a crucial role in MPB as it is thought to be particularly important in the degradation of membrane receptor proteins (Tipton et al., 2018). Assessing metabolic markers (i.e., MuRF1) of these breakdown pathways offers important mechanistic information regarding the role of MPB in muscle remodelling in response to exercise and nutrition; however, assessing MPB in humans is not routinely performed compared to MPS due to technical difficulties. Due to this thesis being focussed on the role of MPS in response to exercise and protein nutrition, we direct readers to Tipton et al., (2018) for a comprehensive review on MPB in response to nutrition and exercise in humans.

1.2.4. Assessment of muscle protein metabolism

Understanding muscle protein metabolism and the mechanisms regulating (e.g.) disease, ageing and inactivity, stems from the ability to accurately quantify the dynamic nature of metabolism underlying the (patho)physiological changes. The use of stable isotope tracer technologies has been at the forefront for the past 80 years, allowing substantial development in understanding physiology and the dynamic nature of muscle protein metabolism. Notably, the application

of stable isotope tracers is not solely limited to AA metabolism, with stable isotope usage being facilitated in also understanding fatty-acid and carbohydrate metabolic regulation (i.e., incorporation, flux, and oxidation, in a tissue-specific and whole-body fashion) in health, disease and response to acute and chronic exercise/inactivity (Wilkinson et al., 2017).

Stable isotopes are elements which occupy the same position in the periodic table, being chemically and functionally identical, but differ in mass due to a different number of neutrons within the atomic nucleus (Wilkinson et al., 2017; Wilkinson et al., 2018; Wilkinson et al., 2021). While naturally occurring, they are far less abundant than their more common endogenous isotopes. The difference in mass makes them analytically distinguishable from one another, allowing them to be “traced” if introduced into a metabolic system (i.e., protein metabolism), usually using the technique of mass spectrometry (Wilkinson, 2018). A mass spectrometer separates atoms and/or molecules based on their mass and/or charge, after undergoing some form of ionisation, as they pass through an electric and/or magnetic field under vacuum (Wilkinson et al., 2017). Traditionally, a mass spectrometer is combined with chromatographic (gas or liquid chromatography) separation techniques to permit the separation of complex mixtures or organic compounds prior to detection in the mass spectrometer (Wilkinson et al., 2017).

There are two types of isotope tracers: radioisotope tracers and stable isotope tracers. Radioisotopes (e.g., ^{14}C and ^3H (tritium)) are, unsurprisingly, radioactive, with their stable isotope (e.g., ^{18}O , ^{15}N , ^{13}C and ^2H) counterparts

being non-radioactive. Stable-isotope tracers are therefore ideal for human research, whereas radioisotope tracers have been limited to animal research (Wilkinson et al., 2017). Typically, stable isotopes are substrate specific, meaning glucose tracers will reflect carbohydrate metabolism and storage, labelled fatty acids will inform on fat metabolism and labelled AA provide information on AA and protein metabolism. The use of stable isotope tracers has led to some of the most important discoveries in the protein feeding and exercise metabolism field. This includes discovering that the EAA constituents of protein food are the key nutrients supporting increases in MPS from feeding alone and in combination with exercise (Smith et al., 1992; Smith et al., 1998; Burd et al., 2011; Pennings et al., 2011a) and the delineation of protein synthetic and breakdown responses to exercise (i.e., when performed in the absence of nutrition exercise is catabolic) (Biolo et al., 1995; Phillips et al., 1997; Kumar et al., 2009).

Traditional stable isotope tracer experiments (like those used in the current project as detailed later) require constant intravenous (IV) infusions requiring cannulation(s), sterile infusions, and multiple (usually muscle) tissue biopsies (see Figure 1.4 for summary). Constant IV infusions of stable isotope tracers require subjects to be studied in a strict, controlled clinical or laboratory environment with measures being traditionally measured over two to eight hours (Wilkinson et al., 2017). Despite the traditional, acute, stable isotope studies providing novel and important insight into (e.g.) protein metabolism, their time-limited and invasive nature has narrowed the application of these studies. Further, acute findings may not always translate into chronic settings.

For example, in young men, acute post-exercise MPS exhibited no correlation to ensuing SKM hypertrophy following RET (Mitchell et al., 2014). Substrate-specific acute tracer techniques have, however, been invaluable to our understanding of basic human metabolism and its responses to stress, nutrition, health, and disease (Wilkinson et al., 2017).

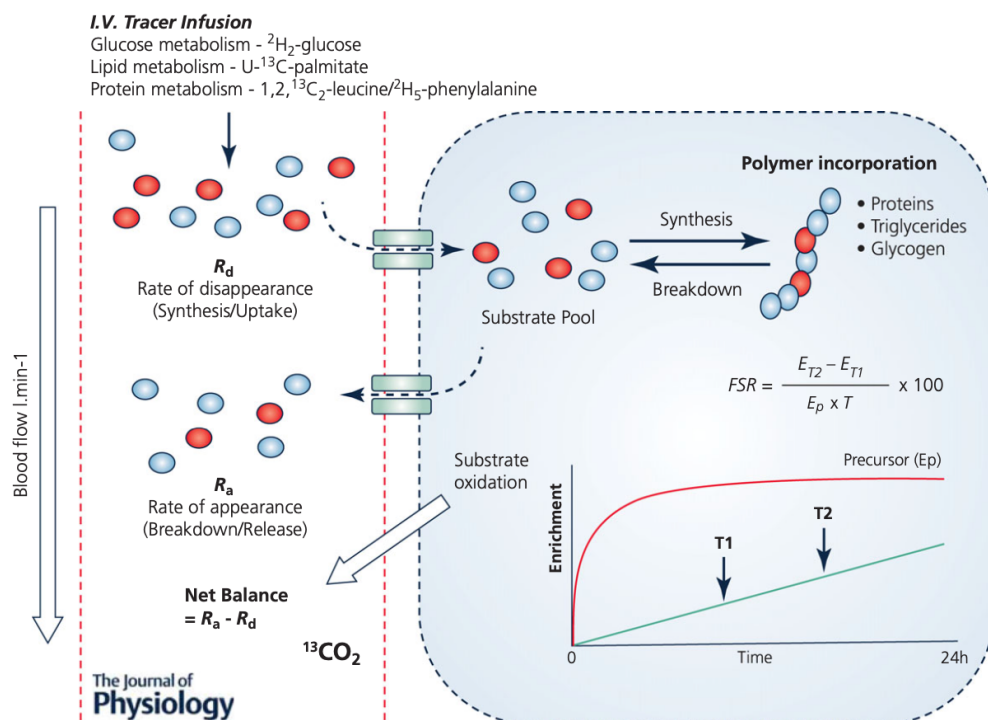


Figure 1.4 Summary diagram of substrate-specific stable isotope tracers for measuring metabolism. Image taken directly from Wilkinson et al., 2017 summarising the typical application of substrate-specific stable isotope tracers for measuring metabolism, via the precursor-product and arterial-venous (A-V) balance techniques. The A-V balance technique involves measurement of the rate of disappearance (R_d) of the stable isotope tracer from the arterial pool (a proxy measurement for synthesis) and the rate of appearance (R_a) of the tracee in the venous pools (a proxy measurement for breakdown). The precursor-

product technique allows measurement of fractional synthetic rate (FSR), or synthesis, by sampling the tissue at two time points in relation to the precursor enrichment. E_{T1} , Enrichment in product at time T1. E_{T2} , enrichment in product at time T2. E_P , enrichment in precursor pool. IV, intravenous.

To address the application to more “real-life” scenarios, such as chronic RET, inactivity and protein feeding over weeks/months, the use of deuterium oxide (D_2O , “heavy water”, or ^2H_2O) has aided and continues to advance this gap of knowledge. Despite being one of the first stable isotope tracers used in metabolic research (Schoenheimer & Rittenberg, 1936; Ussing, 1938), the benefits of D_2O only really began in the late 20th century with the knowledge that D_2O could provide an alternative approach and overcome some of the issues related to the use of traditional substrate-specific stable isotope tracers. D_2O can be administered orally (MacDonald et al., 2013; Wilkinson et al., 2014), as a bolus (Wilkinson et al., 2014), or in the form of regular doses (Brook et al., 2015) as it rapidly equilibrates with body water (~one to two hours in humans (IAEA, 2011)). This creates a labelled precursor pool, which slowly turns over (half-life ~9–11 days (MacDonald et al., 2013; Wilkinson et al., 2014)), thereby overcoming the need for constant IV stable isotope tracer administration. Unlike traditional substrate-specific tracer methods, D_2O allows measurement of multiple substrate pools simultaneously. Furthermore, as the body water pool is turned over relatively slowly, measurement periods can be extended beyond the standard limits for traditional stable isotope tracer studies (i.e., ~24 hours), to days, weeks or even months (Robinson et al., 2011; Gasier et al., 2010;

Wilkinson et al., 2014; Wilkinson et al., 2015; Brook et al., 2015; Wilkinson et al., 2017); providing a vital mechanism for monitoring chronic, cumulative metabolic rates of greater translational relevance. For example, in contrast to findings with acute MPS (Mitchell et al., 2014), chronic MPS derived using D₂O correlated to long-term muscle hypertrophy resulting from RET (Brook et al., 2015), suggesting D₂O can provide a predictive real-life representation of exercise adaptation (Damas et al., 2016).

1.3. Skeletal muscle and protein nutrition

Skeletal muscles are receptive but exhibit temporal regulation to the anabolic effects of EAA in the rested state. Following oral protein feeding, an initial lag of ~45–60 minutes in MPS responses results from oesophageal transit, digestion, absorption, arrival at the target tissue and activation of intracellular anabolic signalling pathways (Atherton & Smith, 2012). MPS then increases ~2–3-fold, peaking between ~1.5–2 hours post-feed, prior to returning to baseline between 90 and 180 mins after initial protein intake (Atherton et al., 2010a). Whilst MPS is stimulated, the anti-catabolic effects of feeding on SKM also occur due to the reflex actions of insulin, leading to MPB being transiently inhibited by ~50% in young muscle, though this effect is significantly blunted in older muscle (Greenhaff et al., 2008; Wilkes et al., 2009). As increases in MPS are far greater than MPB, it is MPS that is the driving force behind nutrient-induced muscle anabolism.

Despite continued EAA availability and elevated intracellular anabolic signalling following feeding, MPS exhibits tachyphylaxis, whereby excess EAA

are directed towards oxidation, intermediary metabolism and eventually to gluconeogenesis and lipogenesis (via recycling of the carbon skeletons through the tricarboxylic acid cycle) rather than incorporation into the muscle or whole-body protein pool. This phenomenon is known as “muscle full” (Bohe et al., 2001; Bohe et al., 2003; Atherton et al., 2010a; Atherton & Smith, 2012), and means a refractory period must, therefore, exist before further stimulation in MPS is achieved. We, and others, have demonstrated that the muscle full state is unable to be overcome by the intake of additional nutrient signals or substrates responsible for regulating MPS, such as LEU (Mitchell et al., 2017), in addition to pharmacological and nutraceutical interventions commonly utilised to enhance nutritive muscle blood flow such as methacholine (Phillips et al., 2014) and cocoa flavanols (Phillips et al., 2016a). Nonetheless, performance of RE can delay the onset of muscle full due to the increased anabolic sensitivity of SKM to dietary AA (Atherton & Smith, 2012). Arguably, as the delay in the muscle full can last at least 24 hours following a single bout of RE (Burd et al., 2011), it is largely irrelevant whether individuals consume protein pre-, during, or post-exercise, rather nutrient sufficiency may be more important to achieve successful hypertrophic adaptation to RET (Atherton & Smith, 2012). This is not to dismiss that some acute performance/recovery benefits may still be afforded by consumption of nutrition in close proximity to exercise, especially endurance exercise (Ferguson-Stegall et al., 2011). Conversely, both ageing and physical inactivity are associated with desensitisation to anabolic stimuli (i.e., exercise and nutrition) resulting in a premature onset of muscle full (Cuthbertson et al., 2005) characterised by a blunted postprandial MPS (i.e., anabolic

resistance) (Breen et al., 2013; Wall et al., 2013b), prompting insulin resistance (Dirks et al., 2016) and muscle atrophy (Kilroe et al., 2020; Wall et al., 2013a).

1.3.1. The importance of protein quantity

Dietary protein recommendations are often expressed per day, and are set as the lowest level of protein intake to prevent protein inadequacies in 97.5% of healthy adults at energy balance (Phillips et al., 2020). Currently, the recommended daily allowance (RDA) is 0.8 g protein/kg body weight/day for all adults (World Health Organization, 2007; EFSA, 2012), although this has been under increasing contention. Indeed, the PROT-AGE study group have recommended healthy older adults to be consuming greater quantities of protein in the regions of 1.0–1.2 g/kg/d in order to maintain and regain lean body mass and function, with those engaged in exercise (endurance or resistance) or otherwise active should be consuming ≥ 1.2 g/kg/d protein (Bauer et al., 2013; Deutz et al., 2014). Further, older adults who present with acute or chronic disease (except those with severe kidney disease) need even more dietary protein in the region of 1.2–1.5 g/kg/d (Bauer et al., 2013; Deutz et al., 2014). The calls for increasing protein intake in such described populations may not come as a surprise due to the current RDA aiming to prevent protein deficiencies; a factor especially relevant to those in developing countries (Wolfe et al., 2017). Resultingly, the lifestyle and goals of a given population (e.g., athletic performance, maintaining function/independence etc) should be considered when identifying minimum and optimal protein intakes (Burd et al., 2019b). The appropriate use and relevance of the current RDA for older adults should arguably be put into question due to the current state of health in older adults

regarding the presence of obesity, cardiovascular disease, type 2 diabetes and/or cancer, as the RDA was established predominantly in healthy individuals (Phillips et al., 2020).

Defining optimal protein intake remains a matter of debate and is dependent on the population of interest as to which method is adopted. For example, with reference to older adults, the current protein RDA was previously derived from meta-analyses based on nitrogen balance studies (the traditional method to determine protein requirements), containing limited data from older individuals ($n = 14$ out of 235), all of which were male, which concluded there was no impact of age on protein requirements (Rand et al., 2003). Similarly, Campbell and colleagues concluded that there were no age-related differences in protein requirements based on nitrogen balance study data from 19 older adults (8 male, 11 female), with the protein requirements identified (0.85 ± 0.21 g/kg/d) not significantly differing from the stated RDA (Campbell et al., 2008).

Conversely, the use of the indicator amino acid oxidation (IAAO) technique, has led to studies deriving a higher safe protein intake of ~ 1.1 – 1.2 g/kg/d (Humayun et al., 2007; Rafii et al., 2015; Tang et al., 2014). Despite this, the “original” RDA (0.8 g/kg/d) is still often treated as the maximum protein intake, even with the potential negative effects on renal and bone health through consuming higher protein intakes, which are commonly cited, being absent of scientific foundation in humans (Traylor et al., 2018). Additionally, the IAAO technique has shown its utility to be used across various age-ranges and in an exercise setting (Moore et al., 2009a; Moore et al., 2015; Mazzulla et al., 2018; Holwerda et al., 2019b),

which should be of great consideration as increasing levels of physical activity (PA) and incorporation of structured exercise regimes, is unquestionably one of the most important lifestyle factors for improved health.

We discuss the influence of protein quantity regarding the observed dose-response to dietary protein and patterns of protein intake across the day in detail in Chapter 3.

1.3.2. The importance of protein quality

A recommended healthy eating pattern consists of ingesting a variety of high-quality protein foods to ensure a sufficient supply of AA for muscle maintenance, growth, and overall diet quality (Phillips et al., 2015). An important consideration of the current protein RDA is that it does not consider the source of dietary protein (i.e., animal or plant protein) despite known geographical differences regarding the source of protein intake (Deane et al., 2020a). To indicate this, in comparison to Western diets which typically contain a high proportion of animal-derived protein (~65% in US populations) (O'Neil et al., 2012), African and Asian populations' primary source of protein intake is plant-derived at 77% and 66% respectively (van Vliet et al., 2015). Due to the increased knowledge and need for environmental sustainability and potential health benefits (Pimentel & Pimentel, 2003), there is a shift in research and emerging interest in the efficacy of plant-derived or other (i.e., blended) protein sources for maintaining/optimising musculoskeletal health (Nichele et al., 2022). Regardless, the AA within a protein food should meet the needs of the consumer and consist of a variety of protein foods to ensure nutrient density

(Burd et al., 2019b). Specifically, the quality of protein foods is dependent on the AA content (especially LEU) and the availability of these AA in circulation, thus influencing their metabolism within different body protein pools (Burd et al., 2019b). We specifically discuss the role of LEU content regarding protein quality in Chapter 3. However, we believe it important to discuss measures to assess protein quality and the generic argument between animal and plant protein consumption below.

1.3.2.1. Assessment of protein quality

The quality of a protein source has typically been assessed via two methods:

- protein digestibility-corrected amino acid score (PDCAAS) and,
- digestible indispensable amino acid score (DIAAS).

Currently, the PDCAAS is the officially recognised assessment method to measure protein quality, dating back to 1991, by the European Union and United States. This is despite the DIAAS being the preferred method to rank protein quality of foods that is recommended by the Food and Agriculture Organisation of the United Nations (FAO) (FAO, 2013). Notably, the DIAAS will not be implemented as the official universal standard to assess protein quality until a more complete catalogue of scores has been tested and verified.

Creation of the DIAAS method resulted from an increasing recognition that protein digestibility (i.e., quality) could not be estimated from faeces, as previously used in the PDCAAS (Burd et al., 2019b; Craddock et al., 2021). The rationale behind the DIAAS method is that protein digestibility estimates should

be based on true ileal digestibility, determined at the end of the small intestine where AA are absorbed, and ideally performed in humans (Burd et al., 2019b). The DIAAS, therefore, aims to determine the limiting AA in circulation after accounting for digestion and absorption to support protein metabolism (Burd et al., 2019b). The major obvious limitation is that it is not feasible to assess ileal digestibility in humans and can only be assessed when a naso-ileal tube is present or following ileostomy surgery (Burd et al., 2019b; Craddock et al., 2021). Human data is therefore very limited, and animal models have subsequently been employed. Where human data is not available, the FAO recommends assays to be performed in growing pigs, followed by growing rats (FAO, 2013), although deriving protein digestibility coefficients from pigs for many food items used in our modern society is not realistic (Marinangeli & House, 2017). While digestion of protein between the mouth and ileum is thought to be similar in pigs and humans (Rowan et al., 1994), important digestive and metabolic differences occur between rats and humans and the lack of direct comparative studies in animals and humans indicate that the application of current DIAAS data to humans should be performed with caution (Craddock et al., 2021). While there are multiple strengths of the DIAAS, many limitations remain which are accentuated in the context of plant-based dietary protein (Craddock et al., 2021). Some of these include limited representation of commonly consumed plant-based foods within the DIAAS scoring framework, inadequate recognition of the increased digestibility of commonly consumed heat-treated and processed plant-based foods, and a focus on individual isolated foods rather than the food matrix (Craddock et al., 2021).

1.3.2.2. The animal vs. plant protein debate

Animal-derived proteins have traditionally been consumed to achieve a maximal muscle anabolic response, with the robust postprandial increase in MPS rates associated with the rapid rise in plasma EAA and LEU concentrations. Animal-derived proteins are, of course, proteins derived directly from animal sources, whether that be in whole and/or isolated foods, such as meat, fish, eggs, and dairy (including whey and casein). Conversely, plant-derived proteins, including (e.g.) soy, pea, and wheat, tend to demonstrate a lesser magnitude of MPS compared to animal proteins, which is often attributed to a lower EAA content or the absence of desired quantities of specific AA such as LEU, lysine and/or methionine (Gorissen et al., 2018). These conclusions have, however, been derived from a small number of investigated plant proteins despite the great diversity in plant-derived protein sources (van Vliet et al., 2015). Furthermore, not all plant proteins are deficient in the same EAA meaning a “blend” (i.e., different sources/types of protein combined to form one nutritional load) of plant protein sources, or with the addition of animal-derived protein, may be generated to create a more optimal isolated supplement which comparatively stimulates MPS to isolated animal-derived supplements (i.e., whey protein (WP)).

There has now been a plethora of acute and chronic studies investigating the effects of animal and plant proteins on SKM anabolism and subsequent increases/maintenance of SKM mass across the lifespan, which have typically given a favourable opinion to animal proteins demonstrating a greater robust response in MPS (see Deane et al., 2020a for a comprehensive review). For

example, men and women with higher intakes of protein from animal sources had a higher percentage of SKM mass regardless of activity status, whereas the benefits of plant proteins were only evident amongst those more physically active (Bradlee et al., 2017). Further, SKM mass estimates from 24-hour urinary creatinine and analysis of food intake, illustrated intake of both total protein and animal protein to be associated with increased creatinine excretion, suggesting greater muscle mass, in both younger and older males and females (Alexandrov et al., 2018).

Differences in the acute SKM anabolic response also arises in response to different fractions of isolated protein sources, such as WP, casein, and soy. In young men, ~20 g (10 g EAA) WP hydrolysate led to the greatest stimulation in MPS rates at rest and in response to acute RE, compared to equal total protein and EAA content of casein and soy isolate (Tang et al., 2009). However, soy protein consumption led to greater MPS rates at rest (64%) and post-exercise (69%) compared to casein (Tang et al., 2009). Similarly, ingestion of 20 g of WP isolate led to greater MPS at rest and post-RE in older adults compared to 20 g micellar casein ingestion (Burd et al., 2012). Both studies also demonstrated a greater and more rapidly achieved peak LEU concentration following WP consumption than both soy and casein (Tang et al., 2009; Burd et al., 2012), which is unsurprising due to WP having a greater LEU content. Again, ingestion of 20 g and 40 g of soy protein stimulated MPS to a lesser extent than WP in the elderly, with 20 g of soy protein displaying no differences in MPS rates from 0 g protein under rested and exercise conditions (Yang et al., 2012). Further, greater LEU oxidation was observed after 20 g of soy protein ingestion

compared to WP, suggesting more AA are directed towards oxidation making them unavailable as a substrate for MPS (Yang et al., 2012). Evidence is not, however, in complete agreement. For example, MPS rates were equivalent between 20 g WP and casein supplementation post-RE (Reitelseder et al., 2011), with the effect of taking casein pre- or post-RE leading to no differences in MPS and equivalent to MPS rates following WP ingestion (Dideriksen et al., 2011). The disparity between research studies above may be a result of the timing of SKM biopsies. As casein is known to have slower digestion rates compared to WP and soy, this may be a strong reason why results differ between investigations. For example, both Tang et al., (2009 and Burd et al., (2012) acquired SKM biopsies at three and four hours, respectively, following protein consumption whereas Reitelseder et al., (2011) and Dideriksen et al., (2011) acquired muscle tissue six hours post-feed, allowing more time for the digestion of casein and sustained MPS stimulation. Furthermore, despite “slow” proteins such as casein thought to potentially inhibit protein breakdown, these data have come from studies investigating protein metabolism at the whole-body level (Borie et al., 1997; Dangin et al., 2001; Dangin et al., 2003), of which muscle makes up 25% (Nair et al., 1988) and turns over at a much slower rate compared to (e.g.) gut proteins (Nakshabendi et al., 1995; Nakshabendi et al., 1999).

Although most research has been directed into investigating SKM anabolism to isolated protein foods (i.e., WP, soy, and casein), whole foods (i.e., eggs, milk, meat) are consumed much more within the general adult diet. Importantly, food processing (e.g., mincing) can impact the matrix and texture of protein feeds, which may cause differences in protein digestibility and absorption in response

to animal-derived protein consumption (Gorissen et al., 2015). For example, beef mince (vs. beef steak) was more rapidly digested and absorbed stimulating a greater more rapid release of AA into blood circulation and enhancing postprandial net protein balance in older males, yet no differences in MPS were observed (Barbe et al., 2014). We previously investigated a bolus vs. “spread” (i.e., pulse) feed, administering 15 g mixed-EAA as a single dose or in 4 fractions at 45-minute intervals, allowing us to mimic a fast compared to slow (i.e., WP vs. casein or minced vs. steak) digested protein (Mitchell et al., 2015b). Despite rapid insulinaemia, aminoacidaemia, and capillary recruitment following the bolus feed only, no differences were exhibited in MPS between the two types of feeds; suggesting the delivery profile of EAA to SKM is not as important of a factor of muscle anabolism (Mitchell et al., 2015b). Other factors which may affect MPS responses via obtaining optimal digestion/absorption in the context of ageing are chewing efficiency (Remond et al., 2007), cooking temperature (Bax et al., 2013) and cooking time (Zhu et al., 2015).

Considering, although in mixed contention, the suggestion that consumption of animal proteins leads to greater MPS, it may be that a greater quantity of plant-derived protein consumption is required to overcome the reduced anabolic response. Although 35 g wheat protein did not stimulate MPS to the same degree as equal quantities of casein, consumption of 60 g wheat protein resulted in a greater MPS response than 35 g WP (Gorissen et al., 2016). Some varieties of plant-derived proteins (e.g., potato and quinoa) contain adequate amounts of all EAA (Gorissen & Witard, 2018) and thus may offer sufficient anabolic alternatives to animal-derived proteins. Indeed, a recent study in young women

found that 25 g of potato protein twice daily for 2 weeks (1.6 g/kg/d total protein) increased integrated MPS, with no increase observed in those consuming a control diet containing 0.8 g/kg/d total protein (Oikawa et al., 2020). Whilst this greater anabolic response could simply be attributed to the greater quantity of protein consumed, it still demonstrates the ability of potato protein to stimulate MPS above a baseline diet already containing the RDA of protein, at least in younger individuals (Deane et al., 2020a). There is, however, a practical challenge of getting older adults to consume greater amounts of plant-derived protein to achieve this must be considered given the reported lack of appetite (Payette et al., 1995) and rapid satiety (Benelam, 2009) in older age. Instead of simply increasing the amount of protein consumed, protein blends may be a way to achieve sufficient quantities of EAA and LEU (Gorissen et al., 2018; Deane et al., 2020a; Pinckaers et al., 2021b; Nichele et al., 2022). For example, in young males, ~19 g protein consisting of sodium caseinate (50%), whey (25%) and soy (25%) protein, elevated aminoacidaemia, mTORC1 signalling and MPS (Reidy et al., 2013), in addition to increasing muscle AA transporter expression to a similar extent as WP (Reidy et al., 2014). Furthermore, MPS increased significantly following 30 g of milk protein, wheat protein or a protein-blend (milk + wheat protein) with no differences in MPS rates between the milk and wheat groups and the milk and protein-blend groups (Pinckaers et al., 2021a). A similar study by the same authors found similar results in which 30 g milk protein led to greater plasma EAA concentrations compared to a 30 g plant-blend (15 g wheat, 7.5 g corn and 7.5 g pea protein), but there were no differences observed in myofibrillar MPS rates (Pinckaers et al., 2022). Subsequently, more human based clinical studies are warranted to assess the anabolic properties of

the various plant-derived proteins to identify factors that may or may not compromise the capacity to stimulate MPS rates, leading to determining whether a more plant-based diet is accompanied by a transition towards greater dietary protein intake requirements (Pinckaers et al., 2021b).

1.4. Skeletal muscle and advancing age

The major successes of modern science and healthcare have led to people living longer than ever before, contributing to a worldwide shift in population demographics. Since the start of the 20th century, global life expectancy has more than doubled, and is now above 70 years of age (Dixon, 2021). Predictions suggest that by 2050, the global population of those aged over 65, 85 and 100 years is set to increase by 188%, 551% and 1004% respectively (McLeod et al., 2016), with it being projected that one in seven people in the UK being over 70 years of age in 2040 (Centre for Ageing Better, 2020). This dramatic shift in population demographics is being driven by a decrease in mortality in young people in less-developed countries, an increase in life expectancy of those 60+ years in high-income countries and a decrease in fertility; all of which contribute to a rapidly ageing populace (Beard et al., 2016). Often, an increase in longevity (i.e., life expectancy) is assumed to be accompanied by an extended period of good health, however, there is a lack of evidence available to suggest that older individuals are experiencing better health, despite living longer (Beard et al., 2016).

An elevated ageing populace will undoubtedly lead to additional strains on worldwide healthcare systems due to heightened prevalence of chronic diseases

associated with ageing. The presence of multi-morbidities (i.e., two or more co-morbidities) has the greatest healthcare needs and expenditure in the healthcare system (Stokes et al., 2021). Approximately one-third of community-based adults present with multi-morbidities (Nguyen et al., 2019) and there has been limited success in developing (cost)effective models of care for multi-morbid patients (Smith et al., 2016; Stokes et al., 2017; Salisbury et al., 2018), likely due to interactions between the different morbidities and treatments for these (Beard et al., 2016). One main consideration of healthy ageing is the number of years an individual can live disability-free (i.e., disability-free life expectancy), with those who present with disability early in life but live to an old age likely have a lesser quality of life compared to an individual who presents with disability later in life (Centre for Ageing Better, 2020). In recent years, disability-free life expectancy has stalled, especially in women, despite great advances in health care(Centre for Ageing Better, 2020). Therefore, developing effective intervention strategies to prevent deterioration in quality of life through the presence of age-related disability is still a hot topic in healthcare and research.

Demographic shifts over the past century have necessitated an increased focus on prevention of noncommunicative diseases through lifestyle interventions such as PA, exercise and nutrition (Chodzko-Zajko et al., 2009). Although age-related functional decline is inevitable and inescapable, avoiding a sedentary lifestyle and performing PA/exercise throughout life alters the rate at which this functional decline progresses and the presence of disease (Chodzko-Zajko et al., 2009). Further, many older adults fail to meet the minimum recommended daily

protein requirements of 0.8 g/kg/d (Bauer et al., 2013) (Murphy et al., 2016a), which is proposed to be even greater in older age to minimise muscle wasting which, along with Vitamin D deficiency (Visser et al., 2003), has been shown to significantly increase the risk of sarcopenia. Regardless of other predisposing factors, diet and lifestyle influence rates of mortality and morbidity throughout life. The World Health Organization (WHO) states a broad and inclusive definition of PA which includes all movement of everyday life including work, recreation, exercise, and sports activities (World Health Organization, 2003). The WHO, like many other organisations and researchers, argue that encouraging PA across the lifespan can benefit both an individual and society (Chodzko-Zajko et al., 2009). Indeed, longitudinal studies confirm that lifestyle and environmental risk factors explain ~75% of the occurrence of coronary heart disease (Magnus & Beaglehole, 2001), in addition to numerous other chronic diseases (Smith, 2007). Although some exercise and sports are arguably not inclusive to all individuals across different socio-economic groups, PA is mostly cheap, safe, and readily available with interventions playing a major role in the prevention, treatment, and management of noncommunicable diseases (Chodzko-Zajko et al., 2009) and sarcopenia (Bilski et al., 2022), thus allowing an avenue for healthy ageing.

Reduced muscle mass and function are some of the most significant and visually apparent changes with ageing (Narici & Maffulli, 2010). The age-related decline in muscle mass and strength, termed sarcopenia, (Rosenburg, 1997), is accelerated by physical inactivity (Doherty et al., 2003) and is a significant predictor of age-related disability and rates of mortality (Cruz-Jentoft et al.,

2010; Cruz-Jentoft et al., 2019). After 50 years of age, the loss of muscle mass becomes noticeable in most individuals, which further accelerates with ageing, reducing muscle mass from ~45% of body mass in younger adult humans to ~27% in older adults (Bilski et al., 2022). The decline in SKM mass, mainly at the expense of fast-twitch type II muscle fibres (Mitchell et al., 2012), does not fully explain declines in muscle function with strength losses being two to five times greater than declines in SKM mass (Mitchell et al., 2012; Lauretani et al., 2003). The age-related decline in neuromuscular function is significant, with those aged over 75 years and the very oldest subjects having less than 50% and 10%, respectively, of their motor units remaining compared to younger counterparts (Tomlinson & Irving, 1977; Doherty et al., 1993; Campbell et al., 1973). Motor unit remodelling, i.e., the rescuing of denervated fibres via axonal sprouting (Piasecki et al., 2016a; Piasecki et al., 2018a; Wilkinson et al., 2018b), may offset declines in muscle size and function with greater success in motor unit remodelling when individuals are physically active, as demonstrated in masters athletes (Piasecki et al., 2021). Subsequently, regular PA has shown promise in preventing and reversing many effects of disease prevalent with ageing, whilst improving functional performance (McPhee et al., 2016).

A plethora of evidence has established RET to be of great benefit to older adults in terms of offsetting the progression of sarcopenia. RET is any movement performed against a specific external force and benefits individuals of all ages, whether the goal is health-related, to improve performance, or wanting changes in body composition. For example, in older adults, RET interventions can partially reverse losses in SKM strength (Fiatarone, 1990; Vandervoort et al.,

2002; Porter et al., 1995; Frontera et al., 1988) and mass (Hurley & Roth, 2000; Tracy et al., 1999). Both muscular strength and endurance can be developed through progressive overload, with increasing the intensity of RET and involving larger muscle groups yielding more significant effects (Beckwee et al., 2019; Yoo et al., 2018; Csapo & Alegre, 2016), although exercising at a lower intensity until “failure” may yield similar benefits (Csapo & Alegre, 2016; Burd et al., 2010). Chronic RET leads to increases in muscle fibre size (i.e., hypertrophy), especially type II muscle fibres, with endurance exercise having a lesser effect on maintaining muscle mass but benefitting cardiovascular health, muscle oxidative capacity and reducing insulin resistance (Erlich et al., 2016; Short et al., 2004). Importantly, it is recommended that RE is started as early as possible due to the potential of SKM hypertrophic response in aged muscle becoming dampened (Csapo & Alegre, 2016; Peterson et al., 2010; Bilski et al., 2022). Undoubtedly, the maintenance of SKM mass and strength throughout older age leads to improved functional capacity and quality of life (Tipton & Wolfe, 2001), with exercise seeming more effective at preventing muscle loss than restoring lost muscle mass (Wolfe, 2006).

The global COVID-19 pandemic has further laid bare the urgency of improving the state of ageing. The pervasiveness of poor health, unsafe and low-quality housing, and a lack of social connections, particularly in the poorest of society, has exacerbated the impact of the pandemic on those who already faced the most challenging prospects later in life (Centre for Ageing Better, 2020). The response to the COVID-19 pandemic also, at times, stoked fear of ageing and the older generations with all those over 70 years being labelled as “vulnerable”,

“dependent”, and “needy” despite the huge variations in health and resilience (Dixon, 2021). The COVID-19 outbreak may indeed have drastically lowered opportunities for structured PA for older adults (Roschel et al., 2020), potentially leading to increased sedentary behaviour which has been associated with negative changes in both physical and mental health (Cheval et al., 2021). Although PA was predicted to be reduced by COVID-19 (Roschel et al., 2020) data thus far appears equivocal. For example, Maugeri et al., observed a reduction in PA and energy expenditure across age groups in Italy (Maugeri et al., 2020) whereas Cheval and colleagues observed increased sedentary time, decreased vigorous PA but an increase in walking time and moderate PA in younger adults (Cheval et al., 2021). Indeed, a reduced step count of just 14 days (75% reduction) has been shown to reduce knee extensor muscle strength by 8% in older adults which was not fully recovered following 2 weeks post-resumption of normal activity (Reidy et al., 2018). This highlights the key detrimental effects of even modest periods of reduced PA and increased sedentary time on older adults let alone the impact of months of reduced PA.

1.4.1. Alterations to skeletal muscle structure and function with age

The age-related loss of SKM mass was coined sarcopenia from the Greek “*sarx*” (meaning flesh) and “*penia*” (meaning loss) (Rosenburg, 1997). Sarcopenia is a muscle disease that presents due to adverse muscle changes characterised through low muscle quantity, quality, and strength, hence predisposing an individual to an increased likelihood of physical disability, falls, fractures, and mortality (Cruz-Jentoft et al., 2019). Large population-based studies have

reported sarcopenia prevalence between 8 and 50% in people over 50 years; with the main characteristics varying depending on ethnicity, setting, age and diagnostic methodology (Abellan van Kan, 2009). The decline in SKM metabolism and function should not be underestimated, with in the UK alone, complications arising from falls in the elderly (with an association to frailty) are estimated to cost the NHS £1.7 billion annually (Age UK).

According to cross-sectional and longitudinal studies, sarcopenia is thought to start in the third decade of life, with a marked acceleration by the fifth decade (Mitchell et al., 2012). Despite some factors which we cannot have influence over, such as the positive correlation between birth weight and lean body mass (Gale et al., 2001), there are several lifestyle factors which we can control to limit the progression of sarcopenia including nutrition, PA, smoking (Szulc et al., 2004) and alcohol consumption (Lang et al., 2003). Importantly, there is no sole contributor to the progression of sarcopenia, more of a combination and integration of multiple factors such as sedentarism, impaired AA sensing, inflammation, lipid infiltration and increased reactive oxygen/nitrogen species (Wilkinson et al., 2018b). Indeed, only recently the European Working Group on Sarcopenia in Older People (EWGSOP) stated that “sarcopenia is a muscle disease that occurs due to adverse muscle changes throughout the lifespan” (Cruz-Jentoft et al., 2019). In addition to this, the EWGSOP also recently updated their sarcopenia definition to include low SKM strength as the primary parameter of sarcopenia; with low SKM quality, quantity and strength suggesting severe levels of sarcopenia (Cruz-Jentoft et al., 2019). Therefore, this steers away from the previously used term “dynapenia” which was proposed to

describe the loss of muscle strength with advancing age (Clark & Manini, 2008; Manini & Clark, 2012). There are now various tools which can be used to characterise sarcopenia depending on (e.g.,) the patient, and resources in a healthcare setting (Cruz-Jentoft et al., 2019) (summarised in Figure 1.5). Initially, questionnaires, such as the SARC-F, were used to obtain self-reports with the use of muscle strength measures (e.g., grip strength), muscle quantity (e.g., via dual-energy X-ray absorptiometry (DXA) or magnetic resonance imaging (MRI), with MRI considered gold standard) and physical performance tasks (e.g., short physical performance battery test (SPPBT) and/or timed-up and go test (TUG)) advancing the diagnosis of sarcopenia (Cruz-Jentoft et al., 2019).

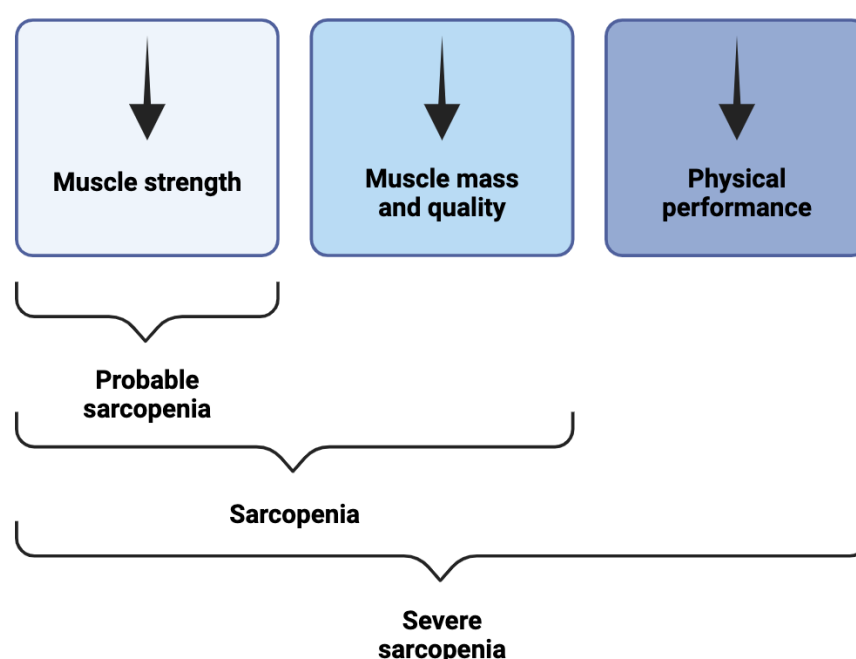


Figure 1.5 Interaction of declines in skeletal muscle strength, mass, and physical performance on the development of sarcopenia. Diagram adapted from European Working Group of Sarcopenia in Older People (Cruz-Jentoft et al 2019). Created in Biorender.com.

With exception of disease states, consistent levels of SKM mass are typically observed during the early years of life until ~50 years where SKM begins to significantly atrophy, with muscle mass decline being ~1% per year in men and ~0.5% per year in women (Mitchell et al., 2012). Around 30% of an individual's peak muscle mass is lost by the age of 80 years, with this loss drastically exacerbated by physical inactivity and poor nutrition (Janssen et al., 2000). In younger individuals, muscle mass also differs between sexes with relation to both absolute (33.0 ± 5.3 vs. 21.0 ± 3.8 kg; males vs. females) and relative (38.4% vs. 30.6%; males vs. females) terms (Janssen et al., 2000). Such differences in SKM appear to be regional, as although males have significantly greater SKM in both the lower (18.1 ± 3.1 kg) and upper (14.1 ± 2.6 kg) body compared to females (lower: 12.2 ± 2.5 kg; upper: 8.4 ± 1.8 kg), this sex difference is less in the lower (33%) compared to upper (40%) body (Janssen et al., 2000). Sex differences in SKM mass and distribution are also consistent with sex differences in muscle strength, with discrepancies in lower body strength (~30%) being smaller than observations for upper body strength (~50%; (Janssen et al., 2000). Further, in older age, longitudinal studies report sex differences in muscle mass decline with those aged ~75 years exhibit muscle mass declines of 0.80–0.98% per year in men and 0.64–0.70% per year in women (Mitchell et al., 2012). Importantly, muscle strength (commonly used as a proxy measure to assess muscle function) is lost more rapidly than muscle mass, with declines of 3–4% per year in men and 2.5–3% per year in women aged ~75 years (Mitchell et al., 2012), with older men (60–74 years) having

significantly lower maximal knee extensor strength compared to middle-aged (35–46 years) men (Izquierdo et al., 1999b). Despite knowledge of sarcopenia and the ageing population, global lifestyle changes such as increasing PA levels and improving nutrition, are progressing at a slow rate and obesity is becoming significantly prevalent, especially in more economically developed countries.

Considering the above, it is no surprise that age-related muscle wasting and loss of function has both severe physiological and psycho-social problems including loss of independence, depression, increased risk of frailty and falls, sedentarism and increased risk of all-cause mortality (Wilkinson et al., 2018b). As such, the scientific underpinning of age-related functional declines is both important and timely to understand, with the potential to aid development of countermeasures to ultimately improve an individual's quality of life.

Extensive scientific literature provides knowledge that the maintenance of muscle mass is regulated by the dynamic relationship between MPS and MPB, leading to a typical protein turnover rate of 1–2% per day in young healthy individuals (Atherton et al., 2010a; Atherton et al., 2016; Breen et al., 2013; Wall & van Loon, 2013). In aged muscle, the anabolic response to such stimuli (protein feeding and exercise) is depressed, resulting in blunted MPS response (Atherton et al., 2010a; Wall et al., 2015) and suppressed inhibition of MPB (Greenhaff et al., 2008); thus, contributing to SKM mass decline. Additionally, as muscle function is achieved as a combination of muscle size and neural mechanisms (Campbell et al., 2019), the contribution of muscle fibre loss and subsequent motor unit remodelling (Piasecki et al., 2016a) should be equally

considered, as it is likely that neuromuscular mechanisms and loss of motor units may explain the additional deficits in age-related functional decline not explained by muscle atrophy (Piasecki et al., 2016a).

Several other factors are thought to influence the development and progression of sarcopenia (discussed in detail in Mitchell et al., 2012). For example, changes to an individual's hormone and cytokine milieu, such as the “andropause” (Ferrando et al., 2002) which includes declines in testosterone bioavailability and subsequent associations with loss of muscle mass and frailty (Gharahdaghi et al., 2019). Indeed, six weeks of testosterone injections alongside whole-body RET led to increases in total, legs, and appendicular fat-free mass with augmentations in vastus lateralis (VL) thickness, fascicle length and quadriceps cross-sectional area (CSA), in addition to muscle strength, being greater with rather than without testosterone injections (Gharahdaghi et al., 2019); thus, demonstrating testosterone coupled with RET to be a short-term intervention to improve SKM mass and function in older non-hypogonadal men. Increased oxidative stress is a further factor which may influence the progression of sarcopenia. Ageing seemingly exposes the cell to greater oxidative stress due to a reduction in antioxidant buffering mechanisms and increases in the generation of free radicals resulting from dysfunction in the mitochondrial respiratory chain (Barreiro et al., 2006; Mitchell et al., 2012). Consequently, this results in damage to DNA, myofibrillar/mitochondrial proteins, the neuromuscular junction, and elements of the sarcoplasmic reticulum responsible for calcium release and subsequent muscle contraction (Mitchell et al., 2012).

Arguably, the varying contributors which may impact the progression of sarcopenia are influenced by environmental factors, namely, protein nutrition and PA, with dietary protein intake and exercise interventions (such as RET) often adopted to attempt to offset the progression of sarcopenia. Below, we discuss the role of muscle protein metabolism which respective to protein nutrition and exercise throughout the lifespan with the overall aim of maintaining quality of life for as long as possible.

1.4.2. Skeletal muscle anabolic resistance

One of the many metabolic features contributing to sarcopenia is the presence of SKM anabolic resistance (see Figure 1.6 for summary). Ground-breaking work more than a decade ago, aided the development of the concept of anabolic resistance in advancing the explanation of age-related declines in SKM mass and function (Volpi et al., 2000). The premise of anabolic resistance is that increases in MPS and suppressions in MPB in response to the key environmental factors regulating muscle maintenance i.e., protein intake (Cuthbertson et al., 2005; Moore et al., 2015) and exercise (Kumar et al., 2009; Wall et al., 2015) are blunted compared to younger individuals. Although some early, small cohort studies reported depressed basal (i.e., post-absorptive) MPS rates in older compared to younger age (Welle et al., 1993), it is now largely accepted that post-absorptive rates of MPS and MPB are unchanged in healthy ageing (Phillips et al., 2012; Breen & Phillips, 2011; Moore et al., 2015; Wall et al., 2015); meaning the gradual loss of SKM mass with age is not related to the dysregulation of muscle protein turnover in the post-absorptive state (Moore et al., 2015). Reflecting this, a plethora of accumulating evidence (Moore et al.,

2015; Wall et al., 2015; Phillips et al., 2012; Marshall et al., 2020; Mitchell et al., 2012; Brook et al., 2016a) suggests that anabolic resistance is likely a major driver of sarcopenia; though that is not to say mixed results engender this contentious (Symons et al., 2009; Chevalier et al., 2011).

The presence of anabolic resistance was eloquently demonstrated by Volpi and colleagues who identified rates of MPS to increase in young but not older adults in response to 40 g crystalline AA (plus 40 g glucose) feeding, given as small 30 mL boluses every 10 minutes across a 3 hour postprandial measurement period (Volpi et al., 2000). Seminal work by Cuthbertson and colleagues demonstrated a maximal MPS response in young adults to 10 g EAA, with older adults having suppressed rates of MPS above 5 g EAA (Cuthbertson et al., 2005). Additionally, a 40 g bolus of EAA failed to elicit a similar MPS response in older adults to that seen in young adults at 10 g EAA (Cuthbertson et al., 2005). Two previous retrospective studies further support the existence of anabolic resistance in aged muscle, with Wall and colleagues reporting depressed post-prandial MPS rates (-16%) in older adults (Wall et al., 2015) and Moore et al. reporting a “rightward shift” whereby myofibrillar FSR (i.e., MPS) plateaus with respect to relative protein intake in older (~0.4 g/kg) compared to young (~0.24 g/kg) men (Moore et al., 2015). Analysis by Moore and colleagues identified protein intake required to maximally stimulate MPS to be ~68% and 140% greater in older compared to younger individuals when expressed relative to body mass and lean body mass, respectively (Moore et al., 2015). Indeed, contractile activity and/or improvements in protein quality (i.e., LEU-enriched proteins) may influence the dosage of protein required for maximal stimulation – as we discuss in our

narrative review (Chapter 3 of this thesis). Conversely, increased sedentarism, disease status and reduced protein quality would likely influence protein requirements to induce maximal MPS, regardless of age (Moore et al., 2015).

Like protein feeding, there is evidence of anabolic resistance in response to exercise in older age. Kumar et al. demonstrated at 1–2 hours post-RE, ranging from 20–90% one repetition maximum (1RM), that MPS responses were blunted in older compared to younger adults, with blunted phosphorylation of p70S6K1 and 4E-BP1, downstream signalling targets of mTORC1, 1-hour post-RE at 60–90% 1RM in older men (Kumar et al., 2009). In a more chronic training study, anabolic resistance to exercise presented in older adults following 6 weeks of unilateral leg RET (75% 1RM), with MPS becoming elevated after 3 weeks in young ($1.6 \pm 0.01\%$ per day) but not older adults ($1.49 \pm 0.08\%$ per day), which was coupled with an absence of thigh fat-free mass gains in older muscle post-training (Brook et al., 2016a). Similarly, reduced sensitivity to AA following endurance type (i.e., walking) exercise at $\sim 40\%$ VO_2 peak was evidenced in older compared to younger adults (Durham et al., 2010). It has been suggested that low-intensity high-volume RET may have comparative effects to high-intensity low-volume RET, a modality which would be of practicable benefit to older individuals (Breen & Phillips, 2011). Indeed, low-intensity RET (20% 1RM) coupled with blood flow occlusion heightened mTORC1 signalling and MPS by 56% above basal levels 3 hours post-exercise, although MPS remained unchanged in the absence of blood flow occlusion (Fry et al., 2010). Interestingly, in younger adults, it was demonstrated that unilateral leg extension exercise at 30% vs. 90% 1RM to failure, was more effective at increasing and

maintaining elevated MPS 24 hours post-exercise (Burd et al., 2010). If RET volume rather than intensity is key for stimulating MPS, a greater portion of older adults may be able to perform RET due to associated sarcopenic comorbidities (e.g., arthritis) limiting performance of high-intensity exercise (Breen & Phillips, 2011); thus, offering a potentially effective intervention to offset the impact of anabolic resistance and age-related muscle functional declines, although this requires further investigation.

Dietary protein, specifically EAA and LEU, acts as both a signalling molecule and substrate for MPS (Bonfils et al., 2012; Han et al., 2012; Moro et al., 2016). Reduced AA delivery to SKM has subsequently been proposed to impair the intracellular signalling and subsequent MPS rates (Moro et al., 2016). As ageing leads to blunting of postprandial macro- and micro-vascular blood flow (Mitchell et al., 2012), AA delivery may become altered; thus, influencing rates of MPS. Previous work from Timmerman and colleagues supports this through evidencing reduced microvascular blood flow impairs MPS and increasing microvascular blood flow (via sodium nitroprusside) led to enhanced MPS (Timmerman et al., 2010a; Timmerman et al., 2010b). Similarly, vasodilation, induced via sodium nitroprusside, during hyperaminoacidaemia in older adults led to increased AA delivery and subsequent MPS rates which were similar to younger counterparts (Dillon et al., 2011). Conversely, more recent studies have demonstrated enhancements in microvascular responses to feeding in older men through use of cocoa flavanols (Phillips et al., 2016a) and pharmaceutical agents (Phillips et al., 2014) did not improve SKM anabolism, meaning AA delivery is an unlikely factor in the presence of age-related anabolic resistance (Wilkinson

et al., 2018b). Furthermore, hyperaminoacidaemia (Koopman et al., 2009a; Mitchell et al., 2015b; Mitchell et al., 2015a) following large doses of protein/AA tends to be higher in older rather than younger individuals, presumably due to compromised clearance of AA by MPS, suggesting that impaired digestion/absorption is not a limiting factor in muscle anabolism.

Impairment in the mTORC1 signalling pathway with advancing age is thought to be the most likely influencer of impaired SKM anabolism, thus contributing to anabolic resistance. For example, during a euglycemic hyperinsulinaemic hyperaminoacidaemic clamp, MPS was robustly stimulated in young but blunted in older subjects with phosphorylation of mTOR and 4E-BP1 being similar between groups yet an absence of p70S6K1 stimulation in elderly subjects (Guillet et al., 2004). Similarly, older adults demonstrated impaired responsiveness of MPS to EAA likely due to decreased intramuscular expression of and phosphorylation of AA sensing and signalling proteins including mTOR, p70S6K1, and eIF2B (Cuthbertson et al., 2005). Further, older adults have demonstrated blunted p70S6K1 signalling to acute exercise (Kumar et al., 2009; Fry et al., 2010), as well as being impaired temporally in response to six weeks RET (Brook et al., 2015; Brook et al., 2016a; Brook et al., 2016b), thus reducing translational efficiency and subsequent muscle accrual. Interestingly, Brook and colleagues investigated aspects of translational capacity, such as RNA content and ribosomal biogenesis, with blunted expression of rDNA transcription factors cMyc and TIF1a, alongside blunted increases in the indices of translational capacity (RNA:DNA and RNA:Protein ratios) being observed in the older adults in response to RET (Brook et al., 2016a). Subsequently, impaired ribosomal

biogenesis, thus reducing the capacity for MPS in older adults, may also be a key factor underlying anabolic resistance (Wilkinson et al., 2018b).

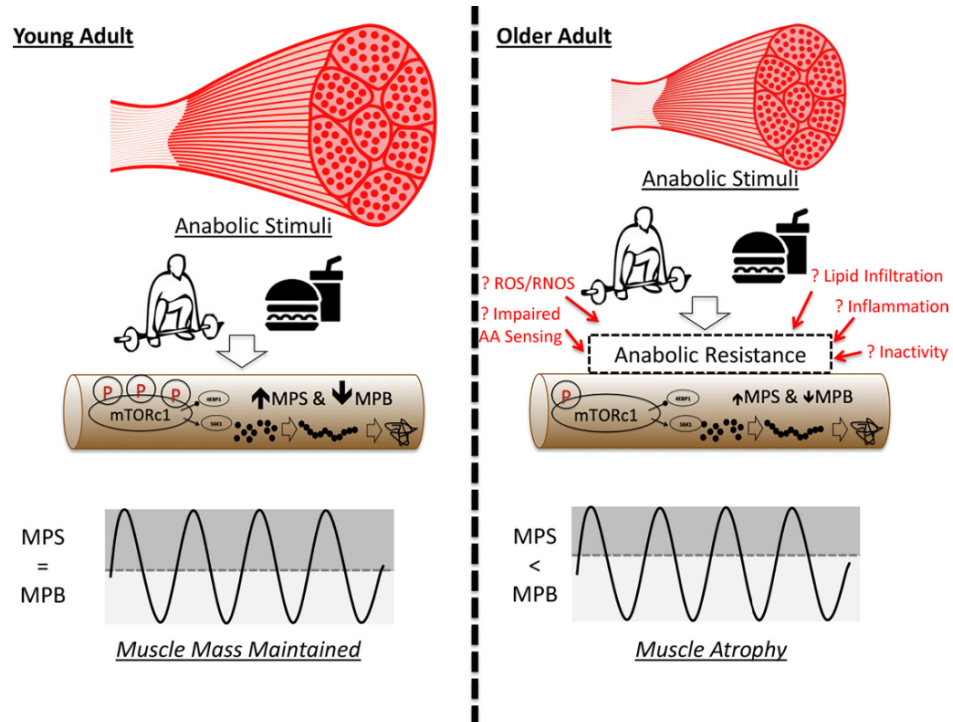


Figure 1.6 Mechanisms driving anabolic resistance and muscle atrophy in older age. Image taken directly from Wilkinson et al., 2018. MPB, muscle protein breakdown. MPS, muscle protein synthesis. ROS, reactive oxygen species. RNOS, reactive nitrogen species.

1.5. Physical (in)activity and nutrition interactions

1.5.1. Physical activity and muscle protein metabolism

Exercise is without a doubt one of, if not the most, important lifestyle factors an individual can integrate into their lives to improve their physical and mental wellbeing. Currently, PA guidelines proposed by the UK government to improve adult health (those aged 19–64 years and 65 years plus) is 150 minutes/week of moderate exercise (e.g., walking or cycling), 75 minutes of vigorous exercise (e.g., running) or shorter durations of very vigorous exercise (e.g., sprinting), with a combination of all exercise intensities often recommended (Physical Activity Guidelines, UK Chief Medical Officer, 2019). Additionally, PA guidelines state muscle strengthening activities should take place at least twice a week and can include heavy gardening, carrying shopping or RE.

As per the response to feeding, that the SKM anabolic response to exercise is of limited duration (Atherton & Smith, 2012). Immediately post-exercise, as with protein feeding, there is a latent period prior to increases in MPS of a duration which seems relative to the magnitude of mechanical stress associated with the exercise (Atherton & Smith, 2012). For example, the latency period following “lower intensity” exercise (6 x 8 repetitions at 75% 1RM) is <1 hour (Kumar et al., 2009), whereas MPS remained unchanged for up to 3 hours following extremely fatiguing and damaging eccentric contractions (Cuthbertson et al., 2006). Following this latent period, MPS sharply rises between 45 and 150 minutes and may be sustained for up to 4 hours in the fasted state (Kumar et al., 2009), with MPS becoming limited by substrate availability. In the fed state, MPS rates may be increased up to 24 hours (Cuthbertson et al., 2006; Burd et al., 2011) and even 48 hours (Miller et al., 2005) post-exercise. With this in mind (i.e., the delay of muscle full lasting up to 24–48 hours), the timing of protein

intake pre-, during- or post-exercise may not be as crucial for muscle anabolism as first thought. Rather the quantity/quality of the protein may be the most fundamental consideration (Atherton & Smith, 2012). The time course of these changes in MPS following exercise is similar within the myofibrillar muscle fraction but also the epimysial and tendon collagen (Miller et al., 2005), demonstrating a high degree of coordination between the tissues of the musculoskeletal system. Despite this, the type of exercise training impacts MPS rates in different muscle fractions. Indeed, in the untrained state, RE increases both myofibrillar and mitochondrial protein synthesis, but following 10 weeks of RET, MPS was only stimulated in the myofibrillar proteins (Wilkinson et al., 2008). Conversely, endurance exercise stimulated mitochondrial protein synthesis in both the untrained and trained state (Wilkinson et al., 2008). Interestingly, muscle hypertrophic remodelling is thought to be the most active during the early stages of RET with increases in MPS evidenced over the first three weeks of RET but not three to six weeks (Brook et al., 2016a). It should be noted that muscle growth is a non-linear process (DeFreitas et al., 2011; Ogasawara et al., 2013), thus this would be reflected in a diminished acute MPS response post-exercise throughout a RET programme (Brook et al., 2016a). For example, MPS was elevated over eight days of RET, yet the magnitude of MPS decreased after just three repeated exercise bouts (Wilkinson et al., 2014), supporting the notion of a progressive desensitisation to the anabolic effects of RET (Brook et al., 2016a).

RE can stimulate MPS rates in both younger and older individuals, with the magnitude of MPS increases seemingly dependent on the workload and intensity

of RE. This was highlighted by Kumar and colleagues who demonstrated no detectable increases in MPS following RE (unilateral leg extension and flexion) in the postabsorptive state at intensities of $\leq 40\%$ 1RM, whereas MPS increased 2- to 3-fold at intensities $\geq 60\%$ 1RM, with no differences between 60–90% 1RM (Kumar et al., 2009). These data confirmed a previous smaller pilot study which demonstrated isometric exercise at 60, 75 and 90% 1RM increased MPS 90–150 minutes post-exercise to a similar extent (Bowtell et al 2003). As per protein feeding, older adults demonstrated a blunted MPS response at all exercise intensities (Kumar et al., 2009). This does not, however, mean that lower-intensity exercise cannot induce anabolic effects. For example, a comparable magnitude of MPS stimulation was evidenced at 30% 1RM compared to 90% 1RM but only when exercise was performed to failure (*not* work matched), likely due to increased recruitment of type II fibres due to the fatiguing nature of the contractions (Burd et al., 2010). Resultingly, the lifting of heavy weights may be escaped by increasing the volume of work at a lower intensity which can overcome the blunted MPS response and may represent an alternative approach to stimulate SKM hypertrophy (Atherton & Smith, 2012). Although eccentric-type exercise training (i.e., lengthening muscle contractions) has previously been shown to result in greater muscle hypertrophy (Roig et al., 2009), assessment of MPS following both concentric (i.e., shortening muscle contractions) and eccentric contractions was found to increase at 6 and 24 hours (not 3 hours) post-exercise, independent of the exercise mode (Cuthbertson et al., 2006). Further, when total work is matched between eccentric and concentric contractions there is no difference in training-induced muscle hypertrophy

(Moore et al., 2012). Thus, the contraction mode of RE may not be as influential on MPS post-exercise as first thought.

The provision of dietary protein is critical for muscle adaption (i.e., maximising muscle hypertrophy) in response to RE. This is exemplified by prolonged elevation in MPB, inducing a negative NPB, when RE is performed in the absence of protein/EAA nutrition (Biolo et al., 1995 Phillips et al., 1997). Sufficient protein/EAA intake must, therefore, complement exercise to promote positive NPB and subsequent muscle hypertrophy via cumulative muscle protein accrual following each bout of RE over time (Atherton and Smith, 2012) (Pennings et al., 2011b; Stokes et al., 2018). RE heightens the anabolic sensitivity of SKM, with both the magnitude and duration of the MPS response enhanced through increasing the availability of dietary EAA post-exercise (Pennings et al., 2011b). For example, during the sedentary state (i.e., at rest), the ingestion of 10 g of EAAs (equivalent to ~25 g of high-quality protein) is required to maximise MPS (Cuthbertson et al., 2005). In comparison, ~8.6 g of EAAs (~20 g of high-quality protein) is required to plateau the postprandial MPS response in the immediate post-exercise period (Moore et al., 2009a). The addition of carbohydrate provision offers no further anabolic effects post-exercise (Staples et al., 2011), highlighting the central role of EAA in maximising SKM anabolism in protein turnover following exercise.

Despite exercise sensitising the muscle to protein feeding, limits do still exist whereby muscle full is still reached and the quantity of protein required to maximise muscle anabolism may be dictated by the type of RE conducted. For

example, in response to unilateral leg-based RE, MPS displays a dose-response relationship to dietary protein ingestion until maximal stimulation with ~20 g protein, with larger doses (e.g., 40 g) leading to irreversible oxidation and excess protein AA being catabolised (Atherton & Smith, 2012; Witard et al., 2014; Moore et al., 2009a). However, in situations of whole-body resistance exercise, protein requirements tend to be greater with 40 g WP shown to stimulate MPS to a greater extent than 20 g in young resistance-trained men (Macnaughton et al., 2016). Interestingly, the mode of exercise may also play a factor in determining the quantities of dietary protein post-exercise. For example, oxidation of endogenous AA may only represent a fraction of total energy provision during exercise (~2–10% depending on CHO availability) but their utilisation increases with endurance exercise intensity (Felig & Wahren, 1971) and duration (Lamont et al., 2001; Burd et al., 2019b). Estimates of LEU oxidation rates during moderate intensity exercise (~60% of $\text{VO}_{2\text{max}}$) are ~8 mg/(kg/h) (Bowtell et al., 1998) with rates increasing to ~10 mg/(kg/h) at higher intensities (~70% $\text{VO}_{2\text{max}}$) in endurance-trained athletes (Mazzulla et al., 2017). Regular endurance exercise training blunts the exercise-induced stimulation of LEU oxidation rates and it has been shown that 24 hours net LEU balance remains unaffected by acute cycling exercise performed twice in a day at ~50% $\text{VO}_{2\text{max}}$ for 90 minutes per session (Forslund et al., 1999). However, it was recently shown that 1 hour of treadmill running at 70% $\text{VO}_{2\text{peak}}$ resulted in a stimulation of LEU oxidation rates and net LEU balance that was more negative when compared to the rested state in athletes (Mazzulla et al., 2017). Net LEU balance remained negative throughout the postprandial period even when providing 18 g whole egg protein immediately post-exercise (Mazzulla et al.,

2017); a commonly recommended quantity of protein (~0.25 g protein/kg per meal) to maximise MPS post-RE (Moore et al., 2009a). Endurance exercise may therefore place more demands on dietary protein due to the need to compensate for exercise-induced AA oxidation losses while also supporting muscle protein remodelling throughout recovery (Burd et al., 2019b). The “optimal” quantity of protein needed to be consumed post-exercise should, therefore, consider exercise mode, intensity, duration, and/or health/performance goals within the recommendation.

We discuss the MPS responses to protein × exercise interactions with specific relation to LEU-enriched supplementation in acute and chronic conditions in Chapter 3.

1.5.2. Physical inactivity and muscle protein metabolism

Periods of physical inactivity often differ in duration and can take many forms throughout an individual’s life. Physical inactivity can happen as a result of injury/illness/surgery (Hortobágyi et al., 2000; Lin et al., 2012; Nash et al., 2004), spaceflight (Sibonga et al., 2008) or merely consequently from increased sedentarism (Evans, 2010). Ultimately increased physical inactivity, regardless of the reason, results decreased SKM mass and function and consequently impairs capacity for activities of daily living and quality of life (Campbell et al., 2019).

Disuse models have been applied throughout the research to investigate the effects of inactivity on SKM such as bedrest (Ferrando et al., 1996; Kawakami et al., 2001), limb immobilisation via casting, bracing or unilateral limb suspension (Clark et al., 2008; Deschenes et al., 2008; Kilroe et al., 2020), and reduced step count (Breen et al., 2013). Specifically for the leg muscles, head-down bedrest induces the greatest rate of loss in CSA, followed by casting, with unilateral leg suspension and knee immobilisation (i.e., bracing) inducing lesser atrophy rates (Phillips et al., 2009). However, the rates of muscle mass loss across all disuse models are marginally faster within the first 30 days of unloading with a mean loss in muscle CSA of $\sim 0.6\%$ /day (Phillips et al., 2009). After the first 30 days, rates of muscle mass loss slow and reach a plateau in both the quadriceps femoris and the soleus (Adams et al., 2003). Interestingly, the rate of muscle strength and functional decline are usually greater and occur faster than the decline in muscle mass (Campbell et al., 2019). Like the ageing process, SKM atrophy cannot solely explain inactivity-induced loss of muscle strength. Whilst muscle fibre CSA is indeed a key factor in determining maximal force-generating capacity, muscle function and strength are also strongly influenced by neural mechanisms (Campbell et al., 2019). It is therefore plausible that changes in neuromuscular function may be responsible for the disproportionately higher loss in muscle strength relative to the reduction in muscle size with physical inactivity.

In humans, it is generally agreed that MPS is downregulated resulting from uncomplicated (i.e., non-pathological) periods of disuse (Glover et al., 2008; Gibson et al., 1988; Paddon-Jones et al., 2006) and alterations in MPS are

facilitative and responsible for most of the observed changes in protein mass (Phillips et al., 2009). In contrast, the relative importance of MPB to muscle loss remains less obvious. MPB is often adaptive to the inactivity-induced fall in MPS and either remains unchanged or declines (Phillips et al., 2009). Indeed, both basal (i.e., fasting) and postprandial rates of MPS decline during periods of disuse. MPS rates in the fasted state occur relatively early in immobilisation (~10 days) and do not appear to decline further with prolonged inactivity (de Boer et al., 2007; Wall et al., 2016). It has been well established that physical inactivity induces a state of anabolic resistance with SKM becoming less responsive to EAA following feeding. Exemplifying this, reducing daily step-count by ~76% for 14 days suppressed postprandial MPS rates by 26% following the ingestion of 25 g protein in healthy older adults (Breen et al., 2013). In young individuals, 14 days of unilateral knee immobilisation suppressed postprandial MPS in the immobilised limb, with the non-immobilised leg demonstrating greater MPS responses to both a low (43 mg/kg/h) and high (261 mg/kg/h) dose infusion of AA (Glover et al., 2008). Again, MPS in older individuals was unresponsive to 12 g (2.5 g LEU) EAA following 5 days of bedrest compared to younger counterparts across 0–3 hours measurement period (Tanner et al., 2015). Although it could be argued 12 g EAA was insufficient to stimulate MPS in older individuals who have not experienced bedrest.

From a clinical perspective, older adults are more likely to experience bouts of inactivity (e.g., hospitalisation/physical debilitation with ensuing bed rest) (Suetta et al., 2009; Arentson-Lantz et al., 2020) and often have longer lengths

of hospitalisation (He et al., 2005). Subsequently, this may induce “acute sarcopenia”, thus contributing (and likely worsening) to the individual’s general progression of sarcopenia (Deane et al., 2020b). Indeed, the associated loss of muscle mass is significant and rapid, with older adults losing detectable muscle mass (~1.5%) within as few as 5 days of unilateral limb immobilisation (Dirks et al., 2014) and losing up to ~6% (~1 kg) of lean mass during 10 days of bedrest (Kortebein et al., 2007). This is purportedly greater than the loss of muscle seen in younger subjects following 14 or 28 days of bedrest (Paddon-Jones et al., 2004; Ferrando et al., 1996). In older individuals, the premature onset of muscle full associated with inactivity may be further confounded by age-related anabolic resistance to nutrition. As such, ageing populations subjected to periods of physical inactivity stand to benefit considerably from optimised dietary protein-exercise intervention.

The ability of protein feeding in both young and older adults to offset the premature muscle full state and minimise SKM mass losses associated with inactivity has produced mixed results. Where protein feeding may not offset the decline in muscle mass alone, exercise interventions such as neuromuscular electrical stimulation, may be thought of as the optimum strategy to minimise muscle mass losses during periods of inactivity. Protein supplementation during these exercise strategies would indeed be additive to this in attempting to minimise mass and functional loss. We discuss the impact of protein, with a focus on LEU, supplementation, and exercise interventions in relation to physical inactivity in detail in Chapter 3.

1.6. Thesis aims

Whilst the field of protein nutrition and exercise is extensive and has improved the knowledge and outcomes of healthy ageing, continued advancements in research to optimise protein nutrition and exercise regimes still need to be made.

In this body of work, we initially reviewed the literature and highlighted the importance of LEU on SKM anabolism in differing physiological states discussed above (Chapter 3). We then aimed to assess the efficacy of a novel WP rich in EAA and LEU, β -lactoglobulin (BLG), compared to an isonitrogenous whey protein isolate (WPI) on SKM anabolism in younger (Chapter 4) and older (Chapter 5) adult males, at rest and in response to acute RE. Further, we also explored different doses of BLG with respect to MPS rates in older adult males (Chapter 6), to investigate whether SKM anabolism can be maximised at low doses of BLG.

Chapter 2

2. Methodology

The current chapter outlines the methodologies utilised for each of the research studies that make up this thesis outlined below:

- **Research Study 1 (Chapter 4):** Investigating the effects of BLG vs. an isonitrogenous WPI on SKM anabolism at rest and following acute RE in young healthy men.
- **Research Study 2 (Chapter 5):** Investigating the effects of BLG vs. an isonitrogenous WPI on SKM anabolism at rest and following acute RE in healthy older men.
- **Research Study 3 (Chapter 6):** Exploring different doses of BLG on SKM anabolism at rest and following acute RE in healthy older men.

All methodologies applied for each research chapter regarding the acute assessment visits and sample analysis were identical apart from the beverage consumed. Importantly, research studies 1 and 2 were double-blind randomised cross-over trials. Resultingly, these research studies involved two acute assessment visits assessing the effects of BLG and WPI supplementation (10 g protein) on muscle anabolism, in young and older adults respectively. Research Study 3 was a single-blind trial involving one acute assessment visit assessing different doses of BLG (5 g vs. 10 g protein) on muscle anabolism in older

adults. The BLG data from research study 2 contributed to the 10 g protein group in research study 3, meaning individuals were recruited for the BLG with 5 g protein group only.

Although not a systematic review, we believe it important to make note of search terms used to gather relevant literature to be included in our comprehensive narrative review (Chapter 3). Importantly, these search terms were not set/defined but were used to aid us address the topic of interest. We performed the literature search in PubMed and Google Scholar. The following search terms were used: muscle protein synthesis, MPS, fractional synthetic rate, FSR, postprandial, protein, leucine, milk, whey, essential amino acids, EAA, egg, ageing, anabolic resistance, hypertrophy, strength, function, training, exercise, resistance, inactivity, disuse, bedrest, immobilisation.

2.1. Ethical approval

All research studies were approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (reference number: FMHS 207-0221), registered at clinicaltrials.gov (registration numbers: NCT05701202 and NCT05700058) and were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to involvement in the study they were recruited for.

2.2. Participant characteristics and screening

A total of 33 participants were recruited across all research studies, with participant characteristics outlined for each research chapter in Table 2.1. Ten healthy young male participants completed research study 1. Twelve healthy old male participants completed research study 2. Twenty-three healthy old male participants completed research study 3 ($n = 12$ from research study 2, meaning an additional 11 participants were recruited). Participants were recruited from the University of Nottingham student population and the general population. Recruitment occurred via word of mouth, social media adverts on the research group pages and adverts in the local community. Participants who had volunteered in previous research studies and had given consent for their contact details to be kept were also invited to participate.

Table 2.1 Participant anthropometric characteristics

		Study 2	
	Study 1	Study 3	
Characteristic	Young men	Older men	Older men
<i>n</i>	10	12	11
Supplement	BLG and WPI	BLG and WPI	BLG
Protein dose (g)	10	10	5
Age (years)	26 ± 2	69 ± 1	70 ± 1
Height (cm)	179 ± 2	179 ± 2	175 ± 2
Weight (kg)	81 ± 3	90 ± 5	86 ± 3.3
Protein intake (g/kg/d)	1.3 ± 0.1	1.0 ± 0.1	1.0 ± 0.05
VL CSA (cm ²)	R: 31 ± 2 L: 30 ± 1	R: 22.3 ± 1.1 L: 20.6 ± 1.1	R: 22.3 ± 1.3 L: 20.8 ± 1.4
VL thickness (cm)	R: 2.6 ± 0.1 L: 2.5 ± 0.1	R: 2.2 ± 0.1 L: 2.2 ± 0.1	R: 2.5 ± 0.1 L: 2.0 ± 0.1
1RM (kg)	65 ± 4	41.1 ± 4.3	40.2 ± 3.3

Data presented as mean ± SEM. BLG, β-lactoglobulin; CSA, cross-sectional area; L, left; R, right; VL, vastus lateralis; WPI, whey protein isolate; 1RM, 1 repetition maximum.

Prior to study enrolment, a screening visit took place to assess participant suitability for the research study. Participants were sent an information sheet via email (Appendix 2) detailing the research study at least 48 hours before attending the screening session. Participants attended the research facility at

~0900 hours, after an overnight fast (from midnight, water *ad libitum*), for ~1.5 hours. During the screening visit, participants had the opportunity to ask any questions regarding the study before providing written informed consent. To examine suitability for the study, a small blood sample to measure health parameters (e.g., liver/kidney function and blood coagulation factors), a discussion regarding medical history, measures of height, weight and blood pressure, and an electrocardiogram assessment were conducted. Participant eligibility was confirmed by a clinician after assessing all screening results and adhering to the inclusion/exclusion criteria (Table 2.2). Finally, a unilateral 1RM assessment was conducted to determine muscle strength of the participant's dominant leg (Leisure Lines LTD, Jan 2000, Iso Lever), of which, 75% was calculated for use during acute RE on the assessment days.

For studies 1 and 2, participants were randomly assigned to consume either BLG or WPI (10 g protein), with the other supplement consumed on the second assessment visit. Each assessment visit was separated by a washout period of 21–42 days. For research study 3, participants were allocated to a BLG group containing 5 g protein.

Table 2.2 Inclusion and exclusion criteria for participant enrolment

Inclusion criteria	Exclusion criteria
• Participant is within the desired age-range. • Participant is willing and able to give informed consent for participation in the study. • Participant is physically able to perform resistance exercise.	• A BMI <18 or >35 kg/m². • Active cardiovascular, cerebrovascular, or respiratory disease. • Any metabolic disease or malignancy. • Clotting dysfunction (including taking blood thinners). • A history of, or current neurological or musculoskeletal conditions. • Lactose intolerance. • Having taken part in a research study in the last three months involving invasive procedures or an inconvenience allowance.

BMI, body mass index.

2.3. Experimental protocol

On assessment days (for all studies), participants reported to the laboratory at ~0800 hours having undergone an overnight fast (≥ 10 hours, water *ad libitum*) and refrained from intense exercise for at least 72 hours. Initially, venous cannulas were inserted into the antecubital fossa for [1,2 ¹³C₂] LEU stable isotope tracer infusion (99 Atoms % of [1, 2 ¹³C₂], Cambridge Isotopes Limited, Cambridge, MA, USA), and another placed retrograde into a contralateral dorsal

vein of the opposite arm for arterialised blood sampling using the “hot-box” technique. Arterialised blood samples occurred at baseline and every 20 minutes thereafter, until conclusion of the assessment visit. The hot-box technique was utilised as a less invasive alternative to direct arterial sampling, to obtain arterialised-venous blood samples. This method involved placing the hand in a heated box at $\sim 55^{\circ}\text{C}$ to create an AV shunt of blood by vasodilation of veins in the fingers, allowing arterialised-venous blood sampling from a retrograde cannula in the dorsum of the hand (Abumrad et al., 1981). Plasma samples were collected for the quantification of AA, insulin and glucose-dependent insulinotropic polypeptide (GIP; Research Study 1 only) concentrations.

Following baseline blood samples, a primed, continuous infusion ($0.7 \text{ mg}\cdot\text{kg}^{-1}$, $1.0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) of $[1, 2 \text{ }^{13}\text{C}_2]$ LEU tracer was initiated and continued for the duration of the assessment visit over 7.5 hours (see Figure 2.3 for protocol schematic).

Bilateral ultrasound scans were performed of the VL to assess muscle CSA and architecture, allowing for participant characterisation in each research study (LA523 probe and MyLabTM50 scanner, Esaote, Genoa, Italy). Bilateral ultrasound scans were performed before the first set of muscle biopsies. All scans were acquired at the midpoint of the line from the greater trochanter to the midpoint of the patella (Franchi et al., 2018). The lateral and medial borders of VL were distinguished using the ultrasound probe in a vertical orientation, with the borders and midpoint of the muscle being marked on the skin. VL CSA was assessed using an extended field of view methodology, with three axial plane

images being taken (Inns et al., 2021). All data collected via ultrasound was acquired as a .bmp image, with offline analysis via ImageJ software (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA) as previously reported (Inns et al., 2021).

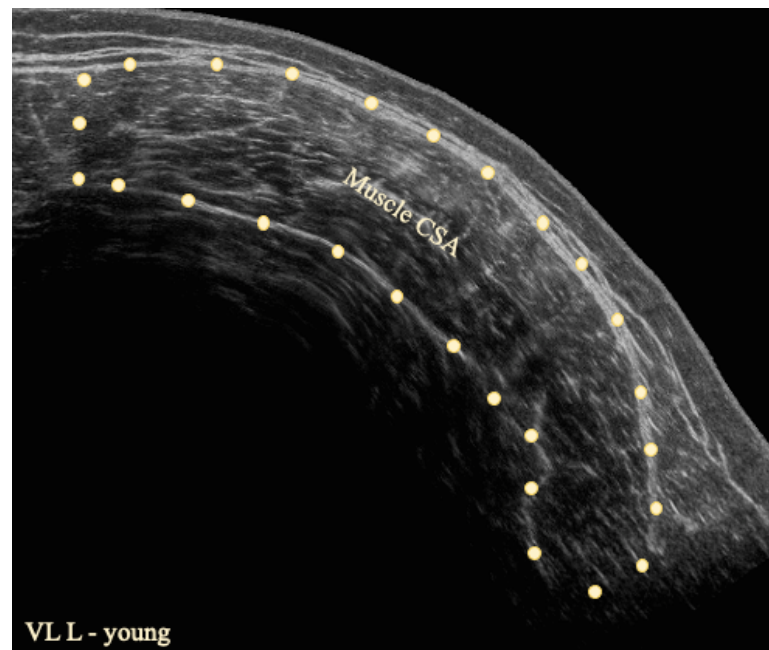


Figure 2.1 Muscle ultrasound scan assessing vastus lateralis (VL) cross-sectional area (CSA). Image is taken from the left leg of a young male participant. Yellow dots indicate borders of VL muscle.

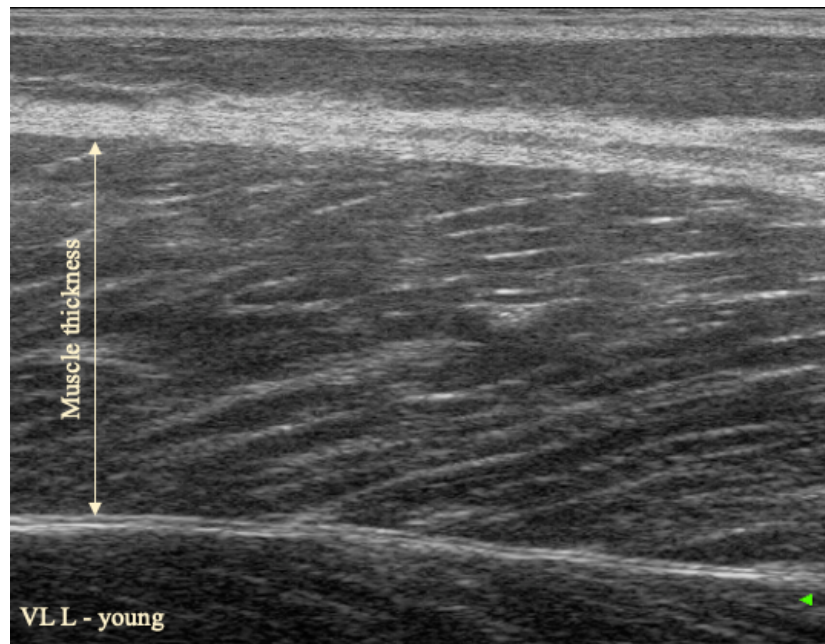


Figure 2.2 Muscle ultrasound scan assessing vastus lateralis (VL) architecture. Image taken from the left leg of a young male participant to assess VL muscle thickness.

At timepoints 0 (i.e., following 1.5 hours of tracer infusion), 3 and 6 hours, after induction of local anaesthetic (~5 mL 1% lignocaine), bilateral muscle biopsies were collected from the VL muscle (~150–200 mg of tissue) under sterile conditions using our standard conchotome technique. The muscle tissue was washed with ice-cold phosphate-buffered saline to remove excess blood, dissected free of visible fat and connective tissue, then snap frozen in liquid nitrogen and stored at -80°C until further analysis. Muscle biopsies at timepoints 0–3 hours allowed for postabsorptive MPS to be quantified. Muscle biopsies at 3–6 hours allowed for quantification of postprandial MPS in the fed, rested state (FED) and the fed, exercised (FED-EX) state. Approximately 20 minutes before the second set of biopsies (i.e., at 3 hours), participants performed a bout of

unilateral RE (leg extension; 6 sets of 8 repetitions at 75% 1RM with a 2-minute inter-set rest period) at a moderate contraction intensity (1–2 seconds concentric, 1–2 seconds eccentric) of the dominant leg (Leisure Lines LTD, Jan 2000, Iso Lever).

Immediately after the second set of biopsies, participants ingested the protein feed that they were randomly assigned. Each protein supplement was dissolved in ~250 mL water and consumed as a bolus within 5 minutes. Most participants consumed the assigned supplement within a minute. To minimise perturbations in plasma isotopic enrichment, beverages were enriched to 8% with [1, 2 $^{13}\text{C}_2$] LEU tracer. The AA composition of the protein supplements is outlined in Table 2.3.

Either absorbable or non-absorbable sutures were used to aid healing following all biopsy incisions. If non-absorbable sutures were used, participants were asked to attend the research facility for checking and removal of suture from biopsy sites at a follow-up visit 5–7 days after each assessment visit. If absorbable sutures were used, participants did not have to attend the research facility but were asked several questions (Appendix 5) to check for the risk of infection. Participants were also invited to send images to the researchers of the incision sites to check the healing status which was checked by a clinician.

Participants were asked to complete a 4-day diet diary recording all food and drink during those 4 days. Instructions and suggestions on how to do so were provided on the first assessment visit, via email and in the diet diary itself

(Appendix 4). Data from the diet diary was inputted into Nutritics Food Management Software to determine dietary protein intake across these 4 days.

Table 2.3 Amino acid composition of β -lactoglobulin and whey protein isolate supplements.

Amino Acid	BLG	WPI
Alanine	5%	5%
Arginine	2%	2%
Asparagine	11%	11%
Cysteine	2%	3%
Glutamic Acid	16%	17%
Glycine	1%	2%
Histidine	1%	2%
Isoleucine	6%	7%
Leucine	15%	10%
Lysine	11%	9%
Methionine	3%	2%
Phenylalanine	3%	3%
Proline	4%	6%
Serine	3%	5%
Threonine	5%	7%
Tryptophan	2%	2%
Tyrosine	4%	3%
Valine	6%	6%

BLG, β -lactoglobulin; WPI, whey protein isolate.

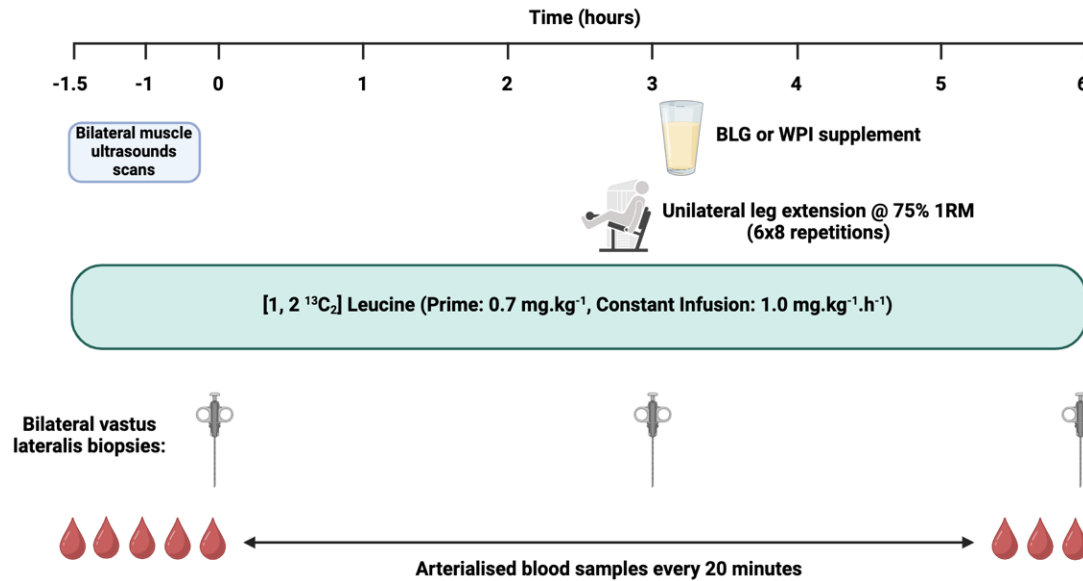


Figure 2.3 Schematic diagram of the acute study protocol. Bilateral ultrasound scans were taken of the vastus lateralis to assess muscle cross-sectional area and architecture. Intravenous infusion of [1,2 ¹³C₂] Leucine over 7.5 hours allowed for assessment of muscle protein synthesis. Arterialised blood was sampled every ~20 minutes. Bilateral muscle biopsies of vastus lateralis occurred at 0, 3 and 6 hours to assess steady state, postabsorptive and feeding/feeding plus exercise MPS responses respectively. Approximately 20 minutes before the second set of biopsies, participants performed a bout of unilateral resistance exercise (leg extension; 6×8 repetitions; 75% 1 repetition maximum (1RM)) with the protein feeds (Studies 1 and 2, 10 g: β-lactoglobulin (BLG) or whey protein isolate (WPI); Study 3, 5 g: BLG or 10 g: BLG) ingested immediately after the second set of biopsies.

2.4. Sample analysis

2.4.1. Plasma insulin and glucose-dependent insulinotropic polypeptide concentrations

Plasma insulin concentrations were measured using a high-sensitivity human insulin enzyme-linked immunosorbent (ELISA) assay (Mercodia AB, Uppsala, Sweden). Plasma GIP concentrations were measured using a human total GIP ELISA (EMD Millipore, Missouri, USA).

2.4.2. Plasma amino acid concentrations

For plasma AA analyses, samples were prepared as previously described by Wilkinson et al., (2018a). Briefly, 10 µL of a mix of stable isotopically labelled internal standards were added to 100 µL of plasma, treated with urease and then deproteinised with 0.5 mL ice-cold ethanol at -20°C for 20 minutes. Following centrifugation (17,000 g for 5 minutes at 4°C), the supernatant was decanted and evaporated under nitrogen to dryness. Following resuspension in 0.5 M HCL, lipids were extracted with 2 mL of ethyl acetate before the lower HCL phase was evaporated and AA were derivatised to their t-butyldimethylsilyl (tBDMS) esters. AA concentrations were quantified against a standard curve of known concentrations using gas chromatography/mass spectrometry (GC/MS; Triple quadrupole MS, TSQ 8000, Trace 1310, Thermo Scientific, Hemel Hempstead, UK). Notably, the EAA were defined as valine, LEU, isoleucine, methionine, threonine, and phenylalanine due to technical difficulties quantifying the other EAA reliably. The NEAA were defined as alanine, glycine, proline, serine,

aspartate, glutamic acid, and tyrosine due to technical difficulties quantifying the other NEAA reliably.

2.4.3. Plasma keto acid enrichments

For keto acid (KA) analysis, 10 μ L of a mix of stable isotopically labelled internal standards were added to 100 μ L of plasma, treated with urease and then deproteinised with 0.5 mL ice cold ethanol at -20°C for 20 minutes. Following centrifugation (17,000 g for 5 minutes at 4°C), the supernatant was decanted and evaporated under nitrogen to dryness. Samples were first derivatised using a quinoxaliny derivative (0.15% OPD in 4 M HCL) and incubated at 90°C for 60 minutes. KA were then extracted with 2 mL of ethyl acetate, with the layers separated and the upper evaporated to dryness before KA being derivatised to their tBDMS esters. α -ketoisocaproate (KIC) enrichments were quantified against a standard curve of known concentrations using GC-MS (Single quadrupole MS, Trace 1300, Thermo Scientific, Hemel Hempstead, UK).

2.4.4. Measurement of muscle protein synthesis

Myofibrillar proteins were isolated, hydrolysed and derivatised using our standard techniques (Wilkinson et al., 2013; Wilkinson et al., 2018a). Briefly, 20–30 mg of muscle biopsy tissue was homogenized in ice-cold homogenization buffer (50 mM TrisHCL (pH 7.4), 50 mM NaF, 10 mM β -glycerophosphate disodium salt, 1 mM EDTA, 1 mM EGTA, 1 mM activated Na_3VO_4 (all Sigma-Aldrich, Poole, UK)) and a complete protease inhibitor cocktail tablet (Roche,

West Sussex, UK) at 10 $\mu\text{L}.\mu\text{g}^{-1}$ of tissue. Following centrifugation at 13,000 g for 15 minutes at 4°C, the resulting insoluble pellet was washed three times with homogenization buffer to remove excess free AA and solubilised in 0.3 M NaOH to aid separation of the soluble myofibrillar fraction from the insoluble collagen fraction by subsequent centrifugation. The soluble myofibrillar fraction was then precipitated using 1 M PCA, pelleted by centrifugation and washed twice with 70% ethanol. The protein-bound AA become released by acid hydrolysis using 1 mL 0.1 M HCL and 1 mL of Dowex ion-exchange resin (50W-X8-200) overnight at 110°C. The free AA were purified, derivatised and the FSR of the myofibrillar proteins was calculated using the precursor-product equation below:

$$\text{FSR (\%/h)} = \left[\frac{\Delta E_m}{E_p \cdot t} \right] \times 100$$

where ΔE_m is the change in enrichment of bound [1,2 $^{13}\text{C}_2$] LEU in two sequential biopsies, t is the time interval between two biopsies in hours, and E_p is the mean free [1,2 $^{13}\text{C}_2$] LEU enrichment in the intramuscular pool.

2.4.5. Immunoblotting for mTORC1 signalling pathway

Immunoblotting was performed using the sarcoplasmic fraction isolated from the MPS preparation. Sarcoplasmic protein concentrations were quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, E-US) and sample concentrations adjusted to 1 $\mu\text{L}.\mu\text{g}^{-1}$ in 3 ×

Laemmli buffer to ensure equivalent protein loading onto pre-cast 12% Bis-Tris Criterion XT gels (BioRad, Hemel Hempstead, UK) of 10 µg/lane. Samples were separated via electrophoresis at 200 V for ~1 hour, followed by wet transfer of proteins to PVDF membrane at 100 V for 45 minutes and subsequent blocking in 2.5% non-fat milk in 1 × Tris-buffered saline/Tween 20 (TBST) for 1 hour. Membranes were incubated in primary antibodies (1:2000 dilution in 2.5% bovine serum albumin (BSA) in 1 × TBST) overnight at 4°C; p-eEF2^{T56}, p-p70S6K1^{T389}, p-RPS6^{S240/244} and p-4EBP1^{T37/46} (Cell Signaling Technology Inc, MA, United States). Membranes were then subsequently washed and incubated in HRP conjugated secondary antibody (Cell Signaling Technology Inc, MA, United States; 1:2000 in 2.5% non-fat milk in 1 × TBST) at ambient temperature for 1 hour, before being exposed to chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA-US) for 5 minutes and bands quantified by ChemidocTM XRS⁺ (BioRad, molecular imager, Hertfordshire, UK). All signals were within the linear range of detection, and loading anomalies were corrected to Coomassie (Welinder & Ekblad, 2011). Blotting data were captured using peak density analysis (BioRad, Image Analysis, Hertfordshire, UK).

2.4.6. Statistical analysis

Data was initially inspected for outliers using the ROUT method with Q set to 1% and checked for normal distribution using a Shapiro-Wilk test.

To offer a comparison of participant characteristics between young and older adult males from sresearch study 1 and 2, VL CSA, VL thickness, knee extensor muscle strength and dietary protein intake were assessed using unpaired t-tests.

Myofibrillar MPS, plasma insulin and GIP concentrations and plasma KIC (studies 1 and 3) enrichments were analysed using a two-way repeated measures ANOVA (supplement $\times$ time) with multiple comparisons analysis using Sidak's correction.

Plasma AA concentrations and KIC (Study 2) enrichments were analysed via mixed-effects analysis (supplement $\times$ time) with multiple comparisons analysis using Sidak's correction. Integrated area under the curve (iAUC) analysis was determined for plasma EAA, BCAA and LEU concentrations and analysed via Student's paired t-test (research study 1 and 2) and unpaired t-test (research study 3).

Immunoblotting data was log-transformed for all studies due to variability in the data and to reduce the skewness of the dataset. Study 1 immunoblotting data was analysed via two-way repeated measures ANOVA with multiple comparisons analysis using Sidak's correction. Research Study 2 and 3 immunoblotting data was analysed via two-way repeated measures ANOVA for targets p-p70S6K1 and p-4EBP1 (with Sidak's correction for multiple comparisons) and Friedman's test for targets p-eEF2 and p-RPS6 (with Dunn's correction for multiple comparisons) due to not meeting normality.

All data analyses were performed in GraphPad Prism (GraphPad Software Inc, San Diego, CA). The alpha level of significance was set at $p < 0.05$. Data is presented as mean $\pm$ SEM unless stated otherwise.

Chapter 3

3.A focus on leucine in the nutritional regulation of human skeletal muscle metabolism in ageing, exercise and unloading states

Throughout the introduction of this thesis, we set the scene as to addressing the importance of SKM health throughout the lifespan and the crucial role dietary protein and exercise play in maximising muscle mass and function as we age. As alluded to, LEU is thought to be the key and most potent AA for stimulating MPS; both in isolation (i.e., free-LEU supplementation) and within protein sources (i.e., WP and/or EAA). We, therefore, performed this narrative review to address the importance of LEU supplementation on SKM metabolism in differing physiological states, in this instance ageing, exercise, and unloading states. We note that this piece of research was performed as a narrative, not systematic, review and is a published article in Clinical Nutrition (<https://doi.org/10.1016/j.clnu.2023.08.010>).

3.1. Background

Occupying ~45–55% of total body mass in healthy adults, SKM health and function across the lifespan is of vital importance for maintaining locomotion and whole-body metabolic health (Wolfe, 2006; McLeod et al., 2016; Deane et

al., 2020b). The maintenance of SKM mass, function and quality is fundamental in dictating healthy ageing and aiding recovery following periods of inactivity/disuse, whereas maximising muscle mass (i.e., hypertrophy) is a common goal for strength/power-based athletes and exercise aficionados (Zaromskyte et al., 2021).

SKM mass is regulated by the dynamic relationship between MPS and MPB. In the postabsorptive state, net MPB exceeds MPS whilst net MPS exceeds MPB in the postprandial state; therefore, achieving net protein balance across diurnal fasted-fed cycles (Burd et al., 2009; Atherton & Smith, 2012). Following a protein feed, MPS increases ~2–3 fold, peaking between ~1.5–2 hours post-feed whilst MPB is transiently inhibited by ~50% in young healthy muscle, though this effect (i.e., increases in MPS/inhibition of MPB) is significantly blunted in older muscle (Volpi et al., 2000; Wilkes et al., 2009). Indeed, both MPS and MPB are responsive to dietary protein feeding, both alone and when combined with various modalities of exercise (i.e., resistance, endurance, and concurrent exercise) (Burd et al., 2009; Atherton & Smith, 2012), but also to periods of inactivity and disease (de Boer et al., 2007; Glover et al., 2008; Wall et al., 2013b; Wall et al., 2016).

The muscle anabolic response to an ingested protein source is dictated by the bio-availability of AA, splanchnic extraction, transport to and uptake of AA by SKM, and subsequent activation of intracellular signalling pathways (e.g., mTORC1-signalling pathway) which upregulates MPS (Gorissen et al., 2015). The muscle anabolic potential of a protein source may therefore be dependent

upon the AA composition of the protein source, the protein digestibility, and possibly the AA absorption kinetics (Zaromskyte et al., 2021; Atherton et al., 2016). Postprandial increases in MPS are dependent on the EAA rather than the NEAA composition of a protein meal (Smith et al., 1998; Tipton et al., 1999; Volpi et al., 2003), with the EAA and BCAA, LEU, thought to be the most potent for stimulating the intracellular signalling cascade that upregulates the mRNA translational initiation process driving nascent MPS (Anthony et al., 2001; Suryawan et al., 2008; Atherton et al., 2010b). As demonstrated *in vitro*, LEU increases phosphorylation of the downstream mTORC1 targets p70S6K1 and RPS6, approximately 3-fold more than all other EAA (Atherton et al., 2010b). However, similar increases in MPS were evident *in vivo* following intravenous flooding of EAA phenylalanine and threonine (Smith et al., 1998), compared to when flooding with LEU (Smith et al., 1992). Despite this, low doses of LEU-enriched protein and/or EAA have been shown to elicit increases in MPS that are comparable to large doses of WP. For example, in older women, 1.5 g of leucine-enriched essential amino acids (LEAA; 0.6 g LEU) demonstrated comparable increases in MPS with 6 g LEAA (2.4 g LEU) and 40 g WP following feeding alone (albeit returning to baseline 2 hours post-feed), which was prolonged when in conjunction with exercise (Wilkinson et al., 2018a). However, comparable increases in MPS elicited by LEU-enriched protein/EAA sources compared to larger WP doses, have often only been measured over 90 minutes with feeding alone, during which time other EAA concentrations begin to fall in muscle, indicating that repeated or long term dosing of LEU alone in isolation is unlikely to promote muscle anabolism, as the other EAA become rate limiting for MPS (Smith et al., 1998). Despite this, supplementing with

LEU-enriched protein/EAA has been of increasing interest to scientists, clinicians, nutritionists, and dieticians to maximise muscle and whole-body protein anabolism in health, exercise, and disease; especially in those who have a suboptimal dietary protein intake.

This review, therefore, focuses primarily upon the anabolic properties of LEU, both in isolation and in enriched protein sources (i.e., EAA and/or protein sources), in the context of i) ageing, ii) exercise (both in acute and chronic interventions) and iii) unloading states. We address what is known about LEU supplementation with respect to acute and integrated measures of MPS, increased or maintained SKM mass in conjunction with exercise or inactivity, respectively, and whether any functional gains are evident with LEU supplementation, in addition to addressing what still needs to be established to maximise muscle protein anabolism in these conditions.

3.2. Fundamental response of skeletal muscle to protein ingestion

The anabolic effects of protein feeding are ultimately driven by the delivery and incorporation of AA from dietary protein sources into SKM proteins. Importantly, SKM exhibits temporal regulation to protein feeding due to being responsive to the anabolic effects of AA for only a short period of time – meaning SKM hypertrophy cannot occur from protein feeding alone (Atherton & Smith, 2012). The kinetics of the MPS response to dietary protein (specifically the EAA components of protein (Smith et al., 1992)) intake have

been well defined and are impacted by dietary protein quantity, the protein source/quality (i.e., whey vs. casein) (Phillips, 2016b; Morgan et al., 2021), and subsequent digestion rates (i.e., slow vs. fast) (Burd et al., 2012; Pennings et al., 2011a; Yang et al., 2012) and also protein-exercise interactions (Biolo et al., 1995; Phillips et al., 1997).

Following oral protein feeding, an initial lag of ~45–60 minutes occurs prior to increases in MPS due to oesophageal transit and digestion and absorption across the splanchnic bed, before arrival at the target tissue (i.e., SKM) and activation of intracellular anabolic signalling pathways (Silk, 1980; Koopman et al., 2009a; Koopman et al., 2009b). MPS then increases ~2–3-fold, peaking between ~1.5–2 hours post-feed, prior to returning to baseline (Atherton et al., 2010a). Thereafter, despite continued EAA availability and elevated intracellular anabolic signalling, MPS exhibits tachyphylaxis, whereby excess EAA are directed towards oxidation, intermediary metabolism and eventually to gluconeogenesis and lipogenesis – via recycling of the carbon skeletons through the tricarboxylic acid cycle – rather than incorporation into the muscle (or whole-body) protein pool. This phenomenon, known as muscle full (Bohe et al., 2001; Bohe et al., 2003; Atherton et al., 2010a; Atherton & Smith, 2012), is based on the “bag full” hypothesis developed by Joe Millward, whereby muscle protein accretion was thought to be physically limited by the extracellular matrix surrounding each muscle fibre (Millward et al., 1994). Although, now knowingly, muscle collagen synthesis is unresponsive to feeding but is increased in response to exercise (Babraj et al., 2005; Miller et al., 2005). A refractory period must, therefore, exist before further stimulation in MPS is achieved.

Although the duration of this period is as yet definitively unknown, it is thought to be ~3–4 hours, with Witard and colleagues exhibiting maximal MPS following provision of a high-protein breakfast (0.54 g/kg) consumed ~3 hours prior to consumption of a WP (Witard et al., 2014). Importantly, the muscle full state is unable to be overcome by the intake of additional nutrient signals/substrates regulating MPS (Mitchell et al., 2017). This extends to muscle full being refractory to both pharmacological and nutraceutical interventions commonly utilised to enhance nutritive muscle blood flow such as methacholine (Phillips et al., 2014) and cocoa flavanols (Phillips et al., 2016a). Nonetheless, the onset of muscle full can be delayed by RE due to the increased anabolic sensitivity of SKM to dietary AA (Atherton & Smith, 2012). Conversely, both ageing and physical inactivity are associated with desensitisation to anabolic stimuli (i.e., exercise and nutrition) resulting in a premature onset of muscle full (Cuthbertson et al., 2005) characterised by a blunted postprandial MPS (i.e., anabolic resistance) (Breen et al., 2013; Wall et al., 2013b), prompting insulin resistance (Dirks et al., 2016) and muscle atrophy (Kilroe et al., 2020; Wall et al., 2013a).

Whilst MPS is stimulated, the anti-catabolic effects of feeding on SKM also occur due to the reflex actions of insulin, leading to MPB being transiently inhibited by ~50% in young muscle, though this effect is significantly blunted in older muscle (Greenhaff et al., 2008; Wilkes et al., 2009). It should be noted, previous work suggested when sufficient intracellular EAA are present, insulin serves to stimulate MPS, but when intracellular EAA fall, insulin serves to mitigate MPB (Wolfe, 2000); thus, insulin is permissively dependent on

intracellular EAA. This is also acknowledged in a systematic review and meta-analysis by Abdulla and colleagues, who concluded insulin to have an apparent permissive role on MPS when there is sufficient AA availability, but plays a clear and primary role in aiding muscle anabolism through the suppression of MPB, regardless of AA availability (Abdulla et al., 2016). Conversely, early work using the AV balance technique across the arm, demonstrated during hyperinsulinaemia, net protein balance became positive which was ultimately driven by the markedly suppressed rate of appearance of AA, with no change in the muscle disposal of AA (i.e., rate of disappearance) (Gelfand & Barrett, 1987). Even though increases in insulin concentrations to that seen following a mixed meal are evident following provision of protein alone (i.e., without carbohydrate) (Atherton et al., 2010a), it is not insulin *per se* that contributes to anabolic effects of EAA on MPS. This was elegantly demonstrated by Greenhaff and colleagues who, when “clamping” insulin at postabsorptive concentrations (5 uIU/mL) whilst infusing EAA (18 g/h over 3 hours), still observed robust MPS stimulation (Greenhaff et al., 2008). Despite this, insulin still plays a role postprandial muscle anabolism, with increases in insulin concentration to 30 uIU/mL inhibiting leg protein breakdown by ~50% in young individuals, with no further increases seen at higher insulin concentrations (Greenhaff et al., 2008). Further, it was demonstrated at 15 uIU/mL (3 × postabsorptive concentrations) leg protein breakdown was reduced by 47% in young individuals but only 12% in older individuals (Wilkes et al., 2009); highlighting a clear age-related difference in the anti-catabolic role of insulin. Resultingly, it is the EAA that regulate anabolic responses via large increases in MPS, whilst insulin regulates SKM anti-catabolic responses to protein feeding (Atherton &

Smith, 2012). As increases in MPS are far greater than MPB, it is MPS that is the driving force behind nutrient induced muscle anabolism.

3.2.1. The role of leucine in muscle anabolism

Unlike other EAA, LEU can independently activate mTORC1 and downstream anabolic signalling (Anthony et al., 2000a), in addition to the more obvious role as a substrate for newly synthesised proteins. Although knowledge of upstream regulation of the mTORC1 pathway is incomplete, it is now known that LEU binds to an intracellular “LEU sensor” of the mTORC1 pathway, Sestrin-2, leading to subsequent dissociation of Sestrin-2 and GTPase-activating protein (GATOR2) which activates the mTORC1 pathway (Wolfson et al., 2016). Further, it is thought LRS plays a critical role in AA-induced mTORC1 activation by sensing intracellular LEU concentrations and initiating molecular signalling events leading to mTORC1 activation (Han et al., 2012). *In vitro*, LRS has been shown to directly bind to Rag GTPase (the mediator of AA signalling to mTORC1) in an AA-dependent manner and functions as a GTPase-activating protein for Rag GTPase to activate mTORC1, with the mTORC1 pathway rendered insensitive to intracellular levels of AA when a mutation in LRS arose (Han et al., 2012); thus highlighting the critical role of LRS in addition to Sestrin-2. Activation of the mTORC1 signalling pathways leads to a cascade of signals resulting in phosphorylation of 4E-binding protein (4E-BP1), ribosomal protein S6 kinase (p70S6K1) and ribosomal protein S6 (RPS6), stimulating binding of eukaryotic initiation factor (eIF) 4A and eIF4E to eIF4G forming the eIF4E complex (Anthony et al., 2001). Subsequently, eIF4E mediates mRNA

binding to the 43S pre-initiation complex, resulting in upregulation in rates of mRNA translation and, therefore MPS (Anthony et al., 2001).

3.3. Protein quantity and muscle protein synthesis

Often, dietary protein recommendations are expressed per day, and are set as the lowest level of protein intake to prevent protein inadequacies in 97.5% of healthy adults at energy balance (Phillips et al., 2020). Currently, the RDA is 0.8 g protein/kg body weight/d for all adults (World Health Organization, 2007; EFSA, 2012), although this has been under increasing contention. For example, the PROT-AGE study group have recommended healthy older adults to be consuming greater quantities of protein in the regions of 1.0–1.2 g/kg/d in order to maintain and regain lean body mass and function and those who are engaged in exercise (endurance or resistance) or otherwise active should be consuming ≥ 1.2 g/kg/d protein (Bauer et al., 2013; Deutz et al., 2014). Further, older adults who present with acute or chronic disease (except those with severe kidney disease) need even more dietary protein in the region of 1.2–1.5 g/kg/d (Bauer et al., 2013; Deutz et al., 2014). More recently, due to the saturable dose-response relationship (and the presence of muscle full) between the quantity of protein ingested and the MPS response, there has been a growing appreciation for the need to consider protein intake on a per-meal basis (Murphy et al., 2016a).

It has been previously demonstrated that a dose response relationship in MPS presents with increasing dietary protein intake until an “optimal” protein dose is established, whereby there are no further increases in MPS despite increasing the AA availability (Cuthbertson et al., 2005, Moore et al., 2009a; Moore et al., 2015; Atherton et al., 2010a). In healthy young individuals, it has been demonstrated that ~20 g of intact protein (or 0.24 g/kg) (Moore et al., 2009a; Moore et al., 2015) or ~10 g EAA (equivalent to 20g protein) (Cuthbertson et al., 2005) is sufficient to maximally stimulate MPS. Similarly, in young males, ingesting 20 g WP led to greater (and maximal) stimulation of MPS compared to 10 g WP, with 40 g WP failing to increase MPS further but instead elicited a marked increase in the rate of indicators of whole-body AA catabolism, such as AA oxidation and ureagenesis (Witard et al., 2014). Despite this, the optimal protein dose to achieve similar levels of MPS stimulation is much greater (~40 g protein or ~20 g EAA) in older compared to younger individuals (Moore et al., 2015), due to the presence of anabolic resistance. To compare WP intake with whole-food sources, a moderately sized protein meal (e.g., 113 g lean beef providing 30 g protein and ~10 g EAA) was equally effective as a large-sized protein meal (340 g lean beef providing 90 g protein and 30 g EAA) for the stimulation of mixed MPS (Symons et al., 2009).

Interestingly, despite knowledge of a dose-response to dietary protein, protein intake across the day is often reportedly of skewed pattern (see Murphy et al., 2016a) for review on per-meal protein recommendations in ageing) with (e.g.) older adults tending to consume ~40–50% of daily protein intake in the evening meal with smaller quantities consumed at breakfast (~15%, 10–15 g protein) and

lunch (~28%, ~16–22 g protein) (Tieland et al., 2012; Almoosawi et al., 2013; Smeuninx et al., 2020). Subsequently, this would translate to suboptimal protein intakes being achieved at each meal in the older adult population; thus maximal MPS may not be achieved (Murphy et al., 2016a). A dose-response approach for protein intake *per meal* to maximise MPS across the day should potentially be considered due to the saturation in the MPS response to maximise muscle anabolism to protein feeding at rest, following exercise but also in ageing and diseased states. For example, in young adults, providing ~30 g protein at breakfast, lunch and dinner led to ~25% greater mixed-MPS measured over a 24 hour period, compared to when protein was consumed in a skewed manner (~10 g, ~15 g and ~ 65 g at breakfast, lunch and dinner, respectively) (Mamerow et al., 2014). Similarly, 4 × 20 g WP doses (total of 80 g protein) spread evenly throughout the day increased 12 hour myofibrillar MPS and whole-body net protein balance to a greater extent than consuming 2 × 40 g doses following resistance exercise (Areta et al., 2013; Moore et al., 2012). Conversely, adopting a “grazing” approach to protein feeding (i.e., 8 × 10 g protein every 1.5 hours), compared to ingesting three meals, demonstrated greater AA oxidation which is likely undesirable when the main aim is to incorporate ingested AA into SKM (El-Khoury et al., 1995). Therefore, it seems, in younger individuals, the optimal approach to protein consumption is consuming a balanced distribution of protein (~20 g per meal) to have the most anabolic response to protein feeding across the day at rest and following exercise (Murphy et al., 2016a).

Despite the above demonstrating the presence of a dose response to protein intake, it is becoming increasingly clear that protein or AA dose response studies

believe the importance of the presence of highly anabolic EAA. For example, LEU is a major driver of MPS to the extent that enriching lower and suboptimal doses of protein/EAA with LEU robustly stimulates MPS compared to larger (20–40 g) dose of WP (Churchward-Venne et al., 2012; Bukhari et al., 2015; Atherton et al., 2017), in addition to just ~3 g isolated LEU in absence of other exogenous EAA achieving maximal MPS stimulation (Smith et al., 1992, Wilkinson et al., 2013). For example, we have demonstrated that LEAA stimulates MPS in older women to a similar extent as a larger (20 g) bolus of WP despite the latter providing more than 3-fold the amount of EAA/kg of LBM (Bukhari et al., 2015). Again, this is somewhat supported by the fact both WP and LEAA achieved the same leucinaemia peak, despite WP having a great total EAA/AA content and achieving a prolonged rise of leucinaemia, no more of a trophic advantage was observed in MPS despite this (Bukhari et al., 2015). Therefore, the ability to enrich suboptimal protein/EAA with LEU indeed seems to challenge the presence of a dose response relationship to protein feeding.

3.4. Protein source/quality and muscle protein synthesis

Although, in young individuals, the optimal protein intake to maximise MPS seems to be at ~20 g or 0.25 to 0.3 g/kg/body mass of protein; the MPS response can vary substantially between the type or source of dietary protein (Gorissen et al., 2018). Many studies have assessed the postprandial MPS response to ingestion of dairy, meat, and plant-based proteins with increasing interest in maximising MPS from non-animal sources due to increased sustainability and

lower production costs (Gorissen et al., 2018). It is outside the scope of this review to discuss in detail the differences between animal, plant, whole-foods and protein isolates and readers should be directed to reviews by Gorissen et al., (2018) and Deane et al., (2020a). However, we believe it important to briefly discuss differences in protein sources on MPS especially with regards to the LEU content of different protein sources.

Typically, animal derived proteins have given greater robust postprandial increases in MPS rates being associated with the rapid rise in plasma EAA concentrations, and leucinaemia in particular, compared to plant-based proteins (Gorissen et al., 2018). This has been attributed to a lower EAA content, such as LEU, lysine and/or methionine within the majority of plant-based proteins (World Health Organization, 2007; Young & Pellet, 1994; van Vliet et al., 2015). Despite this, several studies examining the composition of whole proteins have found that the type of protein, and not just the AA composition, can differentially modulate the SKM anabolic response (Tang et al., 2009). For example, milk has been suggested to better whole body nitrogen retention at rest (Bos et al., 2003) and greater SKM protein accretion after RE compared with soy protein (Wilkinson et al., 2007). Following the ingestion of whey, casein and soy, greater blood concentrations of EAA, BCAA and LEU was observed following WP ingestion than either soy protein or casein (Tang et al., 2009). At rest, MPS was greatest following WP and soy protein ingestion, with WP demonstrating to be the most anabolic following exercise and achieving significantly greater leucinaemia compared to soy protein despite all supplements containing ~10 g EAA (~2 g LEU) (Tang et al., 2009). Similarly, WP stimulated MPS to greater

extents at rest and following exercise in older adults compared to casein (Burd et al., 2012). In older males, when comparing different doses (20 g vs. 40 g) of WP and soy protein, MPS was greater following 20 g WP compared to 20 g soy protein with no differences in MPS seen between 0 g protein and 20 g soy protein at rest and following exercise (Yang et al., 2012). Again, 40 g WP gave a greater anabolic response compared to 40 g soy protein post-exercise, despite plasma LEU concentrations being elevated to levels normally associated with maximal stimulation of MPS (Yang et al., 2012). More recently, despite the same quantities of LEU (2.4 g), 30 g milk protein induced greater leucinaemia compared to 30 g plant-blend (containing 15 g wheat, 7.5 g corn and 7.5 g pea protein), MPS increased to similar levels with no differences between the supplements (Pinckaers et al., 2022), demonstrating protein sources may be manipulated (i.e., in a blend) to improve the quality of the protein in an attempt to maximise muscle anabolism. Conclusively, it seems higher quality dietary protein (i.e., having a greater EAA/LEU content and greater digestibility) elevates postprandial (from feeding alone and RE-induced) MPS to a greater extent than lower-quality dose matched protein (Morgan et al., 2021).

Interestingly, the majority of studies investigating protein nutrition on the stimulation of postprandial MPS have focused on isolated protein sources (i.e., WP, soy protein etc); despite whole-foods being the more commonly consumed protein source (Burd et al., 2019a). The study of these isolated protein sources (i.e., whey, casein, soy protein) has led to the LEU “trigger” hypothesis being developed. This hypothesis suggests that a rapid rise (~60–90 minutes) in blood LEU concentrations following LEU-rich protein ingestion is likely to be the

most anabolic for stimulating MPS (Burd et al., 2019a). However, this hypothesis remains under contention with low-dose LEAA ingestion demonstrating maximal MPS despite no significant increases in plasma LEU concentrations (Wilkinson et al., 2018a), in addition to other EAA ability to stimulate MPS (Smith et al., 1998). Furthermore, as whole foods are also a potent stimulator of post-exercise MPS, despite not facilitating rapid increases in leucinaemia (Burd et al., 2015), the LEU trigger hypothesis is arguably more applicable to isolated protein sources (Burd et al., 2019a). Despite this, whole foods may be altered (i.e., texture) to improve palatability and enhance digestibility leading to a greater postprandial protein retention (Pennings et al., 2013). For example, in older adults, consuming 135 g beef (~26 g protein, ~2 g LEU) provided as beef mince rather than beef steak, resulted in more a rapid digestion and absorption of the protein, thus increasing AA availability to a greater extent and demonstrated significantly greater positive whole-body protein balance (Pennings et al., 2013); although the effects of feeding on MPB also must be considered when assessing protein balance as it is not just increases in MPS that drive a positive protein balance.

Due to the ability LEU has to directly activate MPS through stimulating mTORC1 and downstream anabolic signalling (Atherton et al., 2010b), the LEU content of ingested protein sources is a key characteristic that modulates activation of MPS following protein feeding (Gorissen et al., 2018). As demonstrated by Gorissen et al. in a study comparing several plant-based proteins to their animal constituents and human muscle, several plant-based proteins fell short of meeting the adult AA requirements for LEU, being 5.9 %

of total protein, outlined by WHO/FAO/UNU (Gorissen et al., 2018; World Health Organization, 2007). Despite this, some plant-based protein sources such as potato and corn had greater LEU contents (8.3% and 13.5% LEU respectively) than casein (8.0%), egg (7.0%) and even WP (11.0%) (Gorissen et al., 2018). It could be argued that plant-based proteins could also provide the same amount of LEU as ~20 g WP, by increasing the dose of the plant protein to achieve this (Gorissen et al., 2018). For example, when increasing the dose of ingested wheat protein from 35 g to 60 g to match the LEU content of 35 g WP (4.4 g LEU), MPS rates were significantly increased above basal rates (Gorissen et al., 2016). It should be noted that substantially increasing the protein dose from plant-based proteins may not be feasible in all populations, especially those with an easily satiable appetite (i.e., older adults). In addition plant-based proteins often have lower true-digestibility values (Moughan & Wolfe, 2019). Instead of simply increasing the amount of protein consumed, protein blends may be a way to achieve sufficient quantities of EAA and LEU (Gorissen et al., 2018; Deane et al., 2020a). For example, in young males, ~19 g protein, consisting of sodium caseinate (50%), whey (25%) and soy (25%) protein, elevated aminoacidaemia, mTORC1 signalling and MPS (Reidy et al., 2013), in addition to increasing muscle AA transporter expression to a similar extent as WP (Reidy et al., 2014); although more research should be directed into investigating plant-based protein blends.

3.5. Ageing and anabolic resistance to nutrition

Sarcopenia, the age-related decline in muscle mass and function (Rosenburg, 1997), is a progressive, multi-faceted condition that occurs due to adverse muscle changes (i.e., myofibrillar protein and muscle fibre losses (Wilkinson et al., 2018b)) throughout the lifespan, and leads to an undisputed poor prognosis in both physiological and pathological situations (Cruz-Jentoft et al., 2010; Cruz-Jentoft et al., 2019). In sedentary individuals aged 50 years and over, muscle is lost at a rate of ~1 – 2% per year, with this rate of muscle mass and strength loss being impacted by several environmental influences such as illness/disease, dietary protein intake and exercise/activity status (Mitchell et al., 2012). Ultimately, the progression of sarcopenia with advancing age contributes to a severe deterioration in an individual's quality of life with increased association of morbidity and mortality (De Bandt, 2016). Preservation and restoration of muscle mass in combination with prevention of muscle wasting (i.e., through utilising nutritional and exercise strategies) are, therefore, major health goals.

One of the many metabolic features contributing to sarcopenia, is muscle “anabolic resistance”. First demonstrated by Volpi and colleagues who identified rates of MPS to increase in young but not older adults in response to intravenous infusions of AA (Volpi et al., 2000), anabolic resistance centres upon the blunting of increases in MPS and/or a suppression in the ability to inhibit MPB to key anabolic drivers, namely muscle contractile activity (i.e., exercise) and nutrition (Moore et al., 2015; Wall et al., 2015). Similar to Volpi et al., Cuthbertson and colleagues demonstrated suppressed rates of MPS in response to 5 g of EAA in older compared to young individuals, with even a large bolus

of EAA (40 g) failing to elicit a similar maximal muscle anabolic response in older adults to that observed at 10 g EAA in young adults (Cuthbertson et al., 2005). In addition, a cross-sectional retrospective study highlighted older adults to have depressed postprandial MPS rates, by ~16%, compared to younger counterparts (Wall et al., 2015). Further, a greater relative protein requirement has been identified in older adults (~0.4 g/kg), to achieve a plateau of myofibrillar fractional synthetic rate (FSR), compared to young (~0.24 g/kg) men (Moore et al., 2015); meaning an 80 kg older individual would have to consume 32 g (vs. ~20 g for a younger individual of the same body mass) protein per-meal. Compared to the age-related deficits in MPS in response to protein feeding, it is largely accepted that postabsorptive rates of MPS and MPB are unchanged with ageing (Kumar et al., 2009; Breen & Phillips, 2011). As with protein feeding, there is evidence of anabolic resistance in the MPS response to exercise in older age. For example, MPS responses were blunted in older individuals 1–2 hours post-RE (20–90% 1RM) (Kumar et al., 2009) with impaired skeletal muscle mTORC1 signalling (a possible mechanism underlying age-related blunting of MPS) which was prolonged up to 24 hours post-exercise (Fry et al., 2010). Further, the presence of anabolic resistance has been highlighted chronically following 6 weeks of unilateral leg resistance exercise training (RET, 75% 1RM), MPS was elevated over 3 weeks only in the young but not older adults, which was supported by the absence of thigh fat-free mass gains in older muscle (Brook et al., 2015). Despite a plethora of evidence (i.e., Volpi et al., 2000; Cuthbertson et al., 2005; Moore et al., 2015; Wall et al., 2015; Breen & Phillips, 2011) suggesting anabolic resistance is likely a major driver of age-related muscle wasting in terms of muscle protein turnover

regulation, it is acknowledged not all studies are aligned (Symons et al., 2009; Chevalier et al., 2011).

As one of the main barriers for older individuals consuming greater quantities of protein is increased satiety following a meal (Marshall et al., 2020). Hence, rather than attempting to increase the quantity of protein consumed, especially if there is an inability to do so, the *quality* of the protein should aim to be improved instead (Traylor et al., 2018). Protein quality has often been determined by the EAA, specifically LEU, content in the protein due to the unique anabolic and anti-catabolic properties as an insulin secretagogue (Marshall et al., 2020). It therefore follows, as discussed below (and summarised Table 3.1), that utilising LEU, often in conjunction with a suboptimal protein/EAA dose, as a nutritional strategy, and alongside exercise (i.e., RET) may be considered as a strategy to maximise muscle anabolic responses to protein feeding, thus overcoming anabolic resistance in aged muscle.

3.5.1. Acute leucine supplementation to maximise muscle protein synthesis in aged muscle

When first examining LEU supplementation in the absence of exercise, we previously have demonstrated that supplementing with 20 g WP offered no trophic advantage compared to a low-dose LEAA (3 g, 1.2 g LEU) in older women, with both supplements comparably stimulating MPS over 0 – 2 hours in response to feeding, abating thereafter, despite markedly greater plasma

insulinaemia and EAA/aemia following the WP bolus (Bukhari et al., 2015). Moreover, just 1.5 g LEAA (0.6 g LEU) stimulated MPS over 0–2 hours, with no trophic advantage of larger LEAA doses (6 g, 2.4 g LEU), or a large (40 g) bolus of WP in older women following feeding (Wilkinson et al., 2018a). Work from others has also demonstrated the positive impact of enriching EAA and/or low-dose protein with LEU to overcome anabolic resistance in aged muscle. Again, in older women, MPS (measured over 0–4 hours) increased to a greater extent following supplementation of a LEU-enriched protein supplement (15 g milk protein, 4.2 g LEU) compared to a control drink (15 g mixed protein, 1.3 g LEU) in response to feeding ($53 \pm 5 \%$ vs. $13 \pm 4 \%$) (Devries et al., 2018b). Further, whilst MPS (measured over 0–3.5 hours) increased following ingestion of both 6.7 g EAA with LEU content matched to WP (1.7 g LEU) and an enriched LEU content (2.8 g LEU) in young individuals, such increases were only observed following ingestion of LEAA in older adults (Katsanos et al., 2006). When looking at a pulse feeding approach across 5 hours where 15 small meals (~50 mL; 0.4 g protein/kg) were provided every 20 minutes, supplementing with LEU (0.052 g LEU/kg) rather than an alanine control (0.071 g alanine/kg) led to significant increases in muscle FSR ($0.083 \pm 0.008 \%/h$ vs. $0.053 \pm 0.009 \%/h$; LEU vs. alanine respectively) in older men over 5 hours (Rieu et al., 2006).

Adding the effects of acute exercise in addition to acute LEU supplementation, a greater anabolic impact is observed. Enriching a sub-optimal (10 g) protein drink with 4.2 g LEU (vs. 4.2 g alanine) led to greater MPS across 4 hours in both younger and older men following RE, with no age-related difference, and

p70S6K1 phosphorylation elevated to a greater extent following the LEU-enriched protein drink (Atherton et al., 2017). Interestingly, MPS in older participants was still elevated at 4 hours post-exercise, highlighting the potential of combining RE with LEU-enriched supplementation to maximise the anabolic response (Atherton et al., 2017). Additionally, although we demonstrated MPS to be equally stimulated over 0–2 hours following feeding alone across varying WP and low-dose LEAA doses, the addition of exercise allowed MPS to remain elevated over 4 hours post-feeding, thus prolonging the anabolic response (Bukhari et al., 2015). Further, Devries and colleagues evidenced a greater MPS response to feeding plus exercise than feeding alone following supplementation of a LEU-enriched protein supplement (FED: 53 ± 5 %; FED-EX: 87 ± 7 %) compared to a control (FED: 13 ± 4 %; FED-EX: 30 ± 4 %) (Devries et al., 2018b); again, demonstrating the potentiating anabolic effects of exercise on top of feeding thus, potentially negating the impact anabolic resistance.

3.5.2. Chronic leucine supplementation to increase skeletal muscle mass/function in aged muscle

Based on the seemingly comparable acute anabolic effects of lower dose LEU-enriched protein/EAA to larger doses of WP, the question arises as to whether LEU supplementation translates to equal or greater gains/maintenance in SKM mass. When observing a longer duration (6 days) of LEU supplementation (10 g milk protein, 3 g LEU) in older women, integrated MPS (i.e., MPS measured over several days) increased to a similar extent in the exercised leg as with a

larger (25 g) WP isolate dose (Devries et al., 2018a). Although integrated MPS remained unchanged following LEU supplementation in the non-exercised leg (but increased with WP), these results perhaps highlight the importance of both improving protein quality (i.e., increased LEU content) and introducing exercise as an anabolic stimulus to maximise MPS gains in situations where sub-optimal protein quantities are consumed (Devries et al., 2018a). To evidence this, following 6 d of a slightly higher LEU-enriched supplement (15 g milk protein, 4.2 g LEU), integrated MPS significantly increased in both the rested and exercise legs ($9 \pm 1 \%$ and $17 \pm 2 \%$ respectively) compared to the control supplement (15 g mixed protein, 1.3 g LEU) which saw significant increases in the exercised leg only ($7 \pm 2 \%$) (Devries et al., 2018b). Similarly, when co-ingesting LEU (5 g LEU/meal) for 3 days during consumption of a “high” (1.2 g/kg/d) or “low” protein (0.8 g/kg/d) diet, integrated MPS was elevated in both protein diets across rested and exercised conditions compared to a NEAA placebo in older men (Murphy et al., 2016b). In sarcopenic women, 3 months of LEAA supplementation (with/without exercise) demonstrated improvements in walking speed, with muscle mass increasing following supplementation plus exercise and exercise alone, whereas knee extension strength increased in the supplementation plus exercise group only (Kim et al., 2012). Conversely, isolated LEU supplementation (7.5 g/d for 3 months, 2.5 g/meal) did not augment skeletal muscle mass or strength in healthy elderly men, suggesting exercise training (e.g., RET) needs to be performed alongside supplementation to observe SKM mass gains (Verhoeven et al., 2009).

Taken together, data surrounding age-related muscle anabolic resistance tends to point towards protein quality (i.e., LEU/EAA content) being of more importance than protein quantity to maximally stimulate MPS in older adults through increasing the LEU proportion present in a mixture of EAA (Wilkinson et al., 2018a; Bukhari et al., 2015; Katsanos et al., 2006) and/or sub-optimal protein doses (Atherton et al., 2017), but not necessarily through chronically supplementing LEU alone (Verhoeven et al., 2009); thereby, demonstrating the importance of the other EAA (and exercise) in promoting MPS chronically. Prolonged stimulatory benefit with low doses of LEU-enriched protein/EAA has been observed post-exercise, suggesting combining nutritional and exercise interventions should be adopted to optimally prolong muscle anabolism, especially in older adults. Based on the evidence discussed above, we suggest in situations where protein quantity becomes limited and cannot be easily increased due to (e.g.,) illness/digestibility issues or food availability/tolerability, thus rendering inadequate protein consumption, supplementing individuals with LEU-enriched protein/EAA in conjunction with RE (wherever possible) may aid in maximising muscle anabolism to limit the progression of sarcopenia through overcoming anabolic resistance. In contrast, where protein intake is adequate LEU supplementation may not be of benefit to individuals due to maximal MPS likely already being achieved.

Table 3.1 The role of leucine supplementation to overcome anabolic blunting in older adults.

Reference	N M:F	Age	Duration	Route feed	AA composition	Dose	Amount of LEU	RE	Duration of MPS	Impact on MPS/function	Comments
Atherton et al., 2017	36 M	Y (n = 18) O (n = 18)	Acute	Oral (bolus)	Slim-fast Optima (PRO + CHO) + LEU or ALA	10 g PRO + 24 g CHO	4.2 g	Unilateral knee extension	1, 2 and 4 h	↑ MPS	Greater MPS with Slim-fast Optima + LEU in both groups MPS did not return to baseline at 4 h in older group
Bukhari et al., 2015	16 F	O (n=8/group)	Acute	Oral (bolus)	WP LEAA	20 g 3 g	2 g 1.2 g	Unilateral knee extension	2 and 4 h	↑ MPS ↑ MPS	LEAA comparably stimulated MPS to WP at 0 – 2 h after FED and 0 – 4 h after FED-EX despite greater plasma insulinaemia and essential aminoacidaemia following WP ingestion
Katsanos et al., 2006	20M:16F	Y (n = 16, 8M) O (n = 20, 12M)	Acute	Oral (bolus)	EAA + 26% LEU	6.7 g EAA	1.7 g	–	3.5 h	↑ MPS	MPS increased following both supplements in young participants but only following enriched LEU (+41% LEU)
					EAA + 41% LEU	6.7 g EAA	2.8 g			↑ MPS	supplement older participants
Rieu et al., 2006	20 M	O	Acute	Oral (pulse)	PRO (casein) + LEU or ALA	0.4 g PRO/kg every 20 min	0.052 g/kg (~3.6 g)	–	5 h	↑ MPS	Whole body protein kinetics not affected by LEU supplementation. MPS greater post-LEU supplementation than ALA. Bolus feeding may not have demonstrated same

Wilkinson et al., 2018	24 F	O	Acute	Oral (bolus)	WP Low-dose LEAA High-dose LEAA	40 g 1.5 g 6 g	4 g 0.6 g 2.4 g	Unilateral knee extension	2 and 4 h	↑ MPS ↑ MPS ↑ MPS	results due to MPS measured after 5 h Both low- and high-LEAA comparably stimulated MPS following FED and FED-EX to a larger WP dose. Plasma insulinaemia and EAA concentrations were greater and more prolonged with WP, although concentrations following high-LEAA peaked at similar concentrations
Devries et al., 2018	22 F	O (n=11/group)	Chronic (6 d)	Oral (×2/d)	WP Milk PRO + LEU	25 g 10 g	3 g 3 g	Acute unilateral knee extension	4 h	↑ acute MPS ↑ integrated MPS ↑ acute MPS ↑ integrated MPS	Dietary PRO controlled at 1.0 g/kg/d (supplements incorporated into this value). Acute MPS increased in both groups with greater increase in LEU group following FED and FED-EX. Integrated MPS increased in both groups in the exercised leg, but only following WP in the rested leg.

Devries et al., 2018	22 F	O (n=11/group)	Chronic (6 d)	Oral (×2/d)	Milk PRO + LEU Mixed PRO (milk + soy)	15 g 15 g	4.2g 1.3 g	Acute unilateral knee extension	4 h	↑ acute MPS ↑ integrated MPS ↑ acute MPS ↑ integrated MPS	Dietary PRO controlled at 1.0 g/kg/d (supplements incorporated into this value). Acute MPS increased in both groups with greater increase in LEU group following FED and FED-EX. Integrated MPS increased in both groups in the exercised leg, but only following Milk PRO + LEU in the rested leg. MPS not assessed.
Kim et al., 2012	155 F	O (LEAA, n=39) (LEAA+RET, n=38) (RET, n=39) (HE, n=39)	Chronic (3 months)	Oral (×2/d)	LEAA	6 g/d	42%	RET + balance	–	↑ walking speed ↑ muscle mass ↑ muscle strength	Walking speed improved following all interventions, whilst muscle mass increased following LEAA+RET and RET and muscle strength increased following LEAA+RET only
Murphy et al., 2016	20 M	O (n=11/group)	Chronic (3 d)	Oral (×3/d)	High PRO diet + LEU/PLA Low PRO diet + LEU/PLA	1.2 g PRO/kg/d 0.8 g PRO/kg/d	5 g/meal 5 g/meal	Acute unilateral knee extension	3 and 6 d	↑ integrated MPS ↑ integrated MPS	MPS similar across each diet with greater MPS in LEU supplementation (vs PLA) group in both rest and exercised conditions. LEU co-ingestion with daily meals is equally effective in increasing MPS in older men who

Verhoeven et al., 2009	30 M	O (n =15/group)	Chronic (12 wk)	Oral (×3/d)	LEU PLA (wheat flour)	2.5 g/meal 2.5 g/meal (7.5 g/d)	7.5 g/d –	–	–	↔ muscle mass ↔ strength ↔ muscle mass ↔ strength	consume daily PRO intakes greater than or equal to the RDA. MPS not assessed, nor RET conducted. LEU supplementation led to no changes observed in skeletal muscle mass, strength and/or glycaemic control. PRO consumption throughout study ~0.99 g/kg/d

Abbreviations: ALA, alanine; CHO, carbohydrate; EAA, essential amino acid; F, female; FED, feeding; FED-EX, feeding plus exercise; HE, health education; LEAA, leucine-enriched essential amino acids; LEU, leucine; M, male; MPS, muscle protein synthesis; O, old; PLA, placebo; PRO, protein; RDA, recommended daily allowance; RET, resistance exercise training; WP, whey protein; Y, young.

3.6. Protein nutrition and exercise interactions: what we already know

Alongside its role in SKM mass maintenance, the provision of dietary protein is critical for muscle adaption (i.e., maximising muscle hypertrophy) in response to RE. This is exemplified by a greater prolonged elevation in MPB, which induces a negative protein balance, when RE is performed in the absence of protein/EAA nutrition (Biolo et al., 1995; Phillips et al., 1997). Sufficient protein/EAA intake must, therefore, complement exercise to promote positive net protein balance and subsequent muscle hypertrophy via cumulative muscle protein accrual following each bout of RE over time (Atherton & Smith, 2012; Pennings et al., 2011b; Stokes et al., 2018).

RE heightens the anabolic sensitivity of SKM, with both the magnitude (~3-fold) and duration (up to 4 hours) of the MPS response enhanced through increasing the availability of dietary EAA post-exercise (Pennings et al., 2011b). The addition of carbohydrate provision offers no further anabolic effects post-exercise (Staples et al., 2011), highlighting the central role of EAA in maximising anabolic responses in protein turnover to exercise. In some cases, the enhanced MPS response due to protein $\times$ exercise interactions can last up to 24 hours (Burd et al., 2011) and even 48 hours (Miller et al., 2005) following exercise, although the magnitude of this response falls over time. With this in mind (i.e., the delay of muscle full lasting up to 24–48 hours), the timing of protein intake pre-, during- or post-exercise may not be as crucial for muscle

anabolism as first thought; rather the quantity/quality of the protein may be the most fundamental consideration (Atherton & Smith, 2012).

Despite this, the quantity of protein needing to be consumed to maximise muscle anabolism may be dictated by the type of RE conducted. For example, in response to unilateral leg based RE, MPS displays a dose response relationship to dietary protein ingestion until maximal stimulation with ~20 g protein, with larger doses (e.g., 40 g) leading to irreversible oxidation and excess protein amino acid being catabolised (Atherton & Smith, 2012; Witard et al., 2014; Moore et al., 2009a). However, in situations of whole-body RE, protein requirements tend to be greater with 40 g WP shown to stimulate MPS to a greater extent than 20 g in young resistance trained men (Macnaughton et al., 2016). Again, as discussed above, utilising a low-dose protein/EAA supplement, enriched with LEU, rather than a large protein bolus, may provide comparable results in stimulating post-exercise muscle anabolism which may be especially beneficial to those who struggle to consume large quantities of protein (e.g., older adults/those who are clinically vulnerable) (Wilkinson et al., 2018a; Phillips et al., 2020; Bukhari et al., 2015; Marshall et al., 2020).

3.6.1. The role of leucine supplementation in the context of acute resistance exercise

Indeed, the literature has and continues to demonstrate the importance of combining RE and AA supplementation to maximise the MPS response and shown that consuming EAA (Rennie & Tipton, 2000) or LEU-enriched protein/EAA after RE augments the contraction induced increase in MPS

(Dreyer et al., 2008). For example, in young males, Dreyer and colleagues previously demonstrated MPS and mTOR signalling to be enhanced following acute RE plus a LEAA + carbohydrate (0.5 g/kg/LM carbohydrate, 0.35 g/kg/LM EAA, 35% LEU) compared to a control group (Dreyer et al., 2008). Further, supplementing low-dose WP (6.25 g) with added LEU (3 g) or EAA mix (total EAA, except LEU (0.75 g), equal to 25 g WP) with no added LEU increased MPS 1–3 hours post-exercise to similar extents as a 25 g WP (~3 g LEU) dose (Churchward-Venne et al., 2012). MPS only remained elevated 3–5 hours post-exercise following 25 g WP consumption, likely due to EAA availability being rate limiting with the other supplements (Churchward-Venne et al., 2012). However, the same authors later demonstrated MPS to remain elevated 1.5–4.5 hours post-exercise to a greater extent following both 25 g WP (~267%) and high-dose LEU-enriched WP (6.25 g WP, 5 g LEU) (~220%) (Churchward-Venne et al., 2014). However, MPS in this study (Churchward-Venne et al., 2014) was calculated over a different time-period (0–1.5 hours and 1.5–4.5 hours post-feed) compared to the previous study (1–3 hours and 3–5 hours post-feed) (Churchward-Venne et al., 2012). Importantly, it should be highlighted that the timings of SKM biopsies following exercise and nutritional interventions are crucial in determining rates of acute MPS. For example, we demonstrate post-exercise MPS responses temporally following the RE and nutritional supplementation (at 1, 2 and 4 hours), where MPS rates rose quickly and peaked over the 1–2 hours post-exercise before showing a downwards trend at 2–4 hours (Atherton et al., 2017). In contrast, Koopman et al., measured MPS over 6 hours post-exercise, thereby potentially missing the peak of MPS rise, and “diluting” the measured MPS response over time, leading to

the apparent reduced MPS response (Atherton et al., 2017; Koopman et al., 2008).

We have previously demonstrated that following acute unilateral RE, providing a suboptimal (10 g) protein drink enriched with 4.2 g LEU, led to a greater MPS response in both younger and older adults (Atherton et al., 2017) compared to an isonitrogenous alanine control. Interestingly, in older men, MPS following post-exercise plus LEU-enriched suboptimal protein did not fully return to baseline after 4 hours, as seen in other groups, suggesting MPS and a positive net balance may have extended beyond 4 hours (Atherton et al., 2017). Comparable results in MPS were also observed in older women who ingested a 20 g WP bolus or 3 g LEAA (40% LEU) following a bout of RE, which remained elevated for 4 hours (Bukhari et al., 2015). Further, again in older women, we highlight as little as 1.5 g LEAA (0.6 g LEU), in conjunction with a bout of RE, was enough to robustly (and perhaps even maximally) stimulate MPS with negligible trophic advantage compared to larger doses of LEU-enriched EAA (6 g) or even 40 g WP (Wilkinson et al., 2018a). In older men, post-exercise myofibrillar MPS rates were elevated following consumption 15 g milk protein concentrate with 1.5 g LEU (compared to without), supporting the thesis that LEU co-ingestion further augments post-exercise muscle anabolism of suboptimal doses of protein (Holwerda et al., 2019a). Although similar effects on MPS occurred 2–5 hours post-exercise following ingestion of 10 g LEAA (1.85 g vs. 3.5 g LEU) in older men, stimulation of MPS above basal levels persisted up to 24 hours post-exercise following ingestion of the higher LEU concentration only, which was associated with an elevated and sustained

increase in muscle mRNA expression of the AA transporters L-type AA transporter 1 (LAT1), cluster of differentiation 98 transporter (CD98) and proton-coupled AA transporter 1 (PAT1) rather than mTORC1 signalling (Dickinson et al., 2014). However, the role of AA transporters in response to dietary AA and RE is questionable, with LAT1 and small neutral AA transporter 2 (SNAT2) protein expression and LAT1 membrane localisation demonstrated to not be acutely altered by dietary AA or RE, nor positively associated with LEU or phenylalanine incorporation in SKM (Mazzulla et al., 2021).

3.6.2. The role of leucine supplementation in the context of chronic interventions of resistance exercise training

It becomes apparent that much of the literature favours the effects of post-exercise supplementation of protein/EAA and LEU-enriched protein sources (often with suboptimal quantities of protein) to augment maximal rates of acute MPS both in younger and older individuals. However, whether prolonged LEU supplementation can enhance muscle hypertrophy or maintain muscle mass during extended periods of RET remains less conclusive.

Following 8 weeks of unilateral RET, increases in quadriceps CSA did not differ in young individuals who were supplemented with either 20 g WP plus 6.2 g LEU compared to a carbohydrate (26.2 g maltodextrin) placebo (Coburn et al., 2006). However, the lack of impact of additional WP plus 6.2 g LEU supplementation compared to the placebo may have been due to sufficient daily

dietary protein intake during the 8-week intervention, as there were no dietary restrictions imposed during the course of the study (Coburn et al., 2006). Further, it is unlikely enriching 20 g WP with 6.2 g LEU would have any additional impact MPS and muscle hypertrophy, due to 20 g WP already maximally stimulating MPS in young individuals (Moore et al., 2009a). For context, 6.2 g LEU is equivalent to giving ~70 g of protein, as 25 g WP gives ~2.5 g LEU, but of course does not provide the full complement of EAA. No differences in whole-body SKM mass between supplement groups were observed in young adults following 12 weeks of RET combined with twice daily supplementation of either free-LEU, WP concentrate, WP hydrolysate, soy protein concentrate (all providing ~3 g LEU) or a maltodextrin placebo (Mobley et al., 2017). Further, following 8 weeks of combined RET and LEU supplementation (3 g/d), vs. placebo, significant but similar increases in muscle CSA of vastus lateralis (LEU: +8.9% vs. placebo: +9.6%) and rectus femoris (LEU: +21.6% vs. placebo: +16.4%) were observed between groups (Aguilar et al., 2017).

Despite an absence of greater gains in muscle mass, thickness or CSA following chronic LEU supplementation and RET where adequate daily protein is consumed, LEU supplementation alongside exercise training may confer some functional (i.e., muscle strength) benefits. For example, a recent systematic review and meta-analysis found an effect of higher protein quality (i.e., greater EAA and LEU content) on muscle strength accretion with longer-term (≥ 8 weeks) RET (Morgan et al., 2021). Further, greater increases in muscle strength were evident following WP plus LEU supplementation (compared to a maltodextrin placebo) in younger adults who underwent 8 weeks RET,

suggesting supplementing with LEU-enriched WP may have additional ergogenicity beyond that seen with RET alone (Coburn et al., 2006). Again, greater strength gains were evident after 12 weeks RET in conjunction with 4 g/day free-LEU supplementation in young untrained males (Ispoglou et al., 2011). Clinically significant gains in isometric leg strength were evident alongside moderate functional improvement during a timed-up-and-go test in healthy older adults when supplementing with a larger dose of free-LEU (10 g/day) alongside 12 weeks RET (Trabal et al., 2015). Conversely, in young untrained males, equivalent improvements in 1RM squat and bench press strength were seen when supplementing with AA thrice daily (0.4 g AA/kg body weight, 27.2 g LEU per 100 g AA, ~8.7 g LEU for an 80 kg individual) compared to placebo following a 4-week training programme (Ratamess et al., 2003). Similarly, increases in muscle strength following 8 weeks RET plus LEU supplementation (3 g/d) in young males were identical to that seen with placebo (Aguilar et al., 2017). More recently, twice daily supplementation of free-LEU (5 g/d) or an alanine placebo, whilst undergoing 12 weeks of twice weekly lower-body RET, led to equivalent increases in muscle strength (LEU vs. placebo: 19.0 ± 9.4 % vs. 21.0 ± 10.4 %) and muscle CSA (LEU vs. placebo: 8.0 ± 5.6 % vs. 8.4 ± 5.1 %) (De Andrade et al., 2020); demonstrating LEU supplementation did not further enhance muscle mass and strength following RET.

The lack of enhanced muscle hypertrophy following RET with LEU-enriched protein/EAA and/or free-LEU supplementation, despite evidence of increased acute MPS responses (as evidenced below in Table 3.2), may not be surprising

since previous research has demonstrated a lack of correlation between acute MPS responses and muscle hypertrophy following RET in both fasted (Mayhew et al., 2009) and fed (Mitchell et al., 2014) conditions. However, it is likely that participants within the studies discussed above (i.e., Mobley et al., 2017; Aguiar et al., 2017; De Andrade et al., 2020) were consuming sufficient (especially when protein intake is ≥ 1.2 g/kg/d), and indeed greater quantities of protein such that any anabolic response was maximised and that LEU supplementation could not further stimulate MPS. Also, small sample study numbers may be confounded by the high and low-responder phenomenon routinely observed in larger studies (Davidsen et al., 2011; Sparks, 2017). Chronic LEU-enriched protein supplementation is likely more beneficial when there is a low/insufficient dietary protein intake, since acute LEU-enriched supplements (often containing a low-dose of protein or EAA) stimulate comparable muscle anabolic responses to larger doses of WP, although the impact of chronic supplementation of low-dose LEU (with/without RE) is yet unknown. The use of low-dose LEU supplements may provide an alternative approach to consuming large doses of protein, which may be difficult for older adults, both in terms of cost and satiety and appetite; or indeed those who are clinically compromised, thus, becoming increasingly important in a critical care/clinical setting.

Table 3.2 The role of LEU supplementation in the context of acute and chronic resistance exercise.

Reference	N M:F	Age	Duration	Type	Route feed	AA composition	Dose	Amount of LEU	Duration of MPS	Impact on MPS/function	Comments
Dreyer et al., 2008	16 M	Y	Acute	RE	Oral (bolus)	LEAA + CHO	0.35 g/kg/LM EAA 0.5 g/kg/KM CHO	35%	1 and 2 h	↑ MPS	Increased MPS in both groups 1 h post-exercise with further increase following LEAA + CHO group at 2 h post-exercise.
Koopman et al., 2008	8 M	O	Acute	RE	Oral (pulse)	WP + CHO + LEU	0.16 g WP/kg/h (0.96 g PRO/kg) 0.49 g CHO/kg/h	0.03 g/kg	6 h	↑ MPS	No differences in MPS between treatments. Ample protein ingested so LEU likely to not have any added anabolic effect.
						WP + CHO	0.16 g WP/kg/h 0.49 g CHO/kg/h	–		↑ MPS	
Holwerda et al., 2019	24 M	O	Acute	RE	Oral (bolus)	Milk PRO + LEU	15 g	1.5 g	6 h	↑ MPS	Providing a suboptimal dose of PRO enriched LEU promoted greater muscle anabolism than milk PRO alone
						Milk PRO	15 g	–		↑ MPS	
Churchward-Venne et al., 2012	24 M	Y	Acute	RE	Oral (bolus)	WP	25 g	3 g	1, 3 and 5 h	↑ MPS	Plasma leucinaemia equivalent between WP and WP + LEU but elevated compared to WP + EAA. MPS was similar across conditions 1 – 3 h in both FED and FED-EX with increased MPS 3 – 5 h for WP
						WP + LEU	6.25 g	3 g		↑ MPS	
						WP + EAA	6.25 g	0.75 g		↑ MPS	

Churchward-Venne et al., 2014	40 M	Y	Acute	RE	Oral (bolus)	WP Low WP WP + low-LEU WP + high-LEU WP + BCAA	25 g 6.25 g 6.25 g 6.25 g 6.25 g	3 g 0.75 g 3 g 5 g 5 g	1.5 h and 4.5 h	↑ MPS ↑ MPS ↑ MPS ↑ MPS ↑ MPS	Blood LEU concentrations greatest in W6 + high-LEU compared to W6 and W6 + low-LEU groups. Increased MPS above basal in all treatment conditions over 0-1.5 h and 1.5 – 4.5 h in both FED and FED-EX with greatest increase following WP and WP + high-LEU. No further increase with LEU in muscle strength or CSA following RET. Participants were consuming ~1.6 g/kg/d PRO pre- and post-intervention. Greater increased in muscle strength in T limb with WP + LEU. Increased muscle CSA with both groups with no difference between groups
Aguiar et al., 2017	10M:10 F	Y	Chronic (8 weeks)	RET (2 d/wk)	Oral (×1/d)	LEU PLA (corn-starch)	3 g/d 3 g/d	3 g/d –	–	↑ muscle strength and CSA ↑ muscle strength and CSA	More than adequate amounts of dietary protein consumed (1.8 ± 0.4 g/kg/d). No further enhancements following LEU in muscle strength or CSA.
Coburn et al., 2006	33 M	Y	Chronic (8 weeks)	RET (3 d/wk)	Oral (×2/d or ×1/d)	WP + LEU PLA (maltodextrin)	20 g 26.2 g	6.2 g –	–	↑ muscle strength in T and UT limb ↑ muscle CSA ↑ muscle strength in T limb ↑ muscle CSA	More than adequate amounts of dietary protein consumed (1.8 ± 0.4 g/kg/d). No further enhancements following LEU in muscle strength or CSA.
De Andrade et al., 2020	25 M	Y	Chronic (12 weeks)	RET (2d/wk)	Oral (×2/d)	LEU PLA (ALA)	10 g 10 g	2 × 5 g –	–	↑ muscle strength and CSA ↑ muscle strength and CSA	More than adequate amounts of dietary protein consumed (1.8 ± 0.4 g/kg/d). No further enhancements following LEU in muscle strength or CSA.

Ispoglou et al., 2011	26 M	Y	Chronic (12 weeks)	RET (2d/wk)	Oral (×3/d)	LEU	4 g/d	4 g/d	–	↑ muscle strength and lean mass	Greater increase in 5-RM strength with LEU (40.8%) compared to PLA (31%).
						PLA (lactose)	4 g/d	–		↑ muscle strength and lean mass	
Mobley et al., 2017	75 M	Y	Chronic (12 weeks)	RET (3d/wk)	Oral (×2/d)	LEU	3 g	3 g	–	↑ muscle strength and type I/II fibre CSA	No differences between groups observed for lean mass gains. Similar increases in total body SKM mass and type I/II fibre CSA increases Satellite cell count ↑ with WPC and WPH post-RET
						WPC	26.3 g	3 g		↑ muscle strength and type I/II fibre CSA	
						WPH	25.4 g	3 g		↑ muscle strength and type I/II fibre CSA	
						Soy PRO	39.2 g	3 g		↑ muscle strength and type I/II fibre CSA	
						PLA (maltodextrin)	0.4 g PRO	–		↑ muscle strength and type I/II fibre CSA	
Trabal et al., 2015	30 M	O	Chronic (12 weeks)	RET (3d/wk)	Oral (×2/d)	LEU	10 g	2 × 5 g	–	↑ muscle strength	Daily protein intake averaged 1.25 g/kg/d and 1.20 g/kg/d for LEU and PLA groups, respectively. Increase in muscle strength in both groups at 4- and 12-weeks RET, with chair stand and timed-up-and-go
						PLA (maltodextrin)	10 g	–		↑ in chair stand and timed-up-and-go performance	

Abbreviations: ALA, alanine; BCAA, branched chain amino acids; CHO, carbohydrate; CSA, cross-sectional area; EAA, essential amino acid; F, female; FED, feeding; FED-EX, feeding plus exercise; LEAA, leucine-enriched essential amino acids; LEU, leucine; M, male; MPS, muscle protein synthesis; O, old; PLA, placebo; PRO, protein; RE, resistance exercise; RET, resistance exercise training; RM, repetition-maximum; T, trained; UT, untrained; WP, whey protein; WPC, whey protein concentrate; WPH, whey protein hydrolysate; Y, young

3.7. The role of leucine supplementation in states of physical inactivity/disuse

Periods of physical inactivity, such as with chronic sedentary behaviour, periods of illness, limb immobilisation (e.g., due to casting) and/or bed rest, leads to inactivity-induced anabolic resistance (de Boer et al., 2007; Wall et al., 2016), promotes insulin resistance (Dirks et al., 2016), thus accelerating SKM atrophy. Unloading-induced atrophy is a relatively uncomplicated form of muscle loss and is dependent almost solely on the loss of mechanical input (Phillips et al., 2009). This is not necessarily the case in diseased states associated with inflammation (e.g., cancer cachexia and burns) where there is a pro-catabolic hormonal and cytokine environment, thus mechanisms governing disuse in inflammatory states may be governed somewhat differently (Phillips et al., 2009). In certain conditions, including ageing and chronic diseases, despite resumption of normal ambulation (Perkin et al., 2016), or even following RET (Suetta et al., 2009), inactivity-induced atrophy is not fully restored. As older adults are more likely to experience extended periods of inactivity (Suetta et al., 2009; Arentson-Lantz et al., 2020), with most hospitalised patients being over 65 years and enduring longer hospital stays (He et al., 2005), there is concern of inducing “acute sarcopenia” during these periods of inactivity/enforced bed-rest (Deane et al., 2020b). In fact, in older individuals, up to 6% (~1 kg) of lower extremity lean mass was lost during 10 days of bed rest (Kortebein et al., 2007), with ~4% leg lean mass being lost during 5 days of bed rest (Tanner et al., 2015). Exacerbating this, is age-related anabolic resistance coupled with reduced daily protein intake, particularly during hospitalisation. For example, older adults in

an acute care facility only consumed ~10 g of a ~40 g serving of protein at each meal (Paddon-Jones, 2017). Ageing populations subjected to periods of inactivity, are further compromised and would therefore benefit from, optimisation of both protein supplementation, including low dose LEU, and exercise interventions (e.g., electrical stimulation and/or physiotherapy) (Deane et al., 2020b).

The question, therefore, arises as to how dietary/supplementary protein and/or EAA may ameliorate inactivity-induced muscle atrophy. The consumption of high-quality protein wholefoods can support SKM throughout the lifespan (Marshall et al., 2020), however, whether protein-dense wholefoods are desirable, especially in older/clinically vulnerable populations who exhibit anabolic resistance of MPS to protein/EAA and exercise, remains an area of debate since these populations often display prolonged rates of gastric emptying (Paddon-Jones et al., 2008; Lonnie et al., 2018) and increased satiety following a meal (Marshall et al., 2020). An alternative to dense high-protein meals may be the consumption of isolated LEU or LEU-enriched protein/EAA to attempt to preserve muscle anabolic capacity and maintain muscle mass during periods of inactivity (as summarised below and in Table 3.3). It should be noted that comparisons of the effect of protein/LEU supplementation on periods of inactivity are inherently difficult to compare, due to differences in population/group studied, the approach taken to induce inactivity (i.e., reduced step count, limb immobilisation, bed rest), differences in the supplementation and methodologies used to assess pre/post outcome measures; all of which influence interpretation of the data. Despite this, we believe it important to

discuss the varying methodologies, modes of inactivity and type of nutritional supplement given, whilst acknowledging this as a limitation.

3.7.1. The impact of physical inactivity on muscle protein synthesis

Generally, it is agreed that in humans, MPS is downregulated as a result of uncomplicated (i.e., nonpathological) periods of disuse (Glover et al., 2008; Gibson et al., 1988; Paddon-Jones et al., 2006) and alterations in MPS are facilitative and responsible for the majority of the observed changes in protein mass (Phillips et al., 2009). In contrast, the relative importance of MPB to muscle loss, remain less obvious. MPB is in fact often adaptive to the inactivity induced fall in MPS and either remains unchanged or declines (Phillips et al., 2009). Indeed, both basal (fasting) and postprandial rates of MPS decline during periods of disuse with the reduced fasted-state MPS occurring relatively early in immobilization (10 days) and does not appear to decline further with prolonged inactivity (de Boer et al., 2007; Wall et al., 2016).

Physical inactivity induces a state of anabolic resistance with muscle becoming less responsive to EAA following feeding. For example, following 14-day unilateral knee immobilisation in young individuals, postabsorptive MPS declined by 27% with postprandial MPS also being suppressed in the immobilised limb with the non-immobilised leg demonstrating greater MPS responses to both a low (43 mg/kg/h) and high (261 mg/kg/h) dose infusion of AA over 4 hours (Glover et al., 2008). Further, it was evidenced that MPS in older individuals was unresponsive to 12 g (2.5 g LEU) EAA following 5-day bed rest compared

to younger counterparts across 0–3 hours measurement period (Tanner et al., 2015). When reducing daily step-count by ~76% for 14 days in healthy older adults, postprandial rates of MPS were reduced by 26% following the ingestion of 25 g protein with a 12% increase in insulin resistance and a 43% decrease in insulin sensitivity (Breen et al., 2013). Further, it was recently shown that 1.6 g protein/kg/d was insufficient to modulate muscle mass, following 3-day unilateral leg immobilisation in healthy young individuals (Kilroe et al., 2021). Conversely, provision of thrice daily EAA (plus carbohydrate) supplementation (16.5 g EAA, 3.1 g LEU), in addition to a mixed meal every 5 hours (59% carbohydrate, 27% fat, 14% protein), attenuated declines in muscle FSR following 28-day bedrest (pre: 0.099 ± 0.008 %/h; post: 0.093 ± 0.006 %/h), although declines in MPS were observed in the control group receiving the mixed meal only (pre: 0.075 ± 0.005 %/h; post: 0.055 ± 0.007 %/h) (Paddon-Jones et al., 2004). When looking at the effect of LEU *per se*, thrice daily LEU supplementation (0.06 g/kg body weight/meal; ~5 g LEU/meal for an 80 kg individual), compared to an alanine control, attenuated declines in postabsorptive MPS following 14-day bedrest in middle-aged adults (English et al., 2016). Conversely, Edwards et al., demonstrated supplementing with ~15g LEU/d, compared to NEAA, led to inactivity-induced anabolic resistance in both groups through suppressed postprandial myofibrillar MPS in the immobilised compared to the control limb (-22 ± 4 %), which was purported to be a driving factor for the decline in leg fat-free mass in the immobilised limb (Edwards et al., 2020).

3.7.2. The impact of physical inactivity on muscle mass and function

Early immobilisation studies utilising nitrogen balance as an indicator of protein loss, found nitrogen retention was improved in healthy young individuals during both 6- (Stein et al., 1999) and 14-day (Stein et al., 2003) bedrest in conjunction with daily BCAA, rather than NEAA supplementation. Further, provision of thrice daily EAA attenuated SKM mass declines following a 28-day bedrest compared to a control group (Paddon-Jones et al., 2004), with thrice daily LEU supplementation attenuated declines in whole-body lean mass following a 14-day bedrest (English et al., 2016). Additionally, knee extension peak torque and body fat percentage was unchanged following a 7-day bedrest in the LEU supplemented group only (English et al., 2016). A more recent study found a reduced loss in leg lean mass in healthy older men and women who consumed isoenergetic diets (55% carbohydrate, 29% fat, 16% protein) supplemented with LEU (0.06 g/kg/meal; 3×/d, ~15g LEU per day) rather than an alanine control, during 7 days of bedrest (Arentson-Lantz et al., 2020). Interestingly, thrice-daily supplementation of a mixed AA cocktail named AXA2678 (constituting both EAA and NEAA; 4 g LEU) attenuated muscle atrophy during 7-day limb immobilisation in young adult males compared to a maltodextrin control (Holloway et al., 2019). However, the treatment group (AXA2678) received an additional ~66.8 g of AA (~46 g EAA) daily compared to the carbohydrate placebo group, which may explain the positive results rather than LEU *per se* (Holloway et al., 2019).

Despite the above seemingly supporting the notion of LEU supplementation attenuating muscle mass declines during inactivity, not all studies agree. During 7-day unilateral knee immobilisation, 1-week (3×/day) of free crystalline LEU supplementation (7.5 g LEU/day) compared to a placebo (7.5 g maltodextrin and 7.5 g dextrose monohydrate) failed to attenuate muscle mass and strength declines in healthy young adults (Backx et al., 2018). In middle-aged adults, despite no changes in integrated MPS, once daily supplementation of 20 g milk protein (inherently containing 2.1 g LEU), also failed to attenuate muscle mass losses following 14-day unilateral leg immobilisation (Mitchell et al., 2018). Further, a recent study utilising high-dose LEU supplementation (15 g/day) compared to a NEAA placebo, found that the LEU supplementation failed to attenuate any functional declines following 7-day of limb immobilisation in young healthy males (Edwards et al., 2020).

Overall, the data would indicate that protein/LEU supplementation during periods of inactivity is insufficient to prevent losses in muscle mass and function. However, a potential confounding factor for assessing the effectiveness of supplementation was that participants were often maintained in energy balance and consumed more protein than the RDA (~1.0 g/kg/d), although even in these studies, declines in SKM mass and function were often evident. It should be noted that the results from these studies may not accurately reflect the nutritional status of many older individuals or those who are clinically compromised who often consume insufficient daily dietary protein and are malnourished (Phillips et al., 2020; Backx et al., 2018) thus, more studies involving periods of physical inactivity need to be in such populations if

feasible. Nonetheless, a full complement of EAA, rather than isolated LEU, may be required to protect inactivity-induced declines in muscle strength, mass and MPS during disuse in younger adults (Edwards et al., 2020), although LEU supplementation may still be an effective strategy in middle-aged and older adults, where underlying age-related muscle anabolic resistance may be further exacerbated by disuse (Arentson-Lantz et al., 2020; English et al., 2016; Edwards et al., 2020).

3.7.3. The influence of muscle contraction during periods of inactivity on maintaining muscle mass

Rather than protein/LEU supplementation, reintroducing muscle contraction during a period of disuse arguably has a more pronounced impact in ameliorating inactivity-induced muscle mass wasting and premature anabolic resistance (Gibson et al., 1988). Physical capacity and access to appropriate facilities are, however, significant confounding factors to utilising exercise as a front-line intervention during periods of disuse, especially in clinical settings. The use of neuromuscular electrical stimulation may overcome these difficulties, offering a clinically relevant, feasible and effective strategy to be used alongside protein/LEU supplementation during periods of prolonged inactivity (Guo et al., 2021). For example, applying electrical stimulation for 1 hour/day for 6 weeks in the immobilised limb attenuated declines in quadriceps CSA and MPS was maintained compared to not using electrical stimulation where declines of 17% and 23% CSA and MPS were observed respectively

(Gibson et al., 1988). Additionally, following 1-day of bed rest in older adults, unilateral neuromuscular electrical stimulation in conjunction with 40 g casein ingestion, led to an ~18% increase in MPS overnight compared to the unstimulated leg (Dirks et al., 2017). Further, maintenance of thigh lean mass and attenuation of catabolic markers (myostatin and MAFbx) was observed following 5-day bed rest when combining neuromuscular electrical stimulation (40 minutes, thrice daily) and protein supplementation (17 g/day) (Reidy et al., 2017). It may be that contractile activity is needed to maintain muscle mass/function during inactivity, with added protein/LEU potentially enhancing the muscle anabolic response to this neuromuscular electrical stimulation. Therefore, the use of neuromuscular electrical stimulation alongside protein/LEU supplementation may have translational ramifications for preserving muscle anabolism, mass, and function during periods of inactivity, especially in older adults in a clinical setting.

Based on the current evidence, we suggest, where possible, dietary protein intake should be increased in situations of disuse, and the use of supplements should comprise a full EAA profile, in addition to containing a high LEU content. Alongside protein feeding/LEU supplementation, re-introducing short bursts of involuntary muscle contraction during the disuse period may further negate inactivity-induced muscle mass loss and the premature onset of anabolic resistance. In particular, the use of neuromuscular electrical stimulation alongside protein feeding has provided promising results for preserving muscle anabolism and muscle mass during periods of inactivity (Gibson et al., 1988; Dirks et al., 2017; Reidy et al., 2017). These studies were primarily in *healthy*

individuals, and therefore, research into the effect of these interventions in *clinical* populations where increased levels of inactivity are encountered, are needed to validate, and optimise these anabolic interventions, as these individuals will often present with other catabolic stressors (e.g., systemic inflammation). Resultingly, acquiring an optimal preventative strategy, encompassing both contractile stimuli (i.e., exercise, neuromuscular electrical stimulation) alongside protein feeding/LEU supplementation, should be prioritised in further attempts to attenuate muscle mass decline and preserve muscle anabolism during prolonged periods of inactivity and bed rest.

Table 3.3 The role of LEU supplementation in states of physical inactivity/disuse.

Reference	N M:F	Age	Type of inactivity	Duration	Route feed	AA composition	Dose	Amount of LEU	Impact on MPS/function	Comments
Arentson-Lantz et al., 2020	14M:6F	O	Bedrest	7-d	Oral (×3/d)	Isoenergetic meals (55% CHO, 29% fat, 16% PRO) + LEU	0.06 g LEU/kg/ meal	~14.6 g LEU/d	↓ lean leg mass	LEU supplementation decreased loss of lean leg mass during bedrest. Limited impact on strength or endurance-based outcomes.
						Isoenergetic meals (55% CHO, 29% fat, 16% PRO) + ALA	0.06 g ALA/kg/ meal (~13.2 g ALA/d)	–	↓ lean leg mass	
Backx et al., 2018	30 M	Y	ULI	7-d	Oral (×3/d)	LEU	2.5 g/meal	7.5 g/d	↓ quadriceps CSA and strength	↓ quadriceps CSA and strength in both groups with no differences between groups. Type I and II muscle fibre types did not change following immobilisation in both groups. PRO intake controlled at 1.0 g/kg/d. LEU failed to prevent decline in all functional measures. Inactivity-induced anabolic resistance evidenced through suppressed postprandial myofibrillar MPS in the immobilised limb.
						PLA	7.5 g maltodextrin, 7.5g dextrose monohydrate	–	↓ quadriceps CSA and strength	
Edwards et al., 2020	16 M	Y	ULI	7-d	Oral (×3/d)	LEU	5 g/meal	15 g/d	↓ MPS, lean mass, and muscle strength in immobilised limb	Inactivity-induced anabolic resistance evidenced through suppressed postprandial myofibrillar MPS in the immobilised limb.
						PLA	15 g NEAA	-	↓ MPS, lean mass, and muscle strength in immobilised limb	

English et al., 2016	19 M	Mi	Bedrest	14-d	Oral (×3/d)	Isoenergetic diet (55% CHO, 30% fat, 15% PRO) + LEU	0.06 g LEU/kg/meal	0.06 g/kg/meal	↓ MPS, lean mass, muscle strength/endurance	Postabsorptive declines in MPS for both groups. LEU supplementation prevented as great declines in knee strength/endurance and losses in whole-body lean mass after 7 d but not 14 d bedrest.
						Isoenergetic diet (55% CHO, 30% fat, 15% PRO) + ALA	0.06 g ALA/kg/meal	—	↓ MPS, lean mass, muscle strength/endurance	
Holloway et al., 2019	10 M	Y	ULI	7-d	Oral (×3/d)	AXA2678	23.7 g PRO (EAA + NEAA)	4 g	↔ muscle mass ↓ peak leg isometric torque and time-to-peak torque	Dietary PRO controlled at 1.0 g/kg/day. AXA2678 attenuated muscle atrophy and prevented increase in muscle fat fraction. However, similar decreases in peak leg isometric torque and time-to-peak torque were observed.
						PLA (maltodextrin)	23.7 g	—	↓ muscle mass, peak leg isometric torque and time-to-peak torque	
Mitchell et al., 2018	30 M	Mi	ULI	14-d	Oral (×1/d)	Milk PRO (80% casein, 20% whey)	20 g	2.1 g	↓ muscle CSA and strength	Dietary PRO controlled at 1.0 g/kg/day. Knee extension strength and thigh muscle CSA declined following immobilisation with no effects of supplementation.
						PLA (maltodextrin)	Isoenergetic to milk PRO	—	↓ muscle CSA and strength	
Paddon-Jones et al., 2004	13 M	Y	Bedrest	28-d	Oral (×3/d)	Liquid meals (59% CHO, 27% fat, 14% PRO) + EAA and CHO	16.5 g EAA 30 g sucrose	3.1 g LEU	↔ muscle FSR and lean leg mass ↓ strength	Mixed muscle FSR in the EAA group were not altered post-bedrest. Lean leg mass maintained in EAA group.
						Control: Liquid meals (59%		—	↓ muscle FSR, lean leg mass and strength	

						CHO, 27% fat, 14% PRO)					Strength loss more pronounced in control group
Stein et al., 1999	14 M	Y	Bedrest	6-d	Oral (×4/d)	BCAA	30 mmol/d LEU (8.9%), isoleucine (4.5%) and valine (5.4%)	8.9% of BCAA	–		Nitrogen balance greater in BCAA group and thought to attenuate nitrogen loss during short-term bed rest.
						NEAA	30 mmol/d glycine, serine, and alanine	–	–		
Stein et al., 2003	12 M	Y	Bedrest	14-d	Oral (×4/d)	BCAA	30 mmol/d of LEU, isoleucine, and valine	3.93 g/d	–		Nitrogen retention greater with BCAA feeding.
						NEAA	30 mmol/d of glycine, serine, and alanine	–	–		No effect of BCAA supplementation on whole body, muscle, or plasma protein synthesis or rate of 3-MeH excretion.

Abbreviations: ALA, alanine; BCAA, branched chain amino acids; CHO, carbohydrate; CSA, cross-sectional area; EAA, essential amino acid; F, female; FSR, fractional synthetic rate; LEAA, leucine-enriched essential amino acids; LEU, leucine; M, male; Mi, middle-aged; MPS, muscle protein synthesis; NEAA, non-essential amino acids; O, old; PLA, placebo; PRO, protein; RE, resistance exercise; RET, resistance exercise training; ULI, unilateral limb immobilisation; WP, whey protein; Y, young

3.8. Conclusion

Dietary protein plays a pivotal role in SKM maintenance, influencing the dynamic relationship between MPS and MPB across diurnal cycles. The EAA content, in particular LEU content, drives the SKM anabolic response to protein feeding which remains transient in nature with a refractory period occurring before the next MPS stimulation is possible due to the muscle-full phenomenon. Ageing and physical inactivity are associated with a desensitising to anabolic stimuli (exercise/protein) resulting in a premature onset of muscle-full, whilst exercise extends the magnitude and duration of the muscle-full response allowing for increased sensitivity of SKM to EAA, thereby facilitating longer-term hypertrophy/remodelling. Supplementing with LEU-enriched protein/EAA seemingly promotes MPS to comparable extents as when supplementing with larger total protein (i.e., WP) doses, through a combination of activation of the mTORC1 signalling pathway via the role of Sestrin-2 and LRS. Supplementation of suboptimal protein/EAA enriched with LEU to maximise muscle anabolism may be most beneficial for older people and the clinically vulnerable, eliminating the need for larger protein feeds which are often unachievable due to implications with digestibility and increased satiety.

To attempt to offer translation of current evidence, in order to maximise muscle anabolism we suggest: i) older adults should consume greater quantities of high-quality protein per meal (1.0–1.2 g/kg/d or ~40 g protein with each main meal) or low-dose LEU-enriched protein/EAA (i.e., at least 3 g LEU within a lower protein dose (e.g., 10 g–20 g)) should be aimed for to attempt to still maximise muscle anabolism, whilst also performing RE in close proximity to the meal (if

possible), ii) a bout of exercise should be complimented with 20 g of protein or low-dose LEU-enriched protein/EAA (at least 3 g LEU), although these recommendations may need to increase with advancing age or whole-body RET, and iii) during periods of physical inactivity, we propose some form of muscle contraction (i.e., neuromuscular electrical stimulation), if feasible, should be a priority and should be complimented with greater EAA/LEU consumption (potentially in combination with NEAA due the potential some NEAA may have in mitigating disuse atrophy through (e.g.) attenuating catabolism and maintaining capillary perfusion (Holloway et al., 2019)) to maximise muscle anabolism and offset declines in SKM mass and function. Future research should be directed to optimising the type of LEU-enriched protein supplements (i.e., the addition of LEU and or protein to ice-cream/yogurt given older patient preference for soft foods) and further addressing the effectiveness of chronic LEU-enriched supplementations with respect to ageing, exercise training, inactivity and with a focus in clinical populations to preserve/increase SKM mass.

Chapter 4

4. Investigating the effects of β -lactoglobulin vs. an isonitrogenous whey protein isolate on skeletal muscle anabolism at rest and following acute resistance exercise in young healthy men.

4.1. Introduction

Bovine milk protein consists of two major fractions, whey (~20%) and micellar casein (~80%). Despite both fractions being among the highest-quality sources of dietary protein based on indexes of protein quality (Wolfe, 2015; Phillips, 2016; Gorissen et al., 2018; Deglaire et al., 2009; Mathai et al., 2017), distinct plasma AA profiles are observed following consumption. Specifically, ingestion of WP results in a rapid and large increase in postprandial plasma EAA and LEU availability; thus, is characterised as a rapidly digested protein (Boirie et al., 1997). Conversely, with a similar EAA but lower LEU content, micellar casein coagulates and precipitates in the low pH environment of the stomach following ingestion (Mahé et al., 1996). This results in slower, moderate but protracted aminoacidaemia (Boirie et al., 1997). Previous research has highlighted the myofibrillar synthetic response to either be superior following WP consumption (Tang et al., 2009; Burd et al., 2012) or with no difference between the protein

sources (Tipton et al., 2004; Reitelseder et al., 2011; Dideriksen et al., 2011), with results often dependent on conduction of exercise, feeding strategy (i.e., pulse/bolus) and the timing of SKM biopsies (i.e., micellar casein MPS rates would typically be sustained compared to WP due to slower digestion/absorption).

BLG is a protein fraction found within WP and accounts for 45–57% of whey proteins found in bovine milk (Tulipano et al., 2011; Mose et al., 2021b; Farrell et al., 2004). Perhaps obviously, like WP, BLG is a protein source naturally rich in EAA, especially LEU. In fact, the LEU content of BLG is superior and indeed exceeds the constituent LEU content of WP (~16% vs. ~12%) (Mose et al., 2021b). LEU is commonly thought of the most potent EAA for stimulating MPS in humans (Wilkinson et al., 2013) and a key nutrient regulator of translation initiation (Anthony et al., 2000b). Subsequently, there has been increasing interest in the role of LEU for enhancing muscle anabolism, and ultimately increasing and/or maintaining SKM mass, especially in the context of ageing, exercise, and inactivity (see Ely et al., (2023) for a comprehensive review on LEU supplementation in these physiological states). Indeed, previous acute studies have highlighted the positive role of LEU for the regulation of MPS (e.g., Katsanos et al., 2006; Churchward-Venne et al., 2012; Churchward-Venne et al., 2014; Bukhari et al., 2015; Wilkinson et al., 2018a), however others have demonstrated no beneficial effect of additive LEU in a mixed EAA/protein solution (Koopman et al., 2008; De Andrade et al., 2020) or following free LEU supplementation aiming to enhance lean mass (Verhoeven et al., 2009; Aguiar et al., 2017), especially in a more chronic setting. Despite this, peak plasma LEU

concentrations and postprandial rates of MPS have previously been strongly correlated in a cohort of older men (Pennings et al., 2011a; Xu et al., 2015), this is absent in young individuals and currently, to our knowledge, not determined in women.

BLG also has a marked insulinotropic response following feeding (Mose et al., 2021b). During induced catabolic conditions, supplementation of BLG (0.6 g/kg) led to AUC for serum insulin to be 62% and 30% greater compared to casein and whey consumption, respectively (Mose et al., 2021b). Further, GIP plasma concentrations increased 70% and 34% compared to casein and WP respectively, with no differences in GIP concentration exhibited between casein and whey (Mose et al., 2021b). It is well known that insulin exerts its regulation of lean muscle mass principally via an anticatabolic effect in reducing MPB, rather than the indirect effects of stimulating MPS (Abdulla et al., 2016). Resultingly, the seemingly superior insulinotropic of BLG compared to, namely whey and casein, may translate to a more significant positive net balance following protein feeding.

Although BLG has a higher LEU content and seemingly superior insulinotropic effects, a “ceiling effect” may exist regarding LEU availability and postprandial stimulation of MPS, as evidenced by a plateau in the dose-response of MPS to ingested whey protein in young healthy men (Witard et al., 2014). Simply, enriching large or already “optimal” quantities of protein with LEU would be ineffective for improving muscle anabolism as rates of MPS would already be maximised. Efforts in research have been focused on studying lower protein

doses that contain higher amounts of LEU, aiming to maximise MPS in individuals who are unable to consume the RDA of protein in order to preserve lean muscle mass. Subsequently, previous research has highlighted that suboptimal doses of protein enriched with LEU can enhance MPS and provide a similar anabolic response to larger doses (e.g., 40 g) of WP in both younger (Churchward-Venne et al., 2012; Churchward-Venne et al., 2014) and older individuals (Bukhari et al., 2015; Wilkinson et al., 2018a). Due to its novelty, the impact of low-dose BLG, compared to other protein sources, on muscle anabolism has not yet been determined.

The aim of the current study, therefore, was to assess the impact of low-dose (10 g protein) BLG compared to an isonitrogenous WPI on measures of MPS (myofibrillar FSR; primary outcome) in addition to plasma AA, insulin and GIP concentrations, and anabolic signalling in young healthy individuals. We hypothesised that BLG supplementation would lead to greater anabolic responses in response to feeding alone and with the addition of exercise.

4.2. Results

4.2.1. Plasma amino acid, insulin and glucose-dependent insulinotropic polypeptide concentrations

Arterialised plasma EAA (main time effect: $P < 0.0001$; time $\times$ supplement effect: $P = 0.0027$; Figure 4.1), BCAA (main time effect: $P < 0.0001$; time $\times$ supplement effect: $P = 0.0021$; Figure 4.3) and LEU (main time effect: $P <$

0.0001; time $\times$ supplement effect: $P < 0.0001$; Figure 4.5) concentrations increased rapidly in both WPI and BLG conditions, peaking at ~ 40 minutes following feeding, before returning to baseline at ~ 100 – 120 minutes post-feed. No differences between supplements regarding iAUC were evidenced for plasma EAA (Figure 4.2), BCAA (Figure 4.4), or LEU (Figure 4.6). In the BLG condition, plasma EAA, BCAA and LEU concentrations experienced a significantly greater rise in the first 20 minutes post-feed, with peak leucaemia also being significantly greater compared to WPI ($396 \pm 20 \mu\text{M}$ vs. $334 \pm 17 \mu\text{M}$; $P = 0.0009$). Plasma NEAA (main time effect: $P < 0.0001$; main supplement effect: $P = 0.031$; time $\times$ supplement effect: $P = 0.0045$; Figure 4.7) significantly increase above baseline following both supplements but returned to baseline values ~ 60 minutes post-feed. Plasma NEAA concentrations were significantly greater 20 minutes post-feed in the BLG group ($1066 \pm 39 \mu\text{M}$ vs. $904 \pm 26 \mu\text{M}$; $P = 0.0024$), but with no differences in peak plasma NEAA concentrations.

Plasma insulin concentrations (main time effect: $P < 0.0001$; Figure 4.8) significantly increased above postabsorptive levels, peaking at $8.66 \pm 1.8 \text{ mU/L}$ and $10.43 \pm 0.96 \text{ mU/L}$ for WPI and BLG conditions, respectively. For WPI, plasma insulin concentrations became significantly lower than baseline values 140- and 180-minutes following feeding (both $P = 0.02$). No differences in plasma insulin concentrations were observed between supplements at any timepoint ($P > 0.05$) or for iAUC (Figure 4.9).

Plasma GIP concentrations (main time effect: $P < 0.0001$; Figure 4.10) increased significantly above postabsorptive levels, peaking at $160 \pm 25 \text{ pg/mL}$ and $177 \pm$

20 pg/mL for WPI and BLG respectively. No differences in plasma GIP concentrations were observed between supplements at any timepoint ($P > 0.05$) or for iAUC (Figure 4.11).

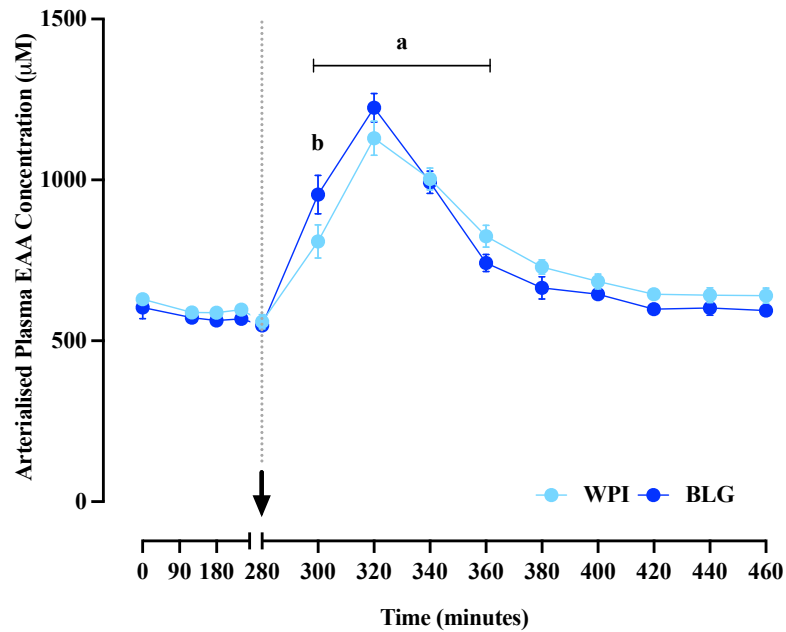


Figure 4.1 Time course effects of WPI and BLG on arterialised plasma essential amino acids (EAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

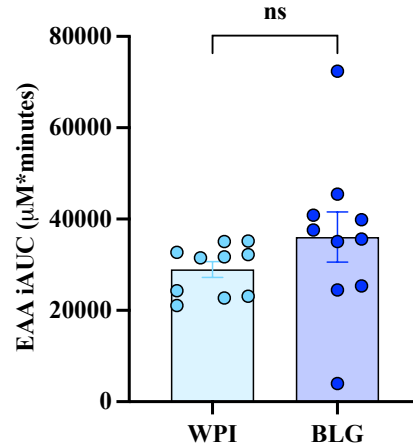


Figure 4.2 Essential amino acid (EAA) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

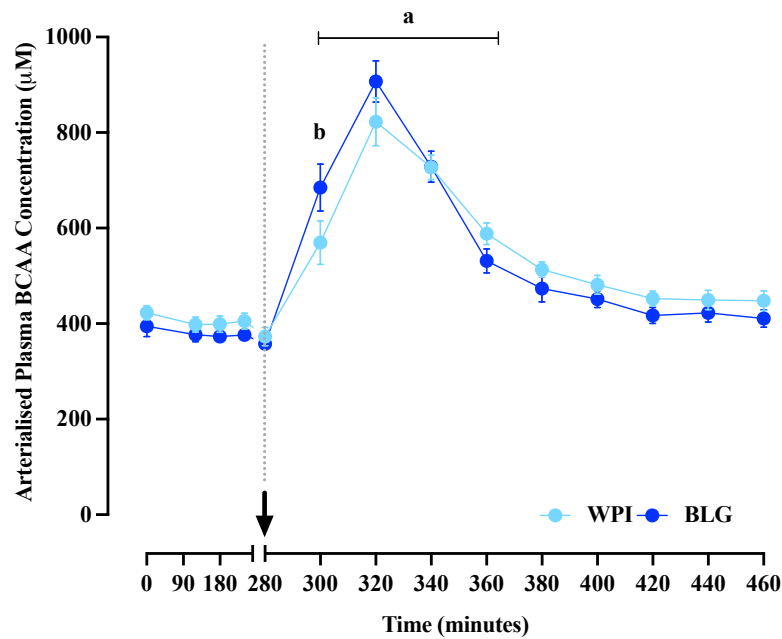


Figure 4.3 Time course effects of WPI and BLG on arterialised plasma branched-chain amino acid (BCAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

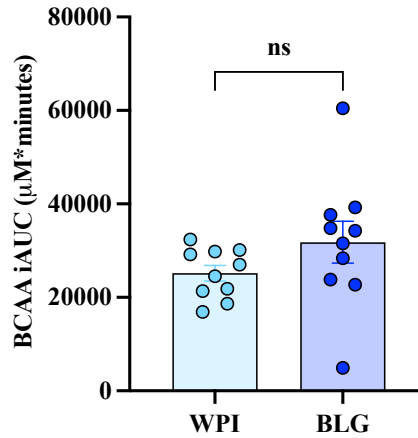


Figure 4.4 Branched chain amino acid (BCAA) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

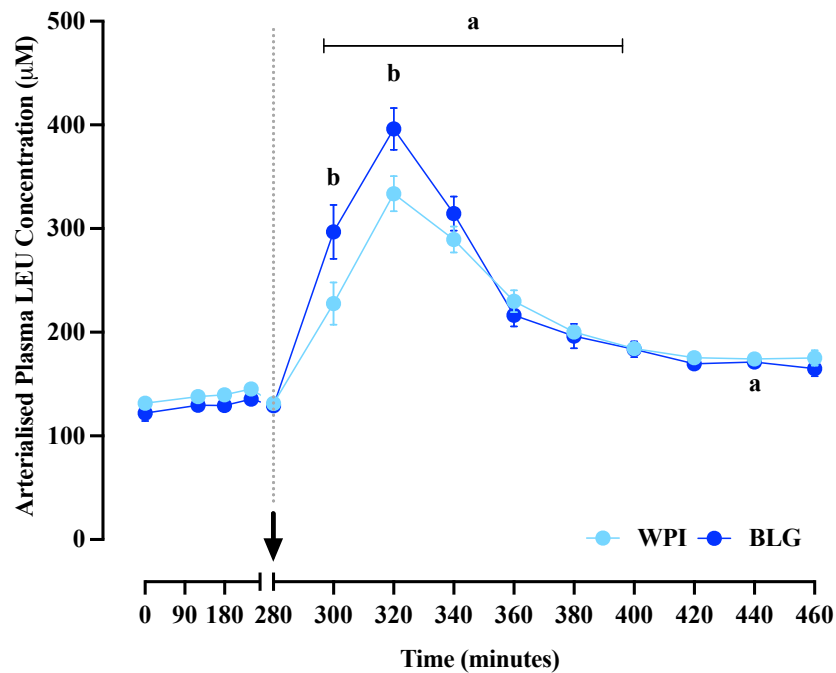


Figure 4.5 Time course effects of WPI and BLG on arterialised plasma leucine (LEU) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

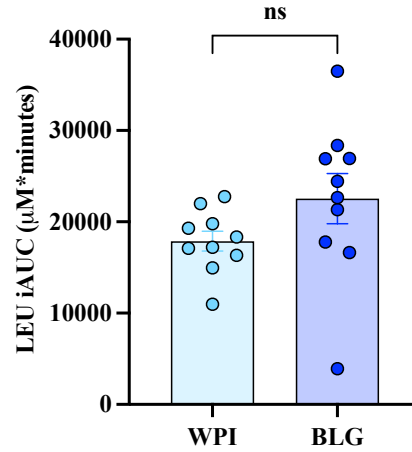


Figure 4.6 Leucine (LEU) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

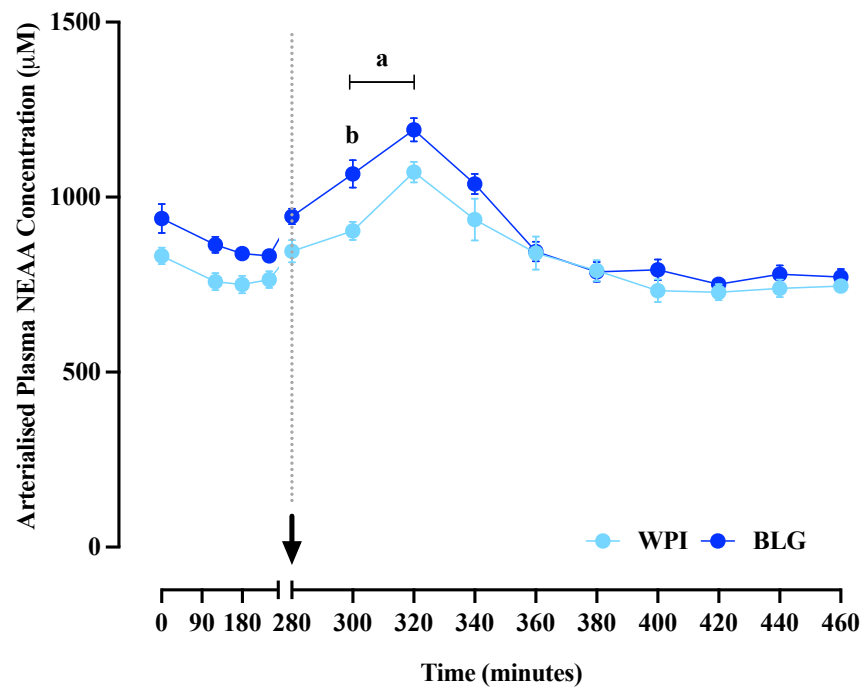


Figure 4.7 Time course effects of WPI and BLG on arterialised plasma non-essential amino acids (NEAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

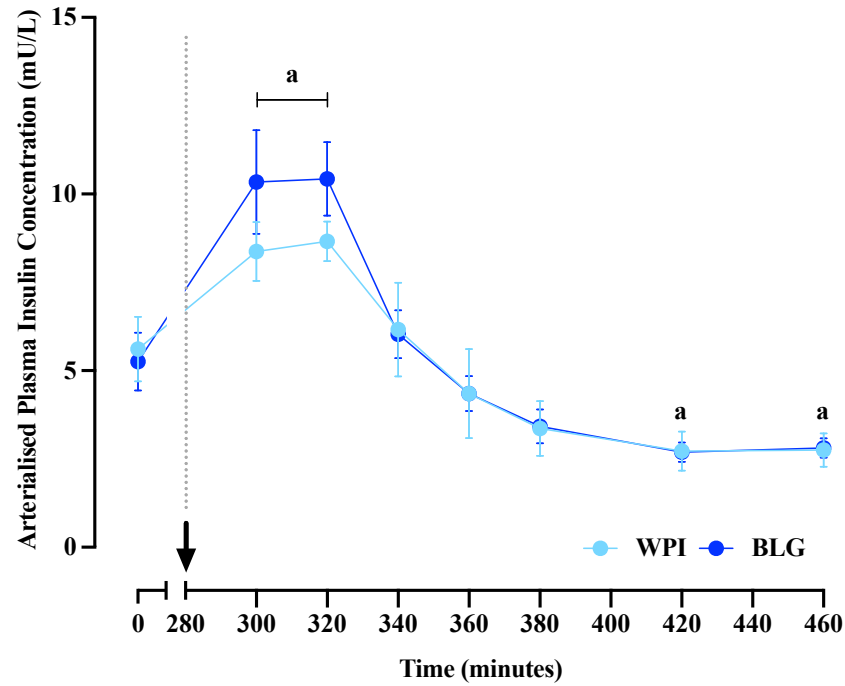


Figure 4.8 Time course effects of WPI and BLG on arterialised plasma insulin concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

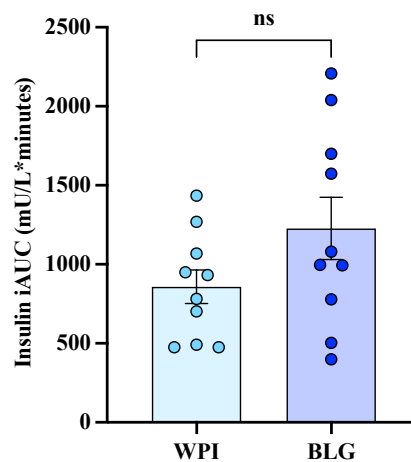


Figure 4.9 Insulin integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

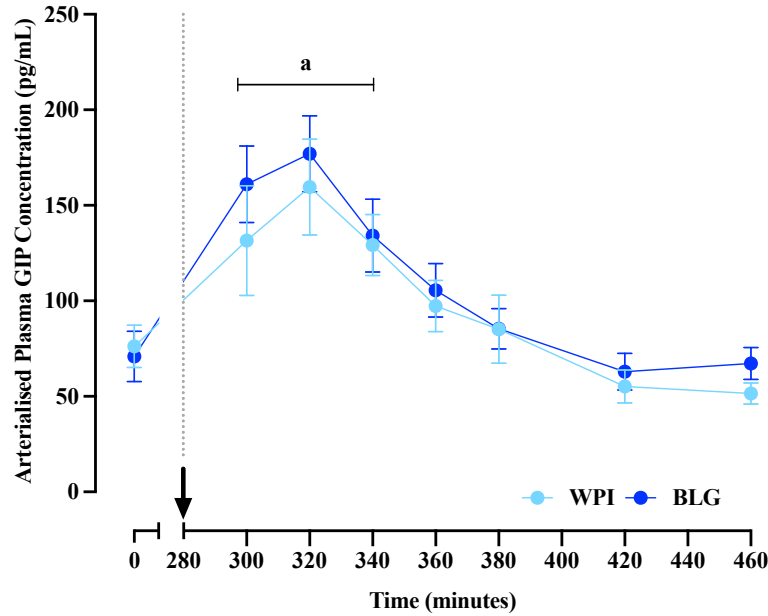


Figure 4.10 Time course effects of WPI and BLG on arterialised plasma glucose-dependent insulintropic polypeptide (GIP) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

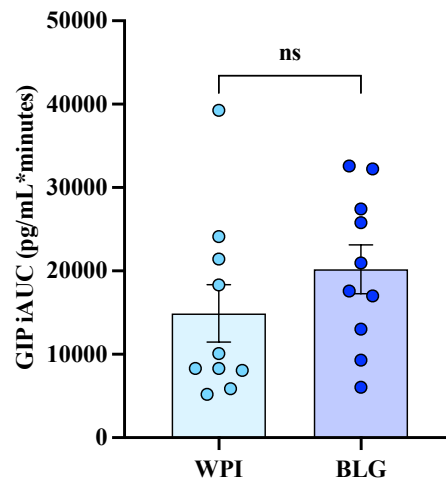


Figure 4.11 Glucose-dependent insulintropic polypeptide (GIP) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

4.2.2. Plasma α -ketoisocaproate enrichments

Plasma KIC enrichment (Figure 4.12) observed a study state following the infusion of 1,2 [$^{13}\text{C}_2$ LEU] (main time effect: $P < 0.0001$). Provision of both protein feeds led to an increase in KIC enrichment which began to return to baseline towards the end of the postprandial period but remained elevated compared to the fasted state following both feeds. No differences in KIC enrichment were observed between supplements.

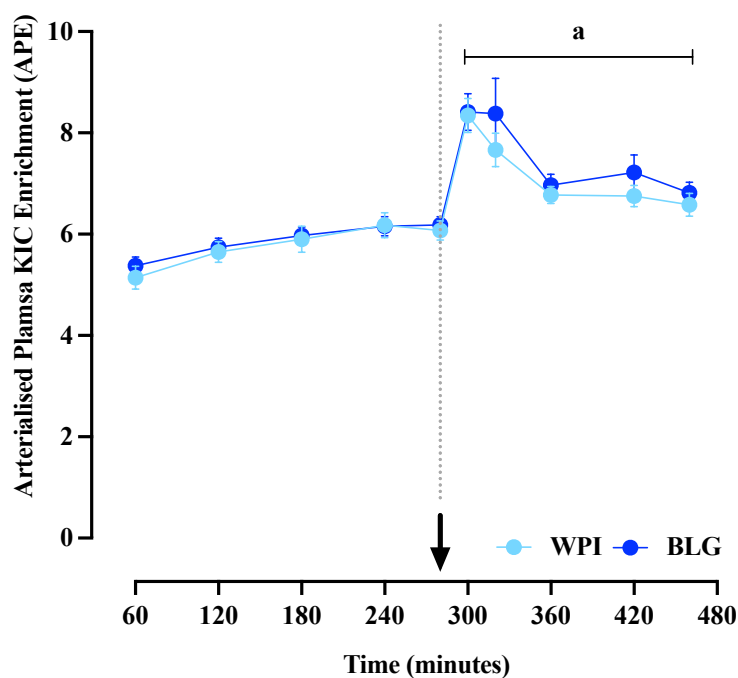


Figure 4.12 Time course effects of arterialised plasma α -ketoisocaproate (KIC) enrichments during WPI and BLG assessment visits. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). APE: atom percent excess. BLG: β -lactoglobulin. WPI: whey protein isolate.

4.2.3. Myofibrillar fractional synthetic rate

Baseline rates of myofibrillar FSR (i.e., MPS) did not differ between legs (i.e., fasted vs. fasted-ex leg) or across conditions (i.e., fasted/fasted-ex legs for WPI vs. BLG groups; $P > 0.05$). FED MPS rates increased similarly (main time effect: $P < 0.0001$; Figure 4.13) to both WPI (0.042 ± 0.006 %/h vs. 0.081 ± 0.009 %/h; $P = 0.0006$) and BLG (0.048 ± 0.006 %/h vs. 0.083 ± 0.008 %/h; $P = 0.0019$) supplementation, with no differences between the supplements ($P > 0.05$). Likewise, FED-EX MPS rates ($n = 8$; main time effect: $P < 0.0001$; Figure 4.14) increased to similar extents following both WPI (0.046 ± 0.006 %/h vs. 0.106 ± 0.008 %/h; $P < 0.0001$) and BLG (0.047 ± 0.007 %/h vs. 0.090 ± 0.005 %/h; $P = 0.0008$) supplementation, with no differences between supplements ($P > 0.05$).

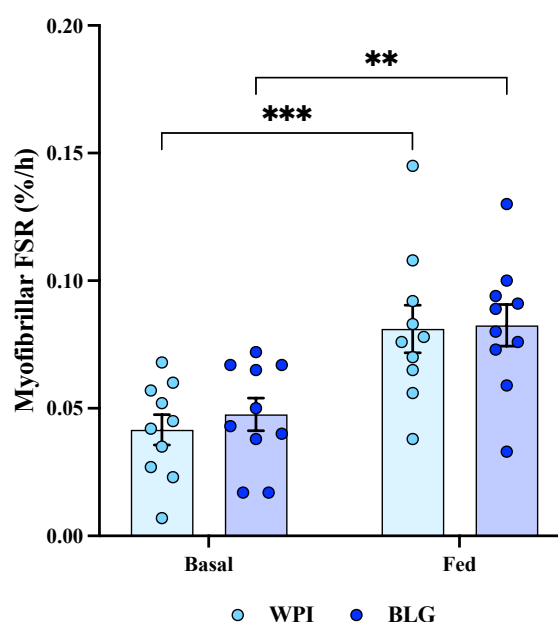


Figure 4.13 The effects of WPI and BLG on skeletal muscle myofibrillar fractional synthetic rate in the FED state. Data presented as mean (bars) $\pm$ SEM,

with individual data points overlaid. BLG: β -lactoglobulin. WPI: whey protein isolate. **: $P < 0.01$. ***: $P < 0.001$.

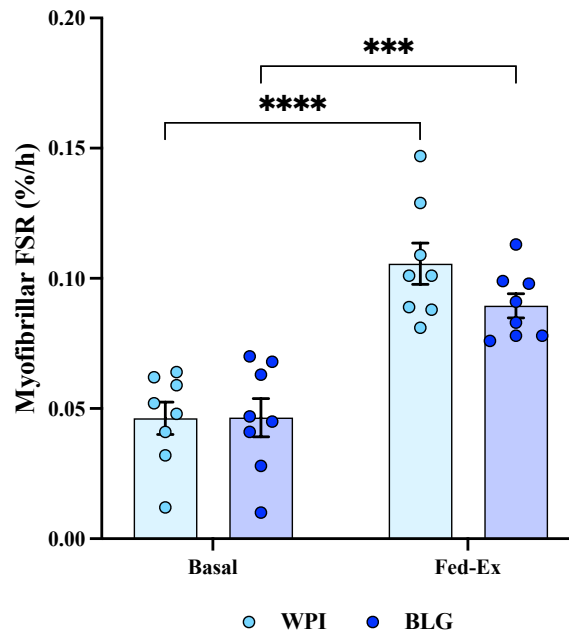


Figure 4.14 The effects of WPI and BLG on skeletal muscle myofibrillar fractional synthetic rate in the FED-EX ($n = 8$) state. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. WPI: whey protein isolate. ***: $P < 0.001$. ****: $P < 0.0001$.

4.2.4. Effects on anabolic signalling

Anabolic signalling relating to the control of MPS was assessed by measuring the phosphorylation status of the mTORC1 translation initiation substrates p70S6K1 (Figure 4.15), 4EBP1 (Figure 4.16), and RPS6 (Figure 4.17) and the phosphorylation status of elongation factor eEF2 (Figure 4.18). No significant differences were observed with respect to anabolic signalling for p-p70S6K1, p-

4EBP1 (main time effect in FED state: $P = 0.0376$), p-eEF2 and p-RPS6 between WPI and BLG supplements in the FED and FED-EX states.

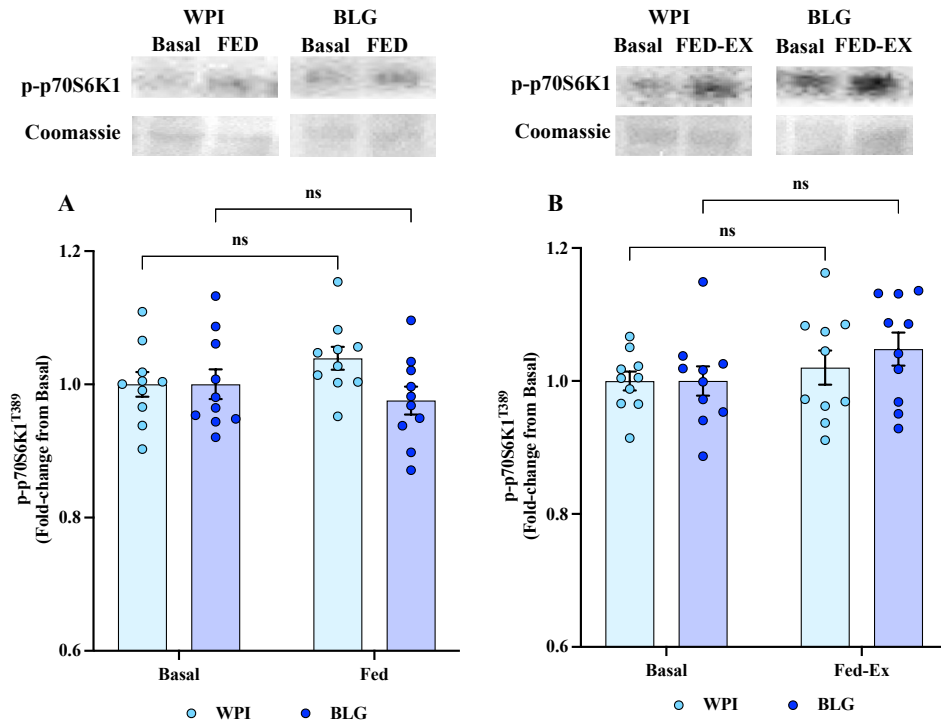


Figure 4.15 The effects of WPI and BLG on muscle anabolic signalling responses of p-p70S6K1 to FED (A) and FED-EX (B). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate.

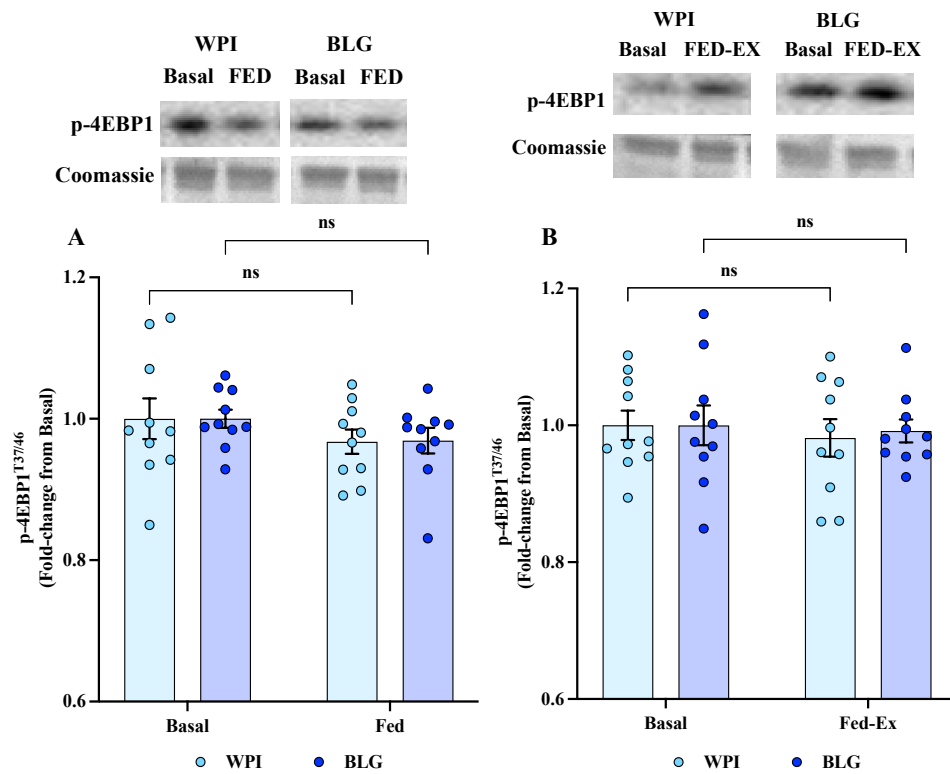


Figure 4.16 The effects of WPI and BLG on muscle anabolic signalling responses of p-4EBP1 to FED (A) and FED-EX (B). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate.

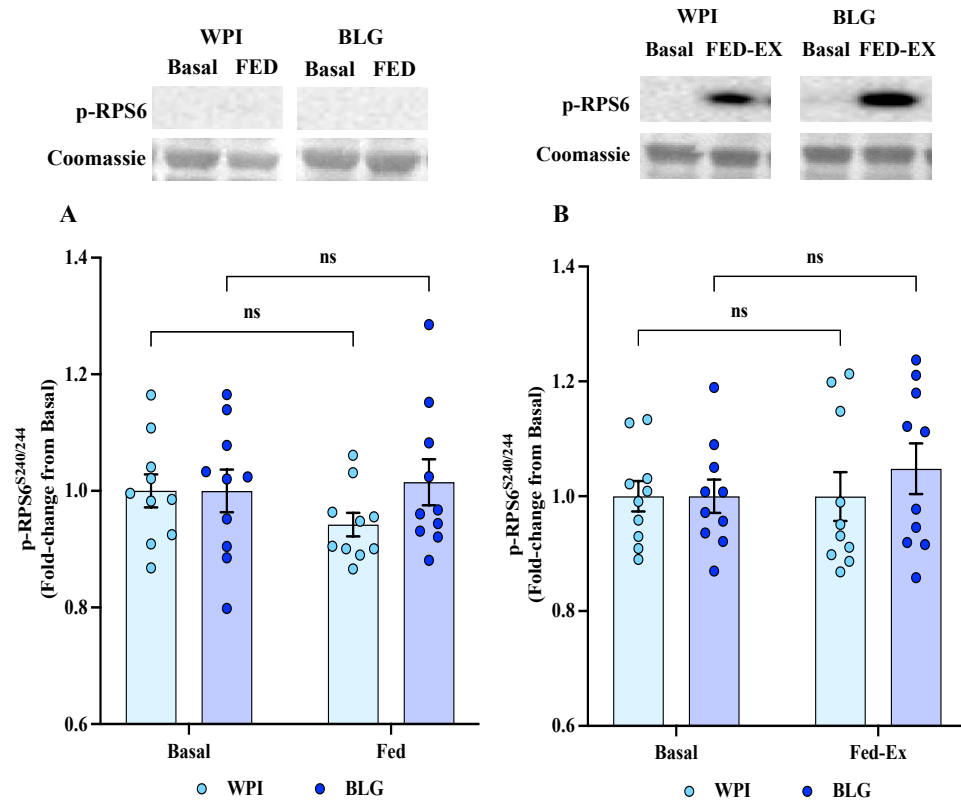


Figure 4.17 The effects of WPI and BLG on muscle anabolic signalling responses of p-RPS6 to FED (A) and FED-EX (B). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate.

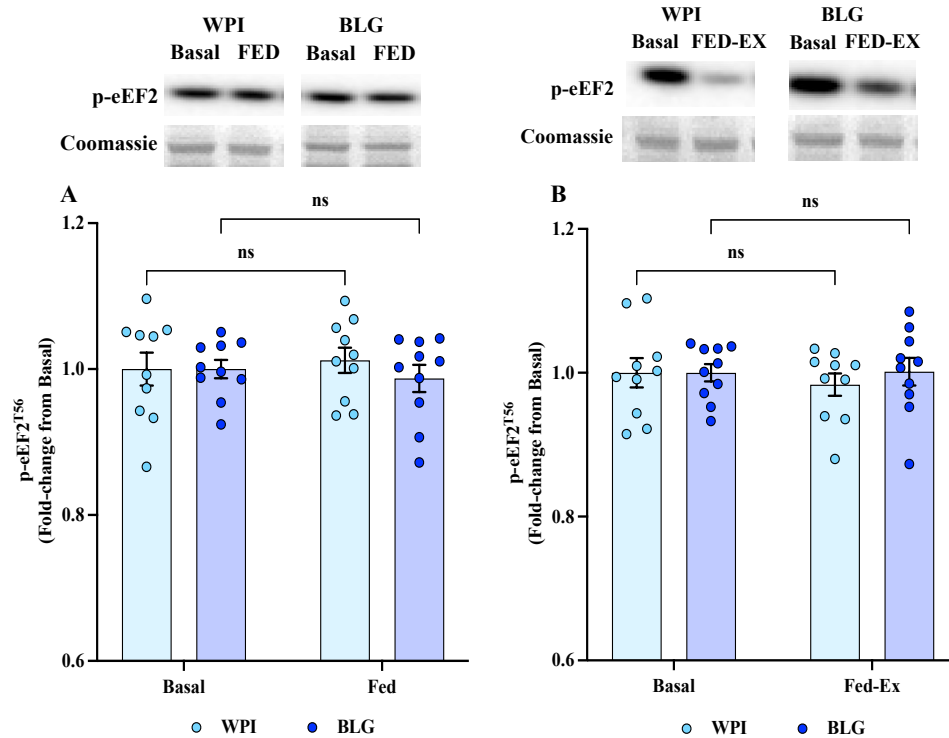


Figure 4.18 The effects of WPI and BLG on muscle anabolic signalling responses of p-eEF2 to FED (A) and FED-EX (B). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate.

4.3. Discussion

The use of LEAA/WP has gained traction over recent years as an alternative way to maximise rates of MPS at lower, suboptimal protein doses. Such research studies often involved the addition of free-LEU to existing WP and/or EAA mixes, rather than a protein feed with a naturally greater LEU content. In the present study, we compared the effects of a novel WP with a naturally superior LEU content, BLG, to an isonitrogenous WPI using what is considered a

suboptimal dose (10 g protein), for muscle anabolism in FED and FED-EX states in young healthy males.

We report the novel findings that BLG supplementation, at a dose deemed suboptimal for muscle anabolism (Cuthbertson et al., 2005; Witard et al., 2014; Moore et al., 2015), significantly increased rates of MPS in both the FED and FED-EX states. Contrary to our hypothesis, however, no differences in MPS were observed between BLG and WPI in both conditions. Previous research has demonstrated that ~10 g EAA in rested conditions (Cuthbertson et al., 2005), or ~8.6 g EAA in exercised conditions (Moore et al., 2009a), is sufficient to stimulate MPS robustly and maximally in healthy young participants. In the current study, both BLG and WPI had lower quantities of EAA than thought optimal (5.2 g vs. 4.8 g EAA, respectively), yet still robust stimulation of MPS was achieved. We note the LEU content of BLG (1.5 g) and WPI (1.0 g) to arguably be similar, allowing for the undistinguishable differences in MPS between BLG and WPI. Similar to our findings, previous studies have demonstrated that a protein dose of 6.25 g WP (0.75 g LEU) robustly stimulated MPS in young individuals (Churchward-Venne et al., 2012). Furthermore, in a study by Katsanos et al., young individuals showed a similar synthetic response when consuming 6.7 g EAA containing 26% LEU (1.721 g) and 41% LEU (2.790 g) (Katsanos et al., 2006). Therefore, we speculate in healthy young individuals, the LEU content provided by WPI (and BLG) appears to be sufficient to activate and robustly stimulate MPS with adequate amounts of the other EAA provided. The other EAA may, indeed, stimulate MPS in addition to

LEU, as previous studies have demonstrated by supplying a flooding dose of valine, phenylalanine and threonine (Smith et al., 1998).

With research utilising LEU-enriched protein supplementation at lower than optimal doses, questions have arisen as to whether a dose-response to protein feeding exists if maximal MPS stimulation can be achieved with LEAA. Indeed, even though suboptimal protein doses with enriched LEU have comparable synthetic responses to larger doses of protein, this is often only in the early phase of MPS stimulation - especially in the absence of exercise (Churchward-Venne et al., 2012; Bukhari et al., 2015; Wilkinson et al., 2018a). Professedly, larger doses of WP have been evidenced to sustain the increase in or lead to greater stimulation in MPS over longer assessment periods (i.e., ~4–5 hours) (Churchward-Venne et al., 2014; Wilkinson et al., 2018a). We, therefore, believe there is a dose-response still evident of MPS to protein, EAA or LEU ingestion (Cuthbertson et al., 2005; Yang et al., 2012; Glynn et al., 2010b), and there is a maximal response to providing an additional AA substrate of around 20 g WP, 10 g EAA, or 3 g LEU in young individuals; even despite stimulation being prolonged by prior exercise (Atherton et al., 2017).

As expected, we observed arterialised plasma EAA, BCAA and LEU concentrations to significantly increase following feeding in both BLG and WPI. In both supplements, we observed peak plasma EAA, BCAA and LEU concentrations to occur at ~40 minutes following feeding, which was expected due to the nature of the protein feed (i.e., protein powder rather than whole food). Although no differences were observed in peak EAA and BCAA concentrations

between the supplements, BLG observed greater peak leucaemia, with EAA, BCAA and LEU concentrations also exhibiting a greater rise in the first 20 minutes post-feed. This mirrors results observed in previous research studies with supplements containing greater LEU content (e.g., Churchward-Venne et al., 2012; Bukhari et al., 2015; Wilkinson et al., 2018a) and in previous studies investigating BLG (Mose et al., 2021b), leading to greater peak LEU concentrations. BLG and WPI also demonstrated an insulintropic response to feeding, but with similar increases in plasma insulin and GIP concentrations following feeding. Our results differ from those reported by Mose and colleagues who highlighted BLG consumption to lead to greater serum insulin concentrations compared to whey and casein protein (Mose et al., 2021b). However, discrepancies in results may be due to the quantity of and how the protein was administered. Mose and colleagues provided 0.6 g protein/kg with one-third being consumed as a bolus and two-thirds consumed as a sip every 20 minutes hereafter (Mose et al., 2021b), whereas the current study provided 10 g protein (regardless of body mass) as a bolus drink. Furthermore, a catabolic state was induced by Mose and colleagues which would vastly impact the metabolic response of protein feeding including (e.g.,) insulin concentrations (Mose et al., 2021b).

With respect to signalling proteins, it has been shown that LEU supplementation in resting and exercised conditions enhances MPS via activating insulin-dependent and insulin-independent mTOR pathway signalling proteins (Drummond et al., 2009a; Wilkinson et al., 2013; Anthony et al., 2000b). In the current study, we did not observe any enhancement in the signalling proteins

examined (p-p70s6k1, p-eEF2, p-RPS6 or p-4EBP1). This may be explained by the timing of the SKM biopsies being 3 hours post-feed meaning signalling would be near/if not at baseline values again. It is well established that anabolic signalling via the mTOR pathway peaks 1–2 hours following feeding (Atherton et al., 2010a). This is corroborated with work from our laboratory demonstrating peak signalling to occur 2 hours post-feed, with baseline values re-established again 4 hours post-feed in older males, despite MPS remaining elevated (Atherton et al., 2017). It is also important to note that MPS rates do not always correspond to cell signalling, therefore cause and effect should be treated cautiously (Atherton et al., 2010a). Moreover, signalling responses are complex and involve many signals outside of those looked at which could be important in regulating the heightened response in MPS following BLG and WPI ingestion (e.g., Sestrin 2 (Wolfson et al., 2016)).

In conclusion, we report that a suboptimal dose of BLG in young individuals leads to significant increases in plasma EAA, BCAA, insulin and GIP concentrations with greater peak leucaemia achieved compared to WPI, in addition to significant stimulation of MPS in the FED and FED-EX states. To advance this research further, a key next step would be to assess the impact of BLG on muscle anabolism in older adults (addressed in Chapter 5), as such individuals seemingly would benefit from the superior LEU content due to their supposed anabolic resistance to protein nutrition and exercise.

Chapter 5

5. Investigating the effects of β -lactoglobulin vs. an isonitrogenous whey protein isolate on skeletal muscle anabolism at rest and following acute resistance exercise in healthy older men.

5.1. Introduction

Global population demographics are shifting towards a more aged populous world. Data from the UK Office for National Statistics (2012) projects an increase in the population aged over 60 years from 17% in 2010 to ~23% by 2035, with the most rapid rise projected for the “oldest” of old (McPhee et al., 2016). Over the next 15 years, those aged 85 years and over is projected to increase from 1.6 million to 2.6 million (3.5% of total population) in the UK (Office for National Statistics (UK) 2024). Chronological age is often a very good predictor of health status and physical capability, although there is considerable inter-individual variability, with some older adults presenting with very good health and others showing disability and frailty (McPhee et al., 2016). Indeed, as early as ~40 years of age, functional deterioration of physiological systems are detectable with associated changes both anatomical and ultrastructural (McPhee et al., 2016). Consequently, declines in health associated

with ageing are becoming an increasingly prevalent major socioeconomic burden.

Of most paramount clinical concern is the age-related decline in muscle mass and function, termed sarcopenia (Rosenburg, 1997). Longitudinal studies demonstrate in individuals aged 75 years, SKM mass is lost at a rate of 0.64–0.70 %/year in women and 0.80–0.98 %/year in men (Mitchell et al., 2012). Muscle strength is lost at a greater rate than muscle mass ($\sim 2\text{--}5 \times$ faster), with declines of 2.5–3 %/year in women and 3–4 %/year in men aged 75 years (Mitchell et al., 2012). Subsequently, musculoskeletal disorders are the most common chronic, disabling conditions, affecting 14% of people aged over 65 years increasing to 30% in those over 75 years (Office for National Statistics (UK) 2013), with this figure being greater following retirement (McPhee et al., 2016) and in those situated in long-term care settings (Cruz-Jentoft et al., 2010; Cruz-Jentoft et al., 2019). Regardless, the consequences of SKM wasting and weakness engender numerous physiological and psycho-social impacts including: i) frailty and increased risk of falls, ii) physical inactivity (i.e., sedentarism), iii) loss of independent living and performance of daily tasks contributing to related depression, iv) increased risk of chronic diseases and v) increased risk of all-cause mortality.

The progression of sarcopenia can be exacerbated by many factors, with PA and nutrition being key regulatory factors in the control of SKM mass throughout the lifespan (Brook et al., 2016b). For more than a decade, work developing the concept of “anabolic resistance” has greatly aided the explanation of age-related

declines in SKM mass (Deane et al., 2020b). Anabolic resistance, centres upon the blunting of increases in MPS and/or suppression of MPB to key anabolic drivers, namely exercise and nutrition (Moore et al., 2015; Phillips et al., 2012; Wall et al., 2015; Marshall et al., 2020). Reflecting this, a plethora of accumulating evidence (Moore et al., 2015; Phillips et al., 2012; Wall et al., 2015; Marshall et al., 2020; Brook et al., 2016a; Mitchell et al., 2012) suggests that anabolic resistance is likely a major driver of age-related muscle atrophy (sarcopenia), though that is not to say mixed results engender this contentious (Symons et al., 2009; Chevalier et al., 2011). Subsequently, optimal nutritional and exercise strategies need to be applied to maximise muscle anabolism in older adults.

There is now strong evidence in the literature demonstrating postprandial rises in MPS in older adults are attenuated compared to young adults (Guillet et al., 2004; Cuthbertson et al., 2005; Smith et al., 2012; Volpi et al., 2000), with reductions as great as 40% in those consuming the same quantity of EAA (Cuthbertson et al., 2005). Indeed, a previous study identified older adults may need a greater quantity (~0.4 g protein/kg) of protein to equal the same anabolic responses seen in younger individuals (0.24 g protein/kg) (Moore et al., 2015). Like protein nutrition, the anabolic effects of both acute (Kumar et al., 2009; Kumar et al., 2012) and chronic (Brook et al., 2016a; Greig et al., 2011) exercise are blunted in older muscle. Kumar and colleagues demonstrated blunted MPS rates in older individuals 1–2 hours post-RE (20–90% 1RM) (Kumar et al., 2009), with impaired skeletal muscle mTORC1 signalling (a possible mechanism underlying age-related blunting of MPS) prolonged up to 24 hours

post-exercise (Fry et al., 2010). Further, following 6 weeks of unilateral leg RET at 75% 1RM, integrated MPS was elevated over 3 weeks in young but not older adults, which was supported by the absence of thigh fat-free mass gains in older muscle (Brook et al., 2015).

One main barrier preventing older adults from consuming greater quantities of protein required to aid the maintenance of SKM mass, is increased satiety following a meal (Marshall et al., 2020). As discussed in detail throughout our review (Ely et al., 2023), rather than attempting to increase the quantity or protein consumed, especially when there is an inability to do so, the quality of the protein should be increased instead (Traylor et al., 2018). Increasing protein quality (i.e., increasing LEU content) may be considered a nutritional strategy to maximise muscle anabolism in older muscle at suboptimal protein doses, negating the need to consume large quantities of dietary protein. For example, 20 g WP offered no trophic advantage compared to a low-dose LEAA (3 g, 1.2 g LEU) in older women, with both supplements comparably stimulating MPS (Bukhari et al., 2015). Moreover, just 1.5 g LEAA (0.6 g LEU) stimulated MPS, with no trophic advantage of larger LEAA doses (6 g, 2.4 g LEU), or a large (40 g) bolus of WP in older women in response to feeding in rested and exercised conditions (Wilkinson et al., 2018a). Whilst MPS increased following ingestion of both 6.7 g EAA with LEU content matched to WP (26% (1.7 g) LEU) and an enriched LEU content (41% (2.8 g) LEU) in young individuals, such increases were only observed following ingestion of LEU-enriched EAA (41% LEU) in older adults (Katsanos et al., 2006).

As discussed in the previous chapter, BLG is a WP fraction rich in EAA and LEU (Mose et al., 2021a; Mose et al., 2021b). We previously investigated the anabolic effects of a suboptimal dose of BLG compared to an isonitrogenous WPI in young healthy males. Based on our results from that study and research highlighting the trophic effects of LEAA/protein, we sought to replicate this study in older adults. Therefore, the current study aimed to assess the impact of BLG supplementation, compared to an isonitrogenous WPI, at a suboptimal dose (10 g protein) in healthy older men at rest and following acute RE. We hypothesised the BLG feed, would correspond to enhanced muscle anabolism compared to the WPI supplement.

5.2. Results

5.2.1. Anthropometric characteristic comparison to young healthy males

To offer a comparison to our young group in the previous chapter and evidence of sarcopenia in our study, we performed additional analyses comparing age, muscle size (VL CSA and thickness), muscle strength and dietary protein intake (Table 5.1). Firstly, our older group were significantly older than their younger counterparts (26 ± 2 years vs. 69 ± 1 years; $P < 0.0001$). VL CSA was significantly greater, for both legs, in the younger compared to older males (young vs. old right VL: 31.0 ± 1.5 cm² vs. 22.3 ± 1.1 cm², $P = 0.0001$; young vs. old left VL: 29.7 ± 1.2 cm² vs. 20.6 ± 1.1 cm², $P < 0.0001$). This was matched with VL muscle thickness being greater in younger (right VL: 2.6 ± 0.1 cm, left VL: 2.5 ± 0.1 cm) compared to older (right VL 2.2 ± 0.1 cm, left VL: 2.2 ± 0.1

cm) muscle, although significant differences presented in the right VL only (right VL: $P = 0.0107$; left VL: $P = 0.0938$). Unsurprisingly, muscle strength (i.e., 1RM) was significantly greater in the young compared to older individuals (65.3 ± 4.4 kg vs. 41.1 ± 4.3 kg; $P = 0.0009$).

When looking at protein intake across 4 days, despite our older group on average consuming greater quantities of protein than the RDA (i.e., 0.8 g/kg/d), younger individuals consumed significantly greater quantities of protein across this assessment period (1.3 ± 0.1 g/kg/d vs. 1.0 ± 0.1 g/kg/d for young and older adults, respectively; $P = 0.0489$).

Table 5.1. Comparison of anthropometric characteristics between young and older adult males

Characteristic	Young men	Older men
<i>n</i>	10	12
Age (years)	26 ± 2	69 ± 1 *
Height (cm)	179 ± 2	179 ± 2
Weight (kg)	81 ± 3	90 ± 5
Protein intake (g/kg/d)	1.3 ± 0.1	1.0 ± 0.1 *
VL CSA (cm ²)	R: 31 ± 2 L: 30 ± 1	R: 22.3 ± 1.1 * L: 20.6 ± 1.1 *
VL thickness (cm)	R: 2.6 ± 0.1 L: 2.5 ± 0.1	R: 2.2 ± 0.1 * L: 2.2 ± 0.1
1RM (kg)	65 ± 4	41.1 ± 4.3 *

Data presented as mean ± SEM. CSA: cross-sectional area; R: right; L: left; VL: vastus lateralis; 1RM: 1-repetition maximum. *: significantly different ($P < 0.05$) between groups.

5.2.2. Plasma amino acid and insulin concentrations

Arterialised plasma EAA, BCAA and LEU concentrations all increased rapidly post-feed in both WPI and BLG supplements, peaking at ~40 minutes. Concerning plasma EAA (main time effect: $P < 0.0001$; Figure 5.1) and BCAA (main time effect: $P < 0.0001$; Figure 5.3) concentrations, no significant

differences were observed between supplements and concentrations returned to basal concentrations within the assessment duration. No significant differences in iAUC were evident for plasma EAA (Figure 5.2) and BCAA (Figure 5.4) concentrations (both $P > 0.05$). Observing plasma LEU concentrations (main time effect: $P < 0.0001$; time $\times$ supplement effect: $P = 0.011$; Figure 5.5), BLG consumption promoted greater peak plasma leucaemia compared to WPI ($454 \pm 26 \mu\text{M}$ vs. $391 \pm 25 \mu\text{M}$; $P = 0.049$), and a significantly greater iAUC for LEU (BLG: $32269 \pm 1695 \mu\text{M} \cdot \text{minutes}$; WPI: $26610 \pm 2792 \mu\text{M} \cdot \text{minutes}$; $P = 0.0072$; Figure 5.6). Additionally, plasma LEU concentrations remained elevated above basal levels at the end of the assessment period in both WPI and BLG supplements. Plasma NEAA concentrations (main time effect: $P < 0.0001$; Figure 5.7) initially fell below basal values in the postabsorptive phase of the study. Post-feed, plasma NEAA concentrations increased significantly above basal and peaked at ~40 minutes (WPI: $1127 \pm 59 \mu\text{M}$; BLG: $1077 \pm 55 \mu\text{M}$; $P > 0.05$), before falling below basal values again towards the end of the assessment period.

Plasma insulin concentrations significantly increased above postabsorptive levels following both protein feeds (main time effect: $P < 0.0001$; Figure 5.8), peaking at $17.51 \pm 0.22 \text{ mU/L}$ and $16.85 \pm 0.24 \text{ mU/L}$ in WPI and BLG supplements, respectively. No statistical differences ($P > 0.05$) were present between the supplements with regards to plasma insulin concentrations or iAUC (Figure 5.9).

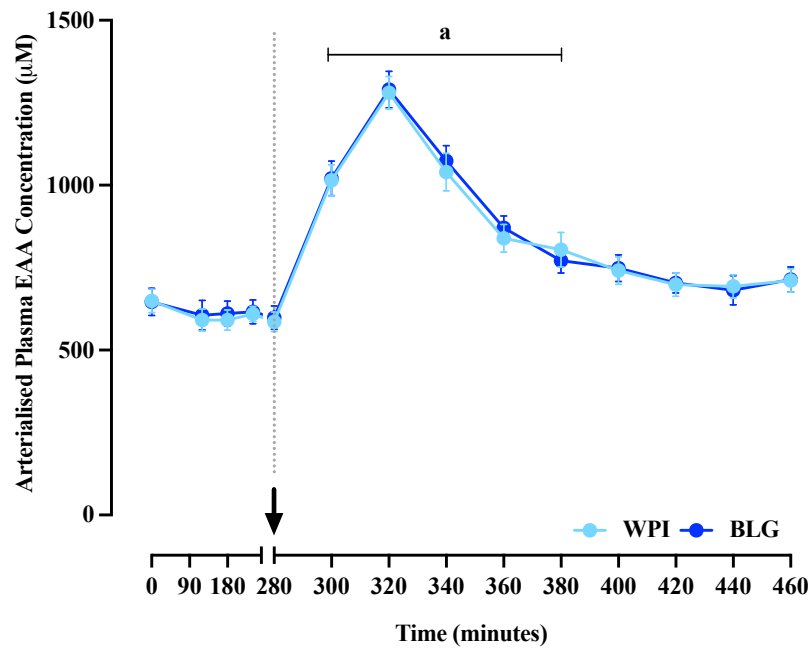


Figure 5.1 Time course effects of WPI and BLG on arterialised plasma essential amino acids (EAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

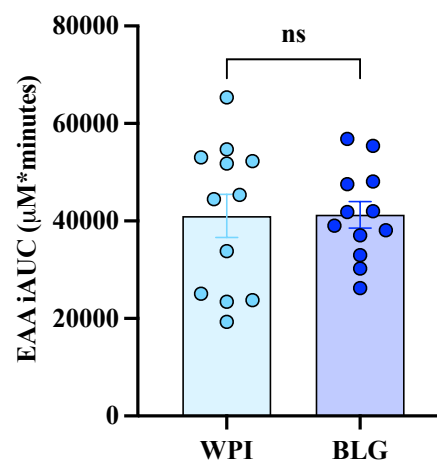


Figure 5.2 Essential amino acid (EAA) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

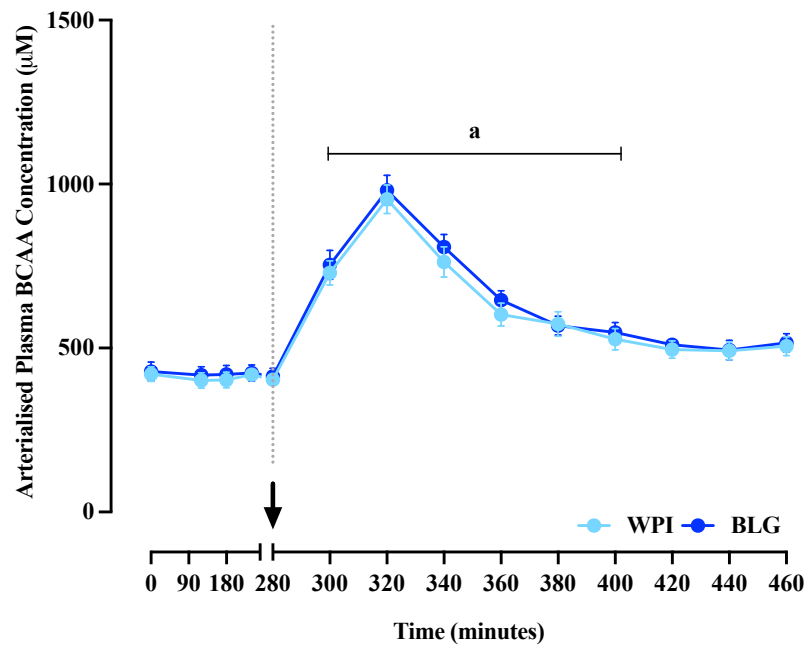


Figure 5.3 Time course effects of WPI and BLG on arterialised plasma branched-chain amino acids (BCAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

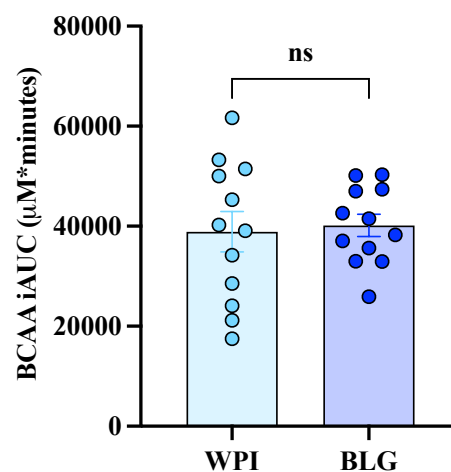


Figure 5.4 Branched chain amino acid (BCAA) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

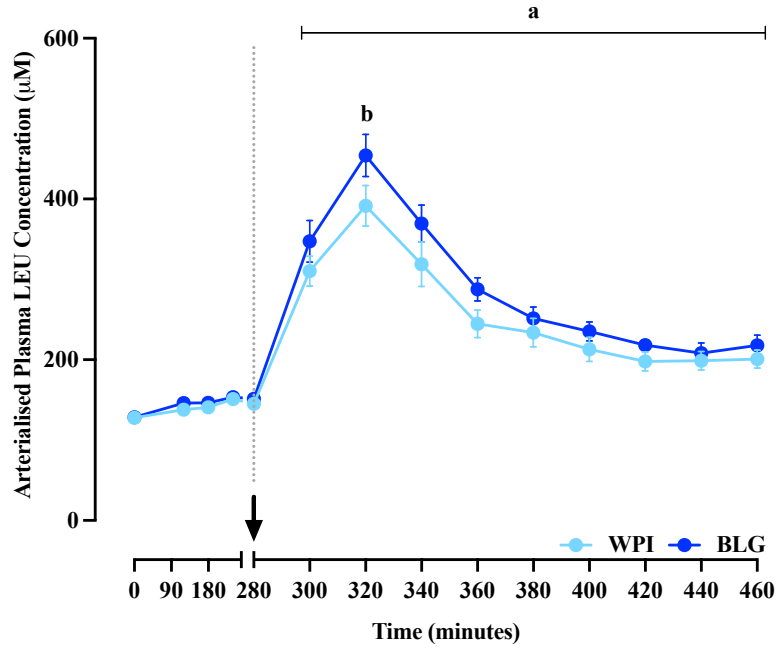


Figure 5.5 Time course effects of WPI and BLG on arterialised plasma leucine (LEU) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

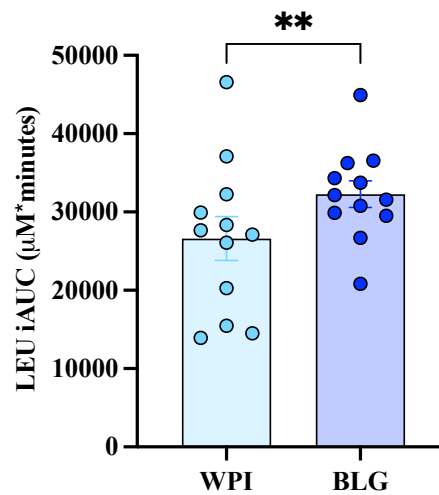


Figure 5.6 Leucine (LEU) integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. WPI: whey protein isolate. **: $P < 0.01$.

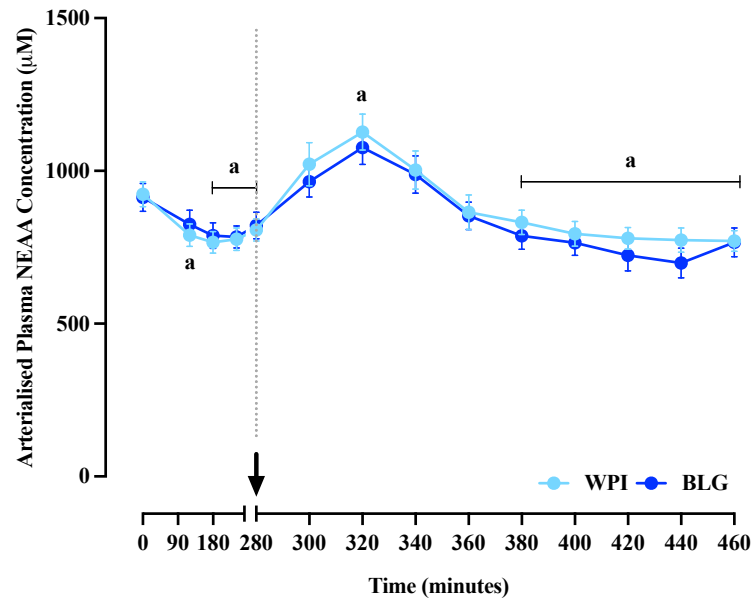


Figure 5.7 Time course effects of WPI and BLG on arterialised plasma non-essential amino acids (NEAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

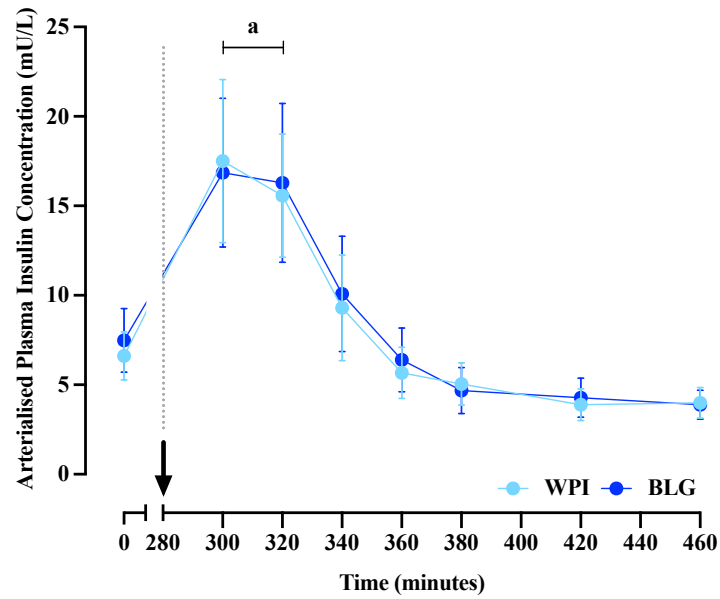


Figure 5.8 Time course effects of WPI and BLG on arterialised plasma insulin concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG: β -lactoglobulin. WPI: whey protein isolate.

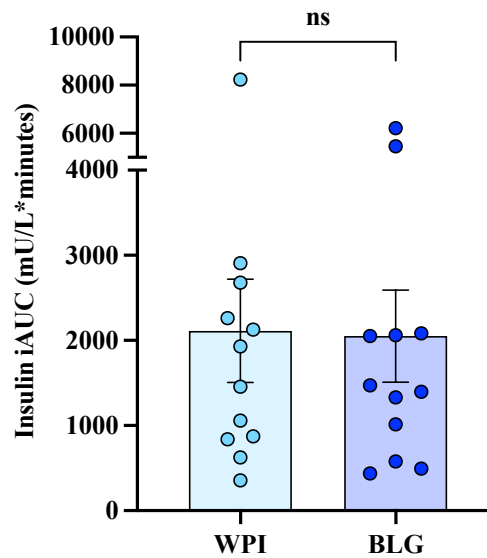


Figure 5.9 Insulin integrated area under the curve (iAUC) for WPI and BLG. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns, non-significant. WPI: whey protein isolate.

5.2.3. Plasma α -ketoisocaproate enrichments

Plasma KIC enrichment (Figure 5.10) observed a study state following the infusion of 1,2 [$^{13}\text{C}_2$ LEU] (main time effect: $P < 0.0001$). Provision of both protein feeds led to an increase in KIC enrichment which began to return to baseline towards the end of the postprandial period but remained elevated compared to the fasted state following both feeds, despite a steady state achieved. No differences in KIC enrichment were observed between supplements.

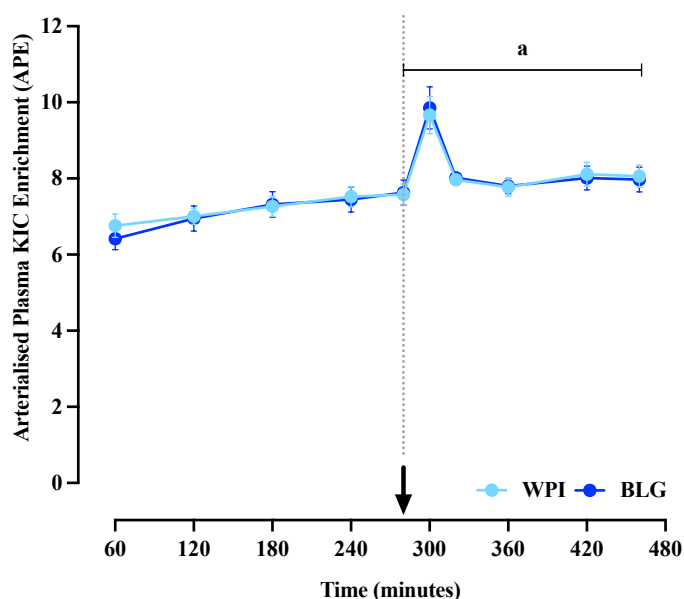


Figure 5.10 Time course effects of arterialised plasma α -ketoisocaproate (KIC) enrichments during WPI and BLG assessment visits. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). APE: atom percent excess. BLG: β -lactoglobulin. WPI: whey protein isolate.

5.2.4. Myofibrillar fractional synthetic rate

Basal myofibrillar FSR rates (i.e., MPS) did not differ between legs (i.e., fasted vs. fasted-ex leg) for WPI and BLG supplements separately or when comparing across conditions (i.e., fasted/fasted-ex leg for BLG vs. WPI). When looking at the FED state ($n = 10$; main time effect: $P < 0.0001$; supplement $\times$ time effect: $P = 0.0716$; Figure 5.11), both WPI (0.044 ± 0.005 %/h vs. 0.069 ± 0.006 %/h; $p = 0.0023$) and BLG (0.046 ± 0.006 %/h vs. 0.089 ± 0.007 %/h; $P < 0.0001$) significantly stimulated MPS from postabsorptive rates. Further, BLG was found to significantly stimulate myofibrillar FSR to a greater extent in the FED state compared to WPI ($P = 0.0464$). Similarly, in the FED-EX state ($n = 7$; main time effect: $P < 0.0001$; supplement $\times$ time effect: $P = 0.0245$; Figure 5.12), WPI (0.044 ± 0.009 %/h vs. 0.095 ± 0.008 %/h; $P < 0.0001$) and BLG (0.051 ± 0.007 %/h vs. 0.075 ± 0.003 %/h; $P = 0.0113$), but with no differences between the supplements.

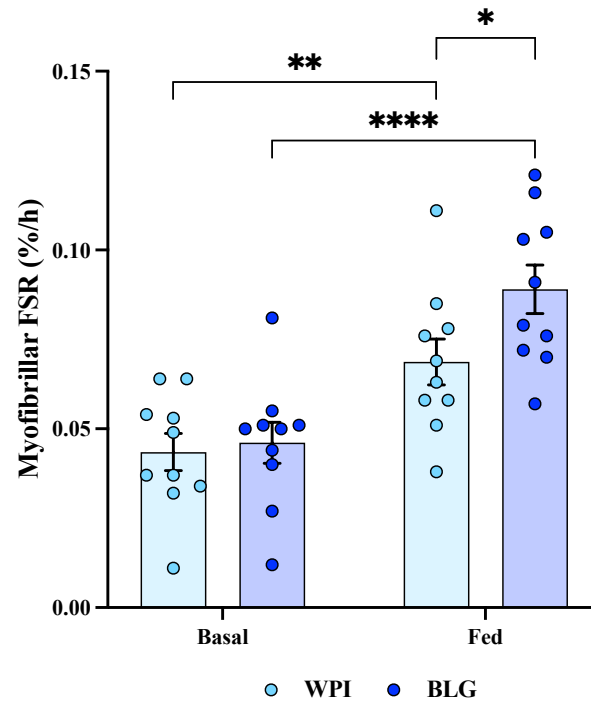


Figure 5.11 The effects of WPI and BLG on skeletal muscle myofibrillar fractional synthetic (FSR) rate in the FED ($n = 10$) state. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. WPI: whey protein isolate. *: $P < 0.05$. **: $P < 0.01$. ****: $P < 0.0001$.

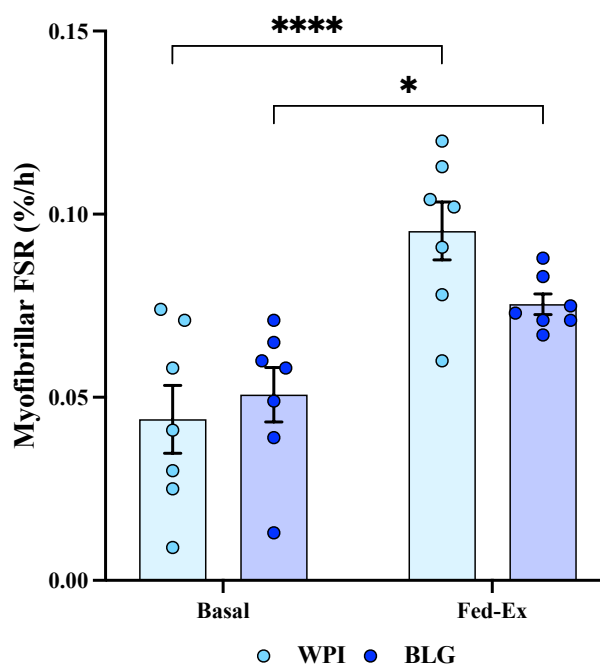


Figure 5.12 The effects of WPI and BLG on skeletal muscle myofibrillar fractional synthetic (FSR) rate in the FED-EX ($n = 7$) state. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. WPI: whey protein isolate. *: $P < 0.05$. ****: $P < 0.0001$.

5.2.5. Effects on anabolic signalling

Anabolic signalling relating to the control of MPS was assessed by measuring the phosphorylation status of the mTORC1 translation initiation substrates p70S6K1 (Figure 5.13), 4EBP1 (Figure 5.14) and RPS6 (Figure 5.15) and the phosphorylation status of elongation factor eEF2 (Figure 5.16). We observed no differences in the phosphorylation status of p70S6K1 and 4EBP1 for both BLG and WPI, in the FED and FED-EX states (all $P > 0.05$). Similarly, no differences between supplements were observed in p-eEF2 or p-RPS6 in the FED state ($P > 0.05$). In the FED-EX state, there was a statistically significant difference in p-

eEF2 depending on the protein feed (Friedman statistic = 10.09; $P = 0.0178$) with multiple comparisons (using Dunn's correction (median [lower 95% CI, upper 95% CI])) revealing significant difference in phosphorylation of eEF2 in the BLG condition (1.012 [0.9639, 1.037] vs. 0.9739 [0.9322, 0.9964]; $P = 0.03$). There was also a statistically significant difference in p-RPS6 depending on the protein feed in the FED-EX state (Friedman statistic = 20.13; $P = 0.0002$), with multiple comparisons revealing a significant difference in phosphorylation of RPS6 following both WPI (0.9850 [0.9257, 1.044] vs. 1.336 [1.146, 1.393]; $P = 0.0031$) and BLG supplements (0.9724 [0.9266, 1.073] vs. 1.233 [1.160, 1.336]; $P = 0.03$).

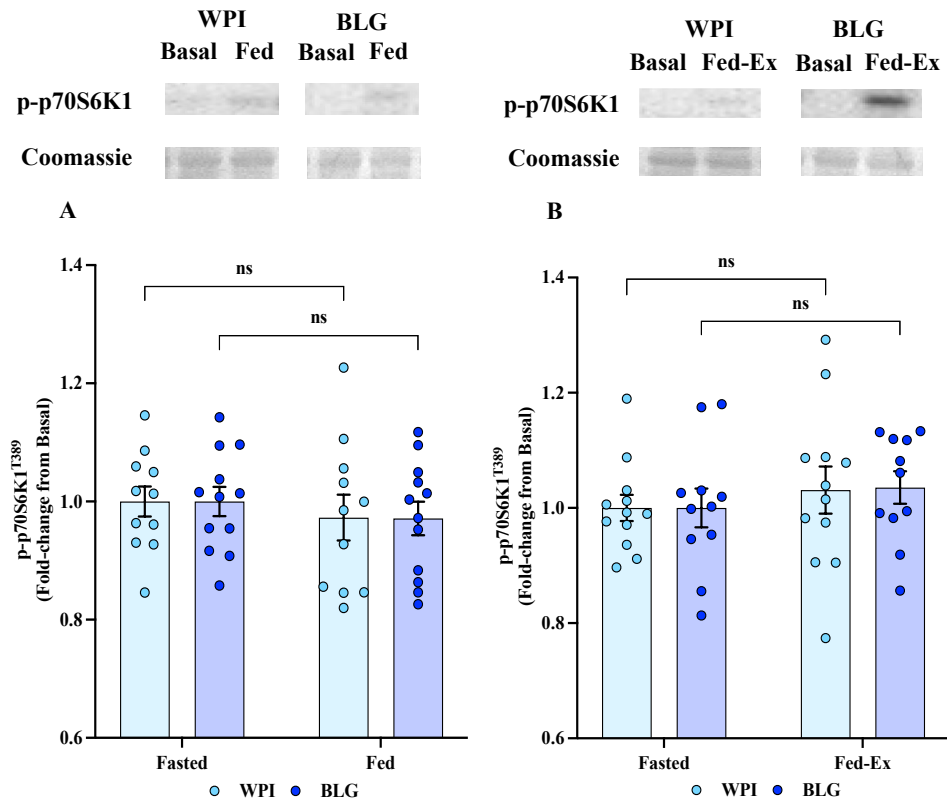


Figure 5.13 The effects of WPI and BLG on muscle anabolic signalling responses of p-p70S6K1 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate.

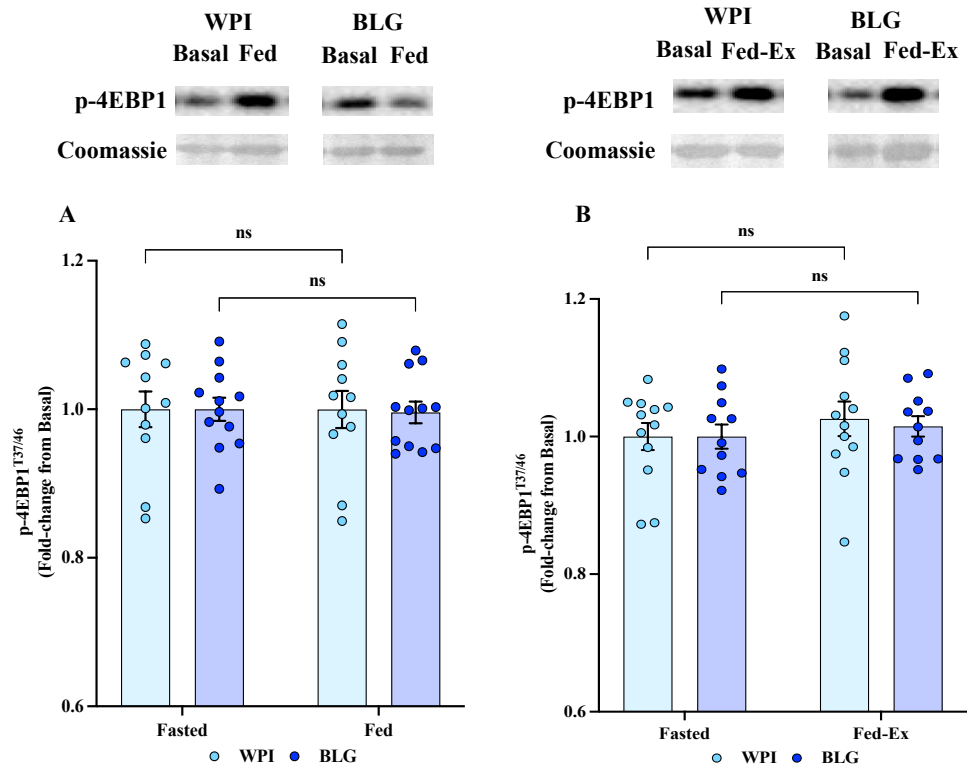


Figure 5.14 The effects of WPI and BLG on muscle anabolic signalling responses of p-4EBP1 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate.

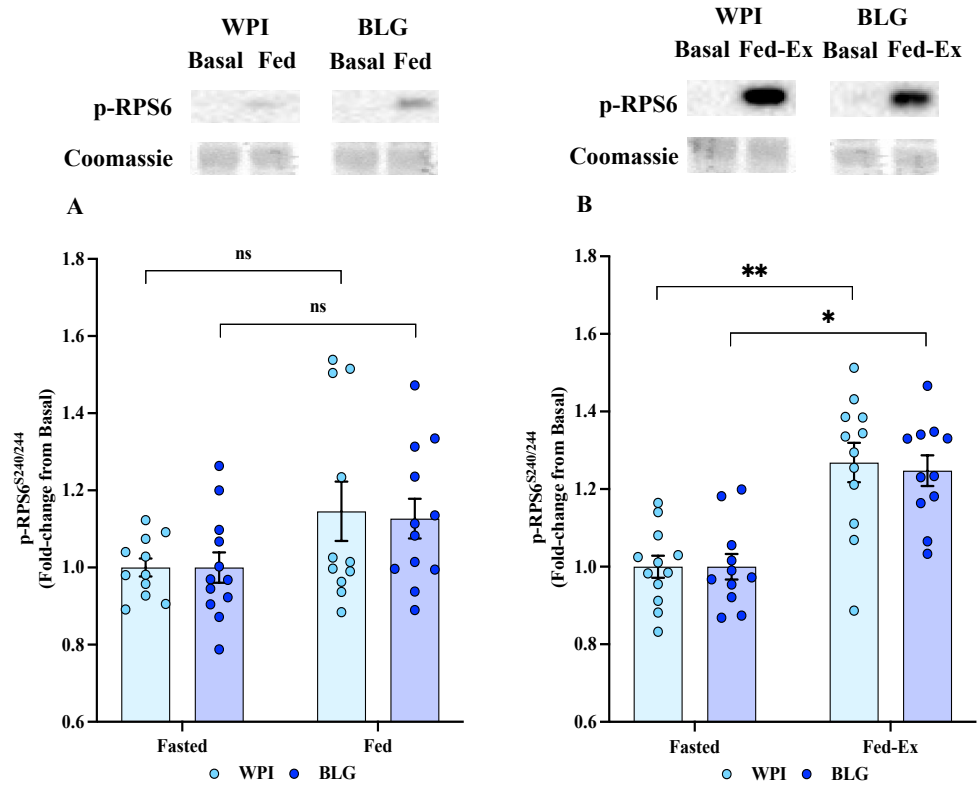


Figure 5.15 The effects of WPI and BLG on muscle anabolic signalling responses of p-RPS6 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate. *: $P < 0.05$. **: $P < 0.01$.

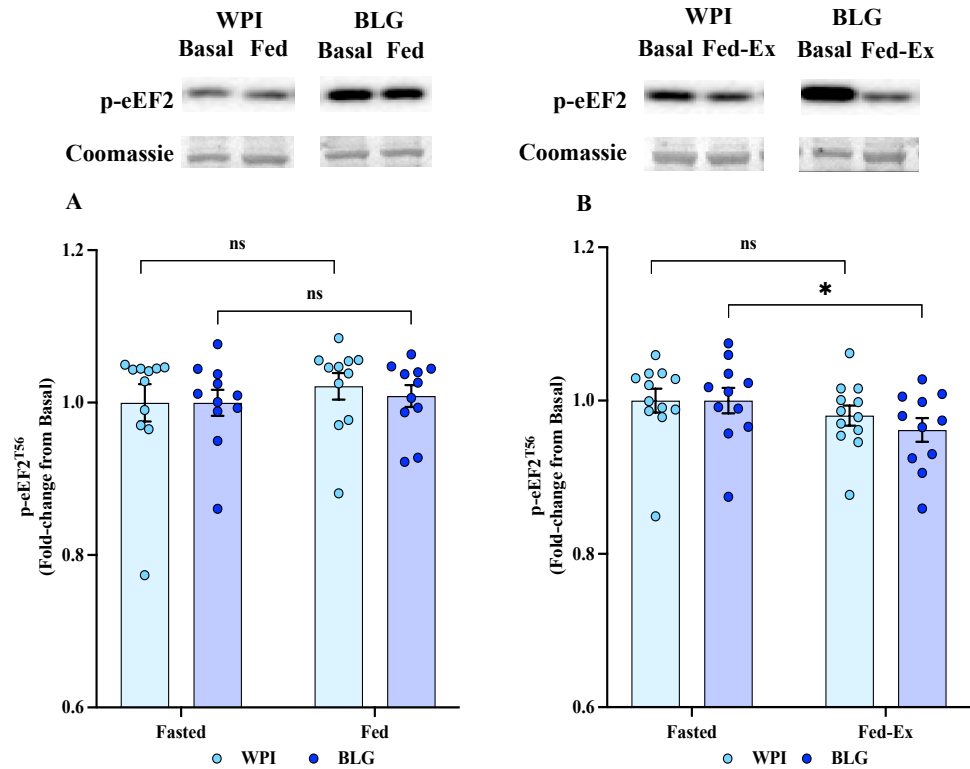


Figure 5.16 The effects of WPI and BLG on muscle anabolic signalling responses of p-eEF2 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG: β -lactoglobulin. ns: non-significant. WPI: whey protein isolate. *: $P < 0.05$.

5.3. Discussion

This study has provided novel insight into suboptimal (10 g protein) BLG supplementation on muscle anabolism in both the rested and exercised states of healthy older adult males. Specifically, we highlight myofibrillar FSR (i.e., MPS) to increase following both BLG and WPI in the FED and FED-EX state, but with greater MPS stimulation following a BLG feed in the FED state only. This builds upon our results in the previous chapter investigating the anabolic

effects of BLG (vs. WPI) in young males, where we observed equivocal stimulation of MPS in both supplements in the rested and exercised state.

Knowledge of EAA, being drivers of the muscle synthetic response has been established for ~30 years (Rennie et al., 1982; Rennie & Tipton, 2000; Smith et al., 1992; Smith et al., 1998) with LEU being central to this. Previously, we demonstrated that a low dose of LEAA (3 g, 40% LEU) comparably stimulates MPS to 20 g WP in older women (Bukhari et al., 2015). Further, as little as 1.5 g LEAA (0.6 g LEU) robustly stimulated MPS to comparable levels as 40 g WP (Wilkinson et al., 2018a), although this was not sustained over the 4-hour measurement period. Indeed, LEU-enriched milk protein (15 g, 4.2 g LEU) stimulated MPS to a greater extent compared to a mixed protein (milk and soy) supplement (15 g, 1.3 g LEU) in both the rested and exercised states (Devries et al., 2018b). Similarly, in older adults, only a LEAA (6.7 g, 2.8 g LEU) supplement stimulated MPS above basal levels compared to an EAA mix without added LEU (6.7 g, 1.7 g LEU), despite both supplements equally stimulating MPS in young adults (Katsanos et al., 2006). Our results somewhat differ from Katsanos and colleagues as we observed a suboptimal dose of WPI to still stimulate MPS despite only having 1% LEU (1 g), compared to 1.5% LEU (1.5 g) in BLG. Despite significant MPS stimulation from WPI, MPS was significantly greater in the FED state following BLG, suggesting it may be more anabolic compared to WPI in older adult males. Based on these results and work from others (Bukhari et al., 2015; Wilkinson et al., 2018a; Mose et al., 2021b), BLG could be considered as a supplement to/or in-between meals to maximise

muscle anabolism across the day, especially in older individuals who often do not meet the RDA for protein (Murphy et al., 2016a).

Several studies demonstrate that in FED (Katsanos et al., 2006; Glynn et al., 2010b) and FED-EX conditions (Koopman et al., 2008), the provision of additional LEU has no further impact upon MPS. Koopman reported that co-ingestion of LEU with carbohydrate and protein (4.7 g LEU) following PA, surprisingly, did not further elevate MPS in older men (Koopman et al., 2008). Such discrepancy in these results is likely explained by the timing of the SKM biopsies. For example, Koopman and colleagues took SKM biopsies at 6 hours post-consumption when peak MPS is captured 1–2 hours following protein ingestion, with 2–4 hours post-ingestion demonstrating a downward trend (Atherton et al., 2017). Thus, caution should be taken when comparing our results to others if biopsy times drastically differ.

PA in the form of RE (Kumar et al., 2009; Churchward-Venne, 2012; Hakkinen et al., 2001; Ivey et al., 2000; Parise & Yarasheski, 2000) or just maintaining habitual movement (Timmerman et al., 2012), is still the best-known countermeasures to combat sarcopenia especially when in conjunction with protein nutrition. This is despite anabolic responses being blunted following acute (Kumar et al., 2009) and chronic (Brook et al., 2016a; Brook et al., 2015) exercise. In the FED-EX state, we observed significant stimulation of MPS following BLG and WPI with no difference between the supplements. This may, indeed, support the notion that exercise sensitises SKM to EAA, potentially ameliorating differences in the protein feeds and prolonging the anabolic

window. For example, acute RE prolonged MPS stimulation following 3 g (Bukhari et al., 2015) and even 1.5 g (Wilkinson et al., 2018a) LEAA, with MPS falling to basal levels in the rested state. Further, 15 g milk protein enriched with LEU (1.5 g free LEU, ~3 g total LEU) led to greater stimulation in MPS 6 hours post-exercise compared to 15 g milk protein alone (~1.5 g LEU) (Holwerda et al., 2019a). Such increases in MPS from LEAA have been evidenced to remain elevated for even longer, with the administration of 10 g LEAA (3.5 g LEU) elevating MPS for 24 hours following exercise (Dickinson et al., 2014). Although we did not assess the duration of MPS longer than 3 hours following BLG supplementation, we speculate BLG may increase MPS for the whole postprandial period (i.e., ~6 hours) following exercise, although this remains to be explored.

Alteration to protein digestion kinetics has been suggested as a mechanism to contribute to age-related anabolic blunting (Mitchell et al., 2016). In the current study, we observed significant increases in basal concentrations in plasma EAA, NEAA, BCAA and LEU. We report no difference between supplements concerning EAA, NEAA and BCAA concentrations, however, peak leucaemia was greater following BLG supplementation. Furthermore, unlike the young group in the previous chapter, plasma LEU concentrations remained elevated above basal values across the whole assessment period in both experimental conditions. Similar results have been evidenced following large doses of WP in young (Moore et al., 2009a) and older (Wilkinson et al., 2018a) adults. Early findings in older adults initially suggested splanchnic metabolism may become altered with age, contributing to increased first-pass extraction of AA by

splanchnic tissues, meaning less AA can enter circulation to be utilised by other body tissues (Volpi et al., 1999; Moreau et al., 2013; Boirie et al., 1997). However, this was subsequently opposed by several studies highlighting small intakes of EAA to achieve similar plasma AA profiles in young and old individuals (Katsanos et al., 2006; Katsanos et al., 2005), whereas larger doses resulted in prolonged hyperaminoacidaemia with a slower return to basal concentrations (Mitchell et al., 2015a; Cuthbertson et al., 2005; Mitchell et al., 2015b; Wilkinson et al., 2018a; Koopman et al., 2009a). Failure to return to basal concentrations following a feed may indicate an inability of older adults to “handle” or utilise larger/certain AA in a metabolically efficient manner (Mitchell et al., 2016). This could be due to several reasons such as older adults having less whole-body muscle mass (Mitchell et al., 2012) and blunted MPS (Atherton et al., 2010a; Mitchell et al., 2015a; Cuthbertson et al., 2005; Mitchell et al., 2015b) so less EAA may be taken up by SKM, thus remaining in circulation. Further, the anticatabolic effects of insulin are reduced in older adults (Wilkes et al., 2009), resulting in a slowing of the clearance of excess AA from the plasma pool (Mitchell et al., 2016). Conversely, Koopman and colleagues highlight no differences between young and older individuals concerning to the rate of appearance of phenylalanine from intrinsically labelled casein and no differences in splanchnic AA extraction (Koopman et al., 2009a). Instead, the authors highlight peak plasma AA concentrations to be ~10–15% higher in older vs. young men (Koopman et al., 2009a; Koopman et al., 2006) which is likely indicative of a reduced whole-body AA flux in elderly humans (Short et al., 2004; Koopman et al., 2006) and/or reduced distribution volume for specific AA (Puthrasingham et al., 1996). Subsequently, this may explain the

elevated plasma LEU concentrations across the whole assessment period we observed in our older, but not young, cohort, with older adults demonstrating peak LEU concentrations to be ~17% greater in the BLG group compared to younger adults.

Phosphorylation of eEF2 and RPS6 were the only anabolic targets to become altered following BLG (both targets) and WPI (p-RPS6 only) supplementation in the FED-EX state. Although there was an absence of anabolic signalling, there was a robust stimulation of MPS following BLG and WPI in the FED and FED-EX states. As stated in the previous chapter, discordance between MPS and anabolic signalling has been previously reported (Atherton et al., 2010a; Greenhaff et al., 2008), with the use of immunoblotting to assess anabolic signalling measurements deemed not sensitive quantitative biomarkers of MPS in response to nutrition and exercise (Wilkinson et al., 2018a). Further, in the current study, the muscle biopsies occurred 3 hours following feeding; thus, we may indeed have missed peak molecular signalling measures, which seemingly occur 1–2 hours post-feed (Atherton et al., 2017; Atherton et al., 2010a). Differences may also have occurred in different targets of the mTORC1 pathway which we did not assess. For example, a recent study applied kinetic modelling to LEU-mediated signalling which highlighted total (rather than phosphorylated) levels of p70S6K1 are a primary determinant of MPS (McColl & Clarke, 2024). Despite this, most experimental studies assess the relative difference in p-p70S6K1 levels between interventions and use these data to infer MPS (McColl & Clarke, 2024). Although p-p70S6K1 indeed controls MPS, results from the model suggest differences in total levels of p70S6K1 may have

a more pronounced influence on MPS (McColl & Clarke, 2024). Resultingly, exploration of total p70S6K1 levels may warrant future examination regarding BLG supplementation.

In summary, our data from the current study expands on our results from younger adults highlighting the positive effects of a novel LEU-enriched whey protein, BLG, on muscle anabolism in older adults. BLG supplementation stimulated MPS in the FED and FED-EX states, with greater MPS stimulation observed in the FED state only compared to WPI. Based on these findings, it would be prudent to examine varying doses of BLG to observe whether there is an optimum dose of BLG for maximising muscle anabolism.

Chapter 6

6. Exploring different doses of β -lactoglobulin on skeletal muscle anabolism at rest and following acute exercise in healthy older men.

6.1. Introduction

SKM protein synthesis is a nutritionally responsive process that is robustly stimulated by dietary protein ingestion and enhanced through exercise. Early work defined the anabolic effects of mixed-meal feeding to be entirely attributable to the EAA component of the meal (Smith et al., 1992), with it shown that SKM is saturable in its response to dietary protein intake (Bohe et al., 2003; Cuthbertson et al., 2005). For example, 20 g WP was sufficient for maximal stimulation of MPS at rest and post-exercise in young men, with doses >20 g stimulating AA oxidation and ureagenesis (Witard et al., 2014). Importantly, in older age, protein quantity/dose differs with respect to achieving maximal MPS following dietary protein/AA consumption. Cuthbertson and colleagues highlighted distinct differences in the postprandial response of protein feeding between young and older males despite basal MPS rates indistinguishable (Cuthbertson et al., 2005). In young men, ingestion of 2.5–10 g EAA stimulated MPS in a dose-dependent manner, with 20 g EAA failing to

elicit any additional stimulation (Cuthbertson et al., 2005). Similarly, older men also demonstrated a dose-dependent increase in MPS, but the anabolic response was depressed and shifted to the right compared to young counterparts (Cuthbertson et al., 2005). For example, in older adults, a larger dose of EAA (40 g) failed to result in the same stimulation of MPS observed at 10 g EAA in young adults (Cuthbertson et al., 2005), highlighting decreased sensitivity and responsiveness of older SKM to dietary protein. Importantly, daily dietary protein requirements are provided relative to body mass, despite the majority of current data from acute metabolic studies are based on a per meal basis (Moore et al., 2009b; Yang et al., 2012; Breen et al., 2013; Burd et al., 2011; Churchward-Venne et al., 2012; Witard et al., 2014), thus not accounting for differences in body mass. Through performing retrospective analysis, Moore and colleagues quantified relative protein intakes from several studies providing absolute doses (Moore et al., 2015). Resultingly, protein intake required to maximally stimulate MPS in older men was identified to be ~68% and ~140% greater than younger men when expressed relative to body mass and LBM respectively (Moore et al., 2015). Thus, the authors suggested older adults may require greater dietary protein intakes (i.e., $3 \times \sim 0.40$ g/kg or ~ 1.20 g/kg/d) than their younger counterparts ($3 \times \sim 0.24$ g/kg or ~ 0.72 g/kg/d) to achieve maximal MPS stimulation throughout the day (Moore et al., 2015; Bauer et al., 2013). Notably, the delivery profile of EAA is not necessarily of major importance with regards to maximising muscle anabolism, with a bolus vs. spread EAA feed leading to identical myofibrillar FSR despite distinct differences in plasma insulinaemia and aminoacidaemia (Mitchell et al., 2015a).

Despite the above suggesting evidence of a dose-response to dietary protein and older SKM desensitising to protein intake meaning more protein/EAA is needed to achieve similar stimulation, consuming greater quantities of protein can be challenging. Older adults generally eat relatively small amounts of meat, eggs and dairy products containing high-quality protein, partially due to economic reasons but also because they are easily satiated and occasionally anorectic (Morley, 1997; Cuthbertson et al., 2005). Another considering factor is that older adults typically have a greater number of hospital stays and many live in clinical care settings where nutrition is often not optimised (Deane et al., 2020b). It would make sense, therefore, for older adults to consume protein to sufficiently raise plasma EAA concentrations to trigger maximal muscle anabolism after a single meal (Cuthbertson et al., 2005). To overcome the problem of satiety, foods with high protein/energy ratios and vitamin/mineral contents (e.g., lean meat or supplements) should be a major part of older adults' diets, especially since their energy requirements fall with age (Millward et al., 1997; Cuthbertson et al., 2005).

It has become increasingly clear that protein or AA dose-response studies support the importance of high-quality protein consumption and subsequent presence of highly anabolic EAA (i.e., LEU). For example, LEU is a major driver of MPS, so much so, that enriching low/suboptimal doses of protein/EAA with LEU robustly (and perhaps maximally) stimulates MPS compared to larger (20–40 g) doses of WP (e.g., Churchward-Venne, 2012; Churchward-Venne et al., 2014; Bukhari et al., 2015; Atherton et al., 2017; Wilkinson et al., 2018a), in addition to ~3 g isolated LEU achieving maximal muscle anabolism (Wilkinson

et al., 2013). Accordingly, we previously demonstrated that LEAA stimulates MPS in older women to a similar extent as a larger (20 g) bolus of WP (Bukhari et al., 2015). Both the LEAA and WP supplements resulted in the same peak leucaemia, despite the latter providing more than 3-fold the amount of EAA/kg of lean body mass (Bukhari et al., 2015). Further, MPS increased to a greater extent following supplementation of a LEU-enriched protein supplement (15 g milk protein, 4.2 g LEU) compared to a control drink (15 g mixed protein, 1.3 g LEU) in response to feeding ($53 \pm 5\%$ vs. $13 \pm 4\%$) (Devries et al., 2018b). Whilst MPS increased following ingestion of both 6.7 g EAA with LEU content matched to whey protein (1.7 g LEU) and an enriched LEU content (2.8 g LEU) in young individuals, such increases were only observed following ingestion of LEAA in older adults (Katsanos et al., 2006). Adding the effects of acute RE in addition to acute LEU supplementation results in a greater anabolic impact. For example, enriching a sub-optimal (10 g) protein drink with 4.2 g LEU (vs. 4.2 g alanine) led to greater MPS across 4 hours in both younger and older men following RE, with no observed age-related difference (Atherton et al., 2017). Therefore, the ability to enrich suboptimal protein/EAA with LEU indeed seems to challenge the presence of a dose-response relationship to protein feeding.

As we have demonstrated throughout the previous research studies, BLG is a high-quality WP with a high LEU content that is able to stimulate MPS in both younger and older individuals. Previous research has demonstrated that low-dose LEAA/protein has given similar MPS rates compared to larger doses, yet the effects of varying BLG doses on SKM anabolism is unknown. We therefore aimed to examine the administration of BLG at different suboptimal doses (5 g

(BLG_5) vs 10 g protein (BLG_10)) on muscle anabolism in older adult males. We hypothesised that both BLG_5 and BLG_10 would lead to significant stimulation of muscle anabolism in older adult males, but with supplementation of BLG_10 contributing to a greater SKM anabolic response.

6.2. Results

6.2.1. Plasma amino acid and insulin concentrations

Arterialised plasma EAA, BCAA and LEU concentrations increased rapidly following both BLG_5 and BLG_10, peaking at ~40 minutes post-feed. Consumption of BLG_5 increased plasma EAA concentrations (main time effect: $P < 0.0001$; time $\times$ dose effect: $P < 0.0001$; Figure 6.1) above basal levels until 60 minutes post-feed, with BLG_10 elevating plasma EAA concentrations above basal until 120 minutes post-feed. Furthermore, BLG_10 led to significantly greater peak EAA concentrations (BLG_5 vs. BLG_10: 1005 ± 49 μM vs. 1290 ± 55 μM ; $P < 0.0001$) and greater iAUC (BLG_5 vs. BLG_10: 26651 ± 2476 $\mu\text{M} \cdot \text{minutes}$ vs. 41286 ± 2721 $\mu\text{M} \cdot \text{minutes}$; $P = 0.0007$; Figure 6.2). BCAA concentrations (main time effect: $P < 0.0001$; time $\times$ dose effect: $P < 0.0001$; Figure 6.3) remained elevated above basal levels for 80 minutes post-feed in BLG_5 group and much of the postprandial period in the BLG_10 group. Greater peak BCAA concentrations were evident in BLG_10 group (BLG_5 vs. BLG_10: 706 ± 29 μM vs. 981 ± 46 μM ; $P < 0.0001$). Consumption of BLG_10 also led to greater BCAA iAUC (BLG_5 vs. BLG_10: 26112 ± 2206 $\mu\text{M} \cdot \text{minutes}$ vs. 40022 ± 2298 $\mu\text{M} \cdot \text{minutes}$; $P = 0.0003$; Figure 6.4). Plasma

LEU concentrations (main time effect: $P < 0.0001$; dose: $P = 0.0021$; time $\times$ dose effect: $P < 0.0001$; Figure 6.5) remained elevated above basal levels for the whole assessment period in both experimental groups ($P < 0.05$), with greater LEU iAUC following BLG_10 (BLG_5 vs. BLG_10: $23075 \pm 935 \mu\text{M} \cdot \text{minutes}$ vs. $30558 \pm 2112 \mu\text{M} \cdot \text{minutes}$; $P = 0.005$; Figure 6.6). Plasma LEU concentrations were significantly greater in BLG_10 group until 100 minutes post-feed ($P < 0.05$), with peak plasma leucaemia concentrations also being significantly greater than BLG_5 (BLG_5 vs. BLG_10: $311 \pm 14 \mu\text{M}$ vs. $454 \pm 26 \mu\text{M}$; $P < 0.0001$). Plasma NEAA concentrations (main time effect: $p < 0.0001$; time $\times$ dose effect: $P = 0.0003$; Figure 6.7) were indifferent between supplements, despite BLG_10 having greater peak NEAA concentrations above basal values (BLG_5 vs. BLG_10: $978 \pm 53 \mu\text{M}$ vs. $1077 \pm 55 \mu\text{M}$; $P > 0.05$). Notably, BLG_5 did not significantly increase plasma NEAA concentrations above basal values. Plasma NEAA concentrations dropped below basal levels in the postabsorptive period at 180 and 240 minutes (both $P < 0.05$) in BLG_10 group, with concentrations dropping below basal levels again in the last 80 minutes of the postprandial period for both groups ($P < 0.05$).

Plasma insulin concentrations (main time effect: $P < 0.0001$; time $\times$ dose effect: $P = 0.038$; Figure 6.8) increased above basal levels in the BLG_10 group only. There were no significant differences in insulin concentrations or iAUC (Figure 6.9) between supplements at any timepoint ($P < 0.05$).

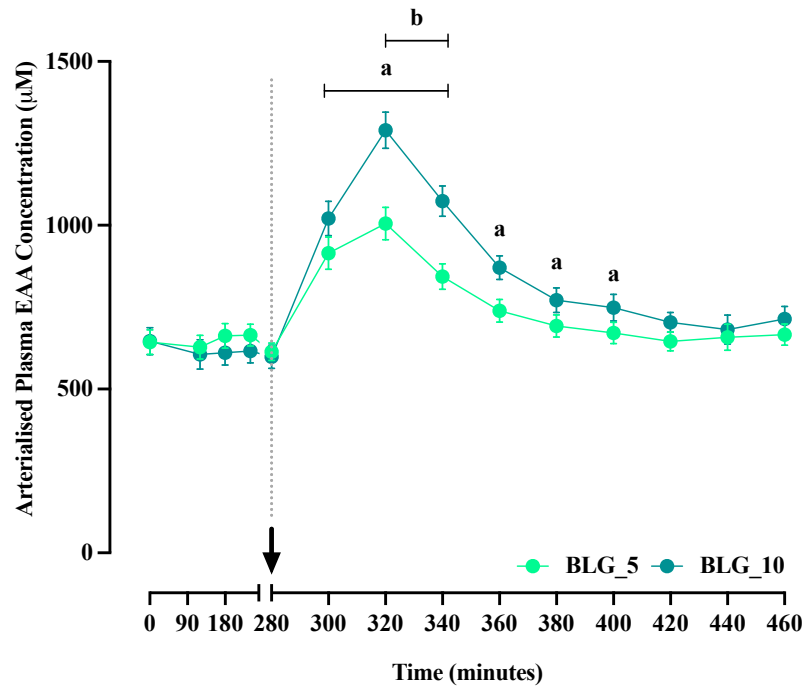


Figure 6.1 Time course effects of BLG_5 and BLG_10 on arterialised plasma essential amino acids (EAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein.

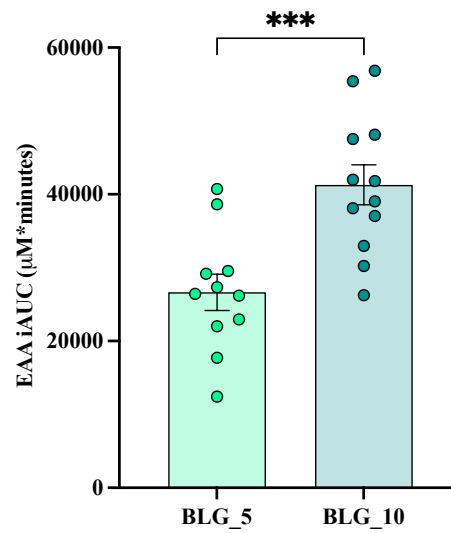


Figure 6.2 Essential amino acid (EAA) integrated area under the curve (iAUC) for BLG_5 and BLG_10. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. ***: $P < 0.001$.

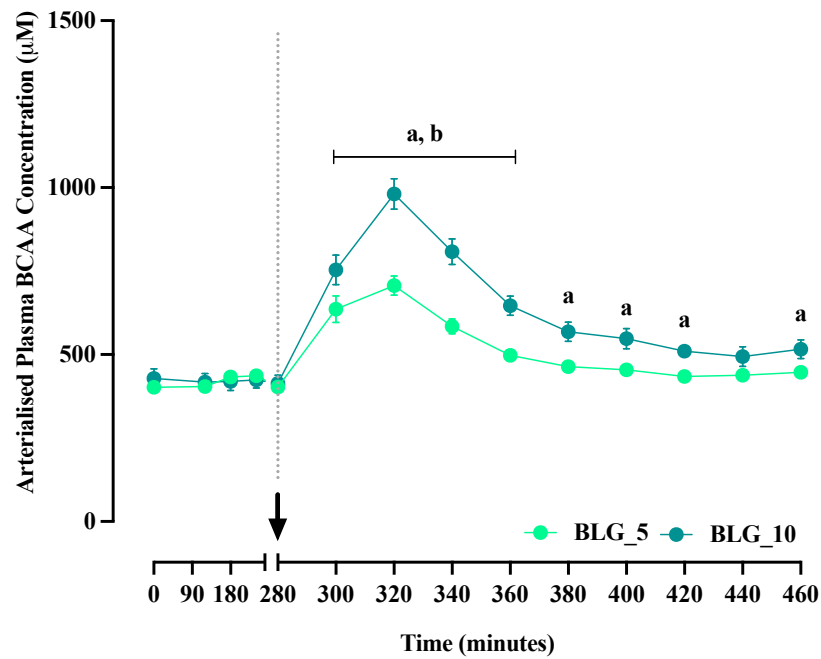


Figure 6.3 Time course effects of BLG_5 and BLG_10 on arterialised plasma branched-chain amino acids (BCAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein.

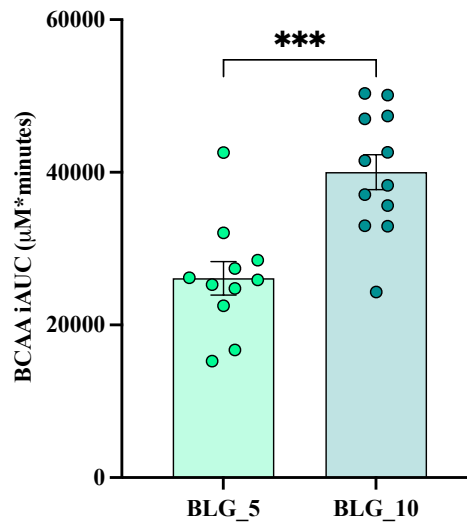


Figure 6.4 Branched chain amino acid (BCAA) integrated area under the curve (iAUC) for BLG_5 and BLG_10. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. ***: $P < 0.001$.

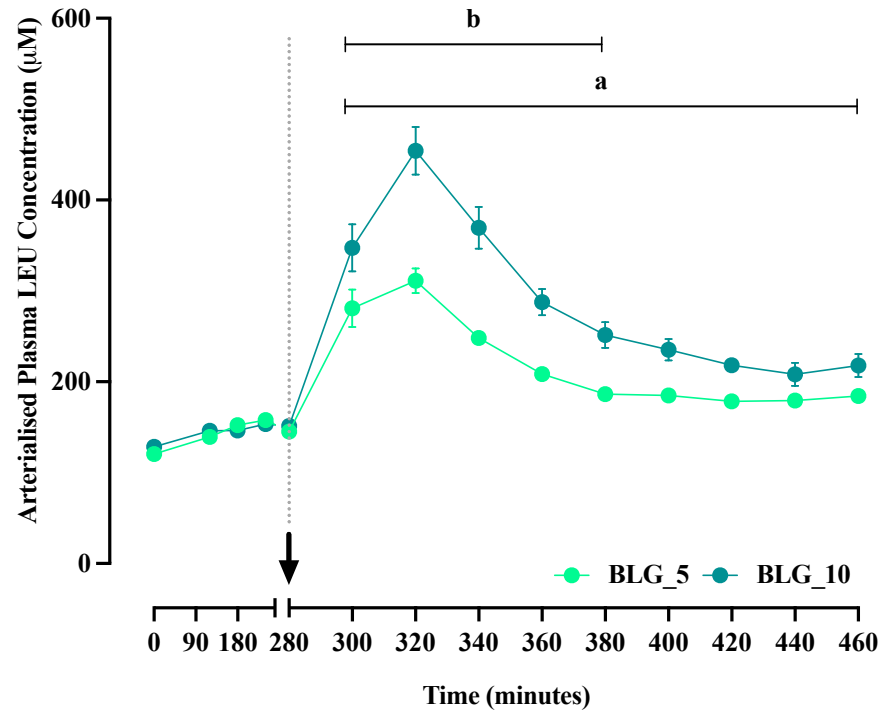


Figure 6.5 Time course effects of BLG_5 and BLG_10 on arterialised plasma leucine (LEU) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). b: significantly different between groups ($P < 0.05$). BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein.

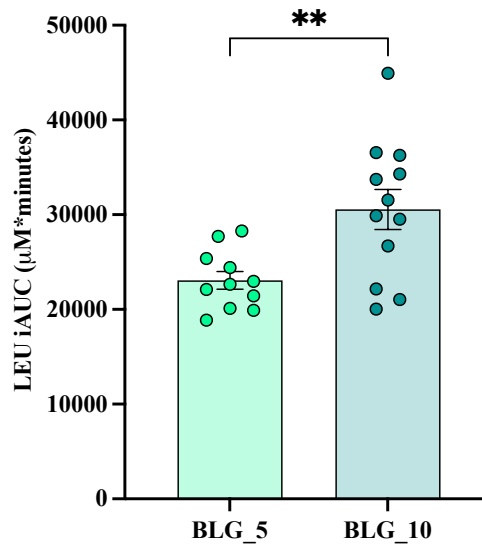


Figure 6.6 Leucine (LEU) integrated area under the curve (iAUC) for BLG_5 and BLG_10. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. **: $P < 0.01$.

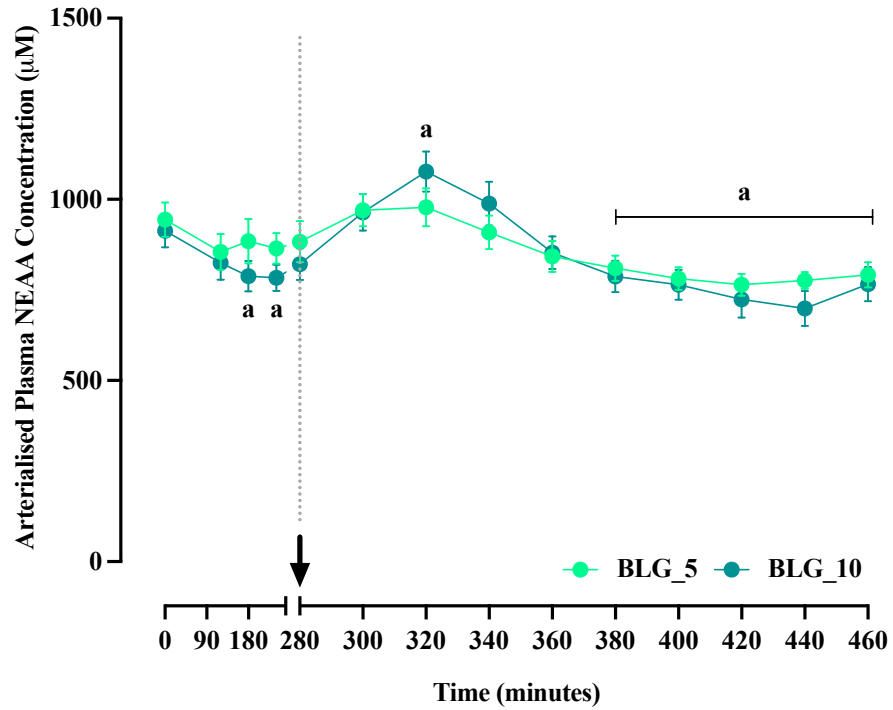


Figure 6.7 Time course effects of BLG_5 and BLG_10 on arterialised plasma non-essential amino acids (NEAA) concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein.

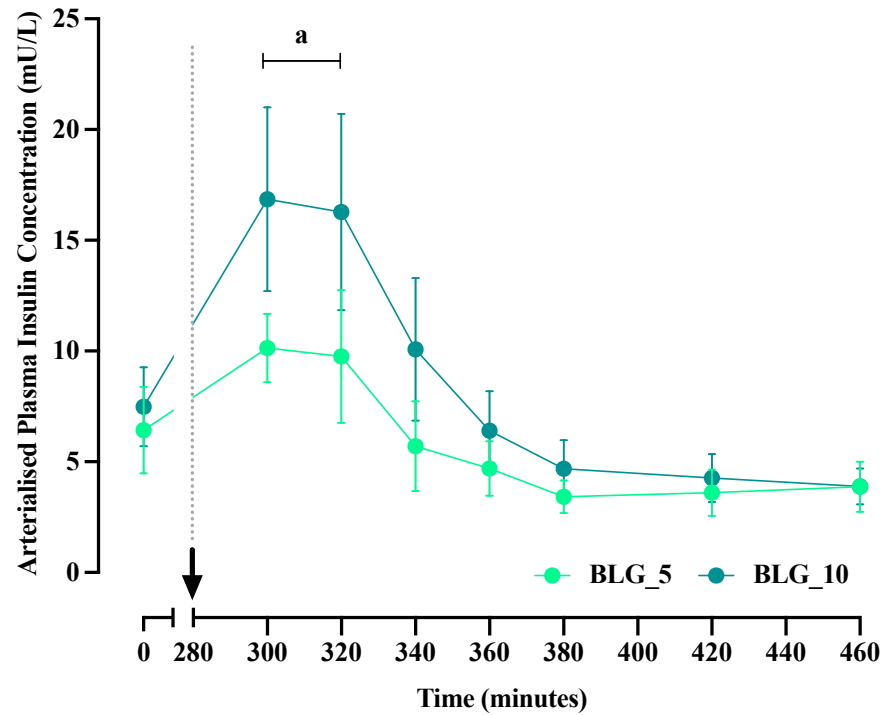
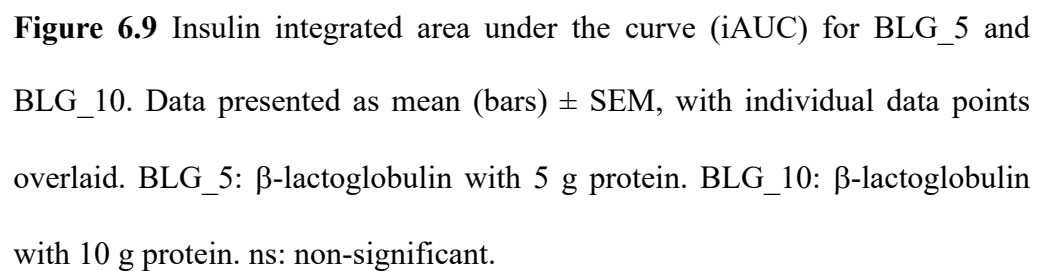


Figure 6.8 Time course effects of BLG_5 and BLG_10 on arterialised plasma insulin concentrations. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. basal ($P < 0.05$). BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein.



Plasma KIC enrichment (Figure 6.10) observed a study state following the infusion of 1,2 [$^{13}\text{C}_2$ LEU] (main time effect: $P < 0.0001$; time x supplement effect: $P = 0.06$). Provision of both protein feeds led to an increase in KIC enrichment which began to return to baseline towards the end of the postprandial period but remained elevated at some timepoints compared to the fasted state following both feeds, despite a steady state achieved. No differences in KIC enrichment were observed between supplements.

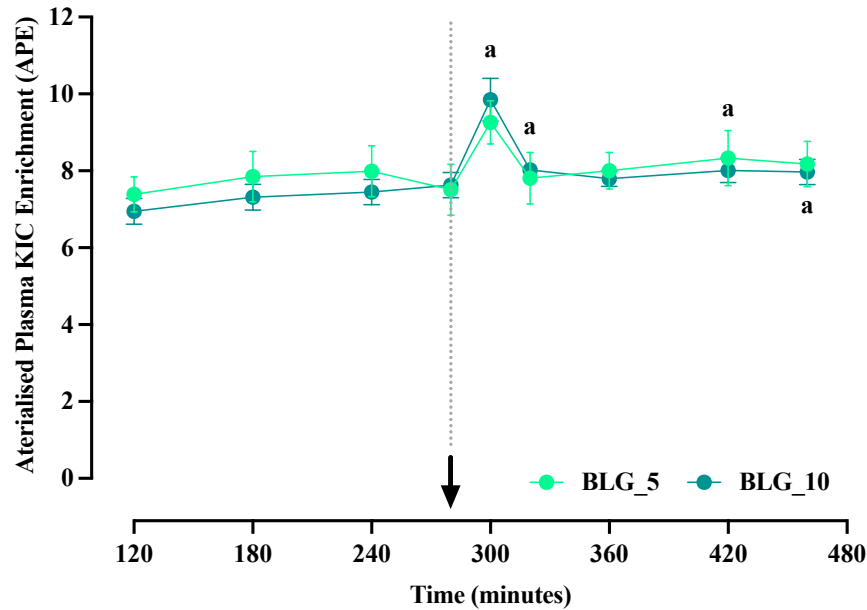


Figure 6.10 Time course effects of arterialised plasma α -ketoisocaproate (KIC) enrichments during BLG_5 and BLG_10 assessment visits. The arrow and dotted line indicate consumption of protein supplements. Data presented as mean $\pm$ SEM. a: significantly different vs. postabsorptive ($P < 0.05$). APE: atom percent excess. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein.

6.2.3. Myofibrillar fractional synthetic rate

Basal myofibrillar FSR rates (i.e., MPS) did not differ between legs (i.e., fasted vs. fasted-ex leg) for BLG_5 and BLG_10 groups separately or when comparing across conditions (i.e., fasted/fast-ex leg for BLG_5 vs. BLG_10). When looking at the FED response ($n = 10$; main time effect: $P < 0.0001$; time $\times$ dose effect: $P = 0.0104$; Figure 6.11), both BLG_5 (0.042 ± 0.006 %/h vs. 0.06 ± 0.006 %/h; $P = 0.0186$) and BLG_10 (0.046 ± 0.006 %/h vs. 0.088 ± 0.007 %/h; $P < 0.0001$) significantly stimulated MPS from postabsorptive rates. Multiple comparisons analysis revealed BLG_10 to significantly stimulate myofibrillar

FSR to a greater extent in the FED condition compared to BLG_5 ($P = 0.0056$). Similarly, in the FED-EX state (main time effect: $P < 0.0001$; supplement $\times$ time effect: $P = 0.0245$; Figure 6.12), BLG_5 ($n = 8$; 0.046 ± 0.006 %/h vs. 0.072 ± 0.003 %/h; $P = 0.0027$) and BLG_10 ($n = 9$; 0.049 ± 0.006 %/h vs. 0.081 ± 0.005 %/h; $P = 0.0003$) significantly stimulated MPS, but with no differences between the supplements.

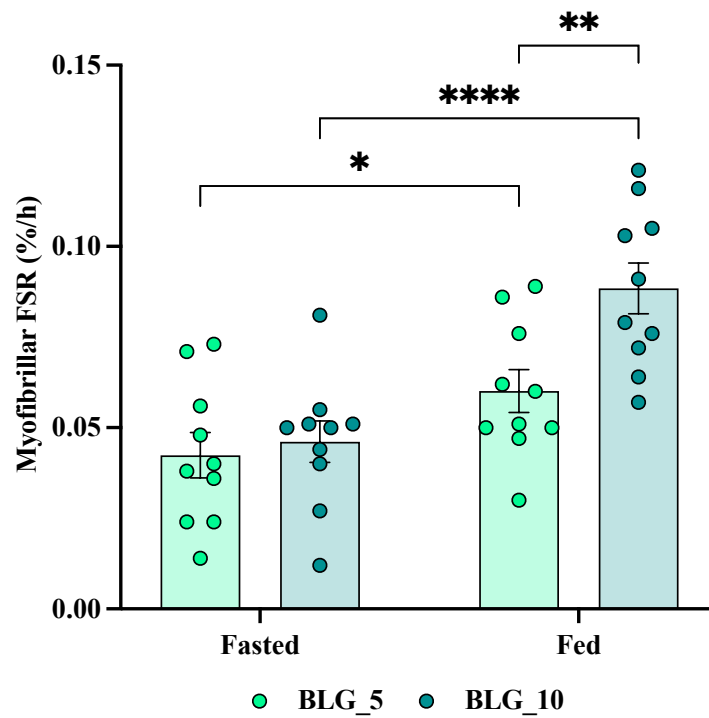


Figure 6.11 The effects of BLG_5 and BLG_10 on skeletal muscle myofibrillar fractional synthetic (FSR) rate in the FED ($n = 10$) state. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. *: $P < 0.05$. **: $P < 0.01$. ****: $P < 0.0001$.

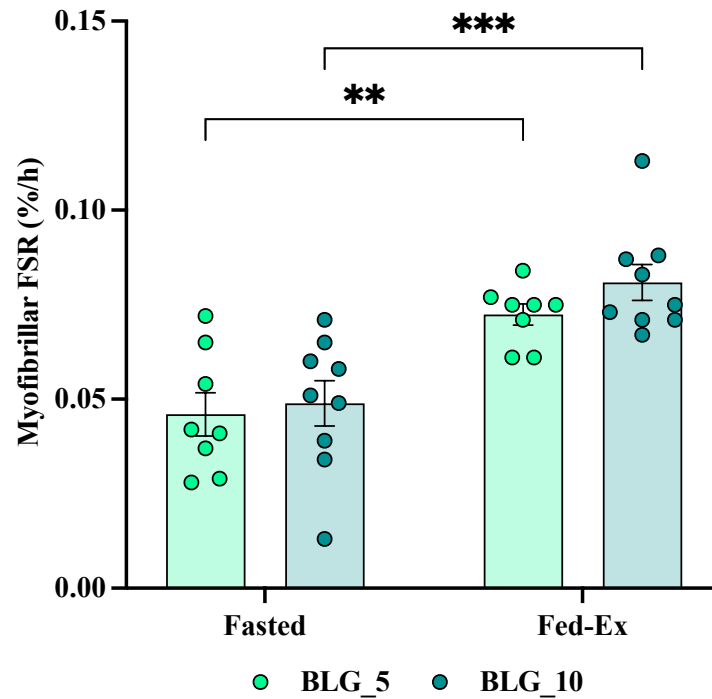


Figure 6.12 The effects of BLG_5 and BLG_10 on skeletal muscle myofibrillar fractional synthetic (FSR) rate in the FED-EX (BLG_5: $n = 8$; BLG_10: $n = 9$) state. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. **: $P < 0.01$. ***: $P < 0.001$.

6.2.4. Effects on anabolic signalling

Anabolic signalling relating to the control of MPS was assessed by measuring the phosphorylation status of the mTORC1 translation initiation substrates p70S6K1 (Figure 6.13), 4EBP1 (Figure 6.14) and RPS6 (Figure 6.15) and the phosphorylation status of elongation factor eEF2 (Figure 6.16). We observed no differences in the phosphorylation status of eEF2, p70S6K1, RPS6 in the FED state, and 4EBP1 in the FED and FED-EX state for both BLG_5 and BLG_10

(all $P > 0.05$). In the FED-EX state, the phosphorylation status of eEF2 (main time effect: $P = 0.0163$) was significantly reduced in BLG_10 ($P = 0.0477$) but not BLG_5 ($P > 0.05$). Phosphorylation status of p70S6K1 significantly increased in BLG_5 ($P = 0.0004$) but not BLG_10 ($P > 0.05$). Phosphorylation status of RPS6 was significantly increased following both supplements (BLG_5: $P < 0.0001$; BLG_10: $P = 0.0008$) with a trend for BLG_5 leading to greater phosphorylation of RPS6 ($P = 0.0625$).

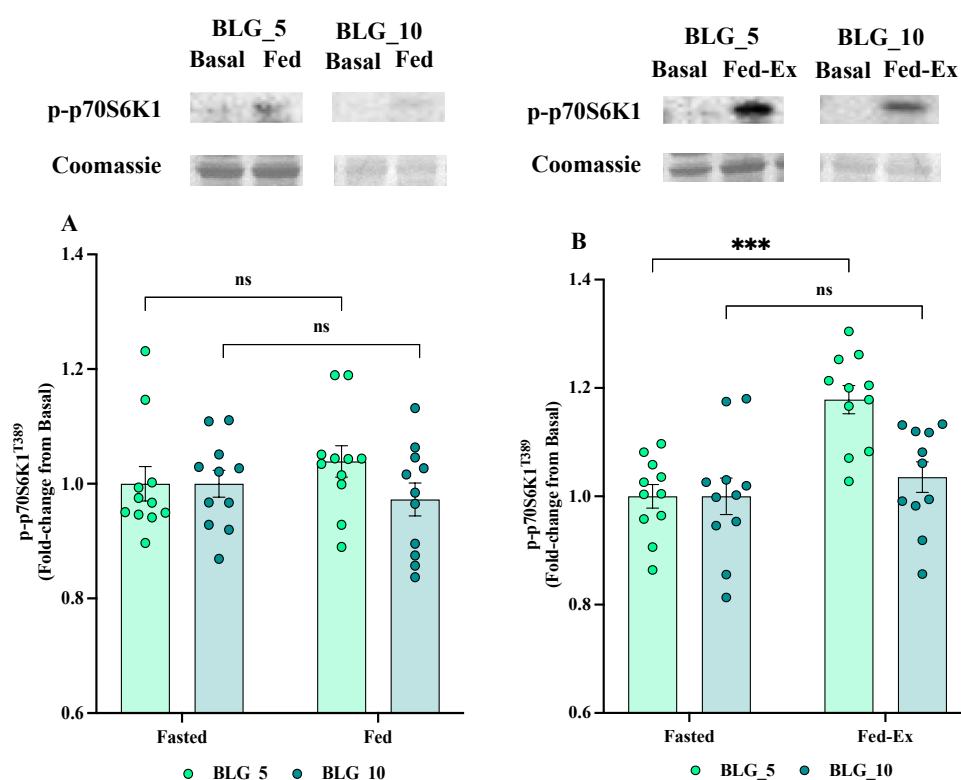


Figure 6.13 The effects of BLG_5 and BLG_10 on muscle anabolic signalling responses of p-p70S6K1 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. ns: non-significant. ***: $P < 0.001$.

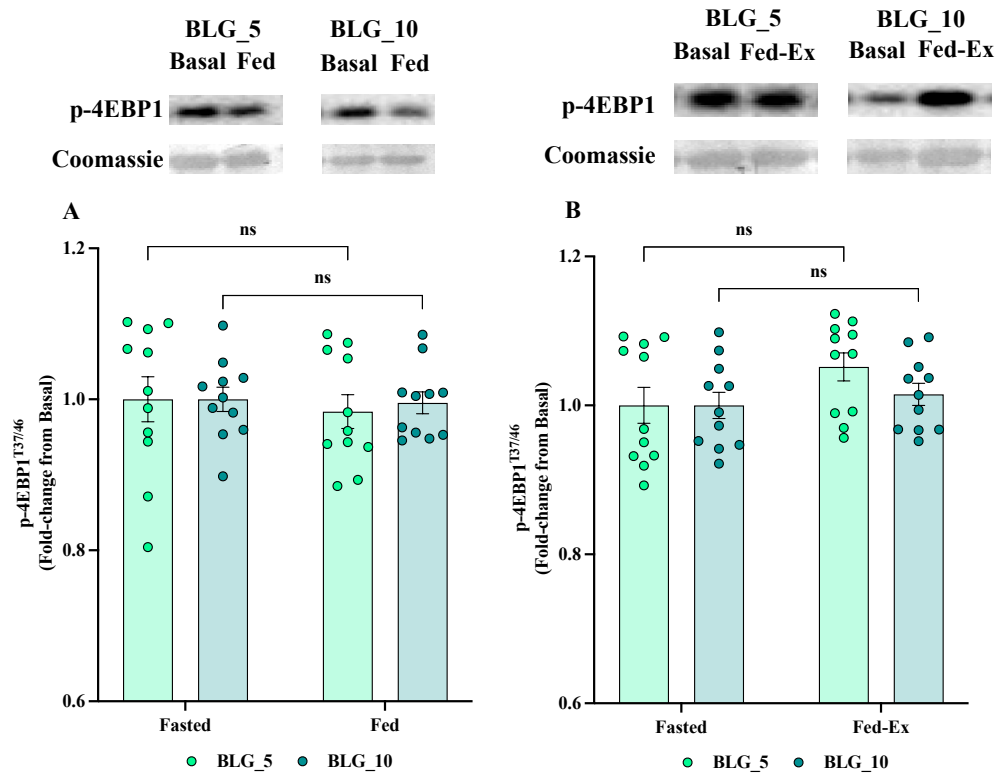


Figure 6.14 The effects of BLG_5 and BLG_10 on muscle anabolic signalling responses of p-4EBP1 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. ns: non-significant.

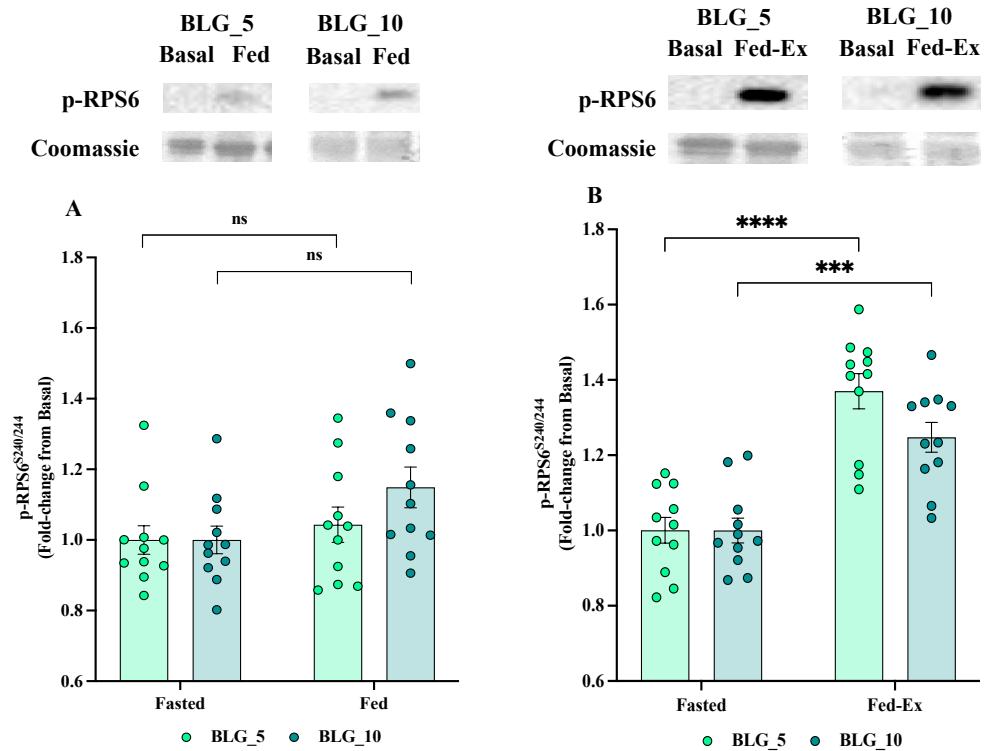


Figure 6.15 The effects of BLG_5 and BLG_10 on muscle anabolic signalling responses of p-RPS6 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. ns: non-significant. ***: $P < 0.001$. ****: $P < 0.0001$.

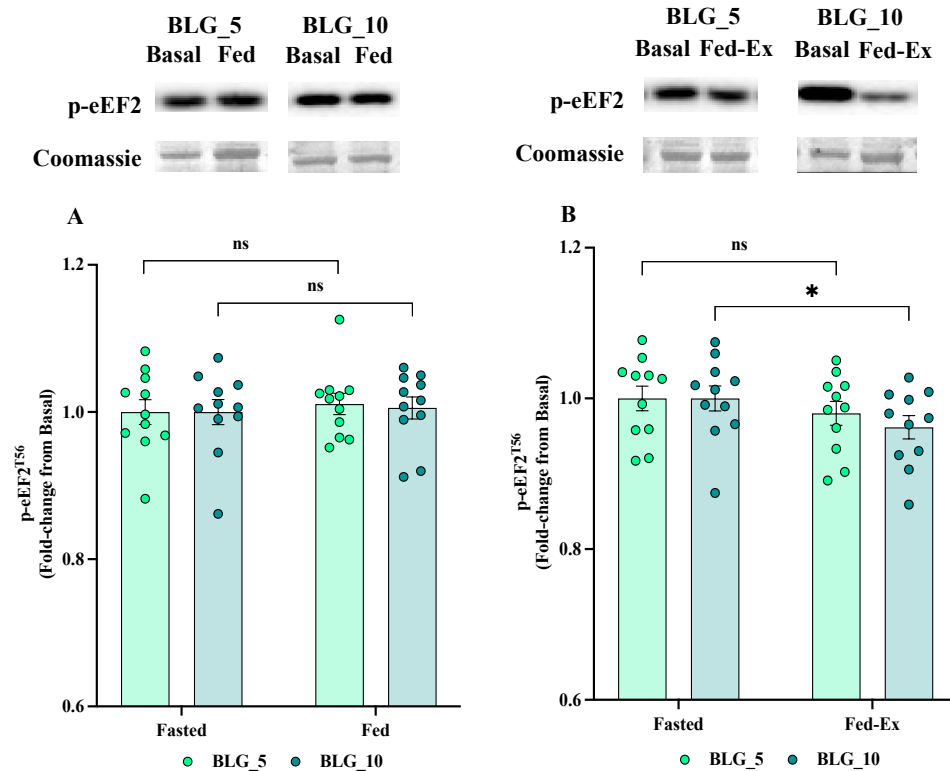


Figure 6.16 The effects of BLG_5 and BLG_10 on muscle anabolic signalling responses of p-eEF2 to FED (A; $n = 11$) and FED-EX (B; $n = 11$). Responses were log-transformed and normalised to basal-fasted biopsy. Data presented as mean (bars) $\pm$ SEM, with individual data points overlaid. BLG_5: β -lactoglobulin with 5 g protein. BLG_10: β -lactoglobulin with 10 g protein. ns: non-significant. *: $P < 0.05$.

6.3. Discussion

The current study aimed to exam different doses of BLG, both deemed suboptimal (5 vs. 10 g protein), on SKM anabolism in older adult males. In the FED state, MPS was significantly stimulated following both BLG doses, but to a significantly greater extent following BLG_10. Significant stimulation of MPS

was also evident in the FED-EX state, this time with the absence of any difference between supplements. Plasma EAA, BCAA and LEU profiles were superior (i.e., greater peak concentrations) following BLG_10, but with no differences between supplements observed in plasma insulin concentrations. Results regarding anabolic signalling were mixed, with differences in signalling responses occurring in the FED-EX but not FED state.

Despite observing significant MPS stimulation following BLG_5, supplementation of BLG_10 led to greater MPS rates suggesting our results support the notion of a dose-response to protein feeding in the FED state. However, this should be treated with caution as we did not examine BLG doses larger than 10 g protein which would be needed to establish a true dose-response relationship following BLG consumption. Similar results were exhibited by Cuthbertson et al. who, despite having a depressed synthetic response, observed older muscles to display a dose-response to consumption of 2.5–40 g EAA (Cuthbertson et al., 2005). However, recent studies have demonstrated the absence of a dose-response relationship through low-dose LEAA equally stimulating MPS to larger doses of WP. For example, in the early stimulation phase (0–2 hours) of the FED state, 3 g LEAA (40% LEU) equally stimulated MPS to 20 g WP in older women (Bukhari et al., 2015), with 40 g WP offering no trophic advantage to just 1.5 g LEAA (0.6 g LEU) (Wilkinson et al., 2018a). Similar results were also evidenced in young individuals with 6.25 g LEU-enriched (3 g LEU) WP stimulating MPS to the same extent as 25 g WP 1–3 hours following feeding (Churchward-Venne et al., 2012). Notably, we may have observed a difference between MPS rates from BLG_5 and BLG_10 in the

FED state due to the timing of SKM biopsies. We speculate that we may have observed similar rates of MPS from both supplements if we sampled SKM earlier in the synthetic response (i.e., 1–2 hours), as MPS exhibits a downward trend at timepoints after 2 hours post-feed (Atherton et al., 2010a).

Layering exercise on top of feeding ameliorated differences in the MPS response between supplements, with equivalent myofibrillar FSR observed between BLG_5 and BLG_10 in the FED-EX state. This is unsurprising, due to the potent effects RE has on sensitising muscle to AA and extending the anabolic window (Burd et al., 2011; Atherton & Smith, 2012). For example, the addition of RE extended the equivocal stimulation of MPS (across 4 hours) following 3 g LEAA vs. 25 g WP (Bukhari et al., 2015). Additionally, 1.5 g LEAA equally stimulated MPS across 4 hours compared to 6 g LEAA and 40 g WP following RE (Wilkinson et al., 2018a). When examining over a longer postprandial period, consumption of 15 g milk protein plus 1.5 g free LEU following RE led to greater MPS rates over 6 hours compared to 15 g milk protein without added LEU (Holwerda et al., 2019a). Indeed, it may be argued that over prolonged postprandial periods (i.e., 6 hours), lower doses of protein/LEAA may not sufficiently prolong stimulation of MPS meaning more regular doses of low-dose protein or larger quantities of protein should be consumed. The former may be of more interest to those in clinical care settings where individuals often cannot consume large quantities of protein. Notably, in such care settings, protein nutrition should be combined with some form of exercise (i.e., RE or neuromuscular electrical stimulation) whenever possible (Deane et al., 2020b).

The robust stimulation of MPS evidenced at such a low quantity of BLG (and LEAA, e.g., Bukhari et al., 2015; Wilkinson et al., 2018a) may be surprising due to previous research demonstrating a dose-response relationship to protein feeding (Bohe et al., 2003; Cuthbertson et al., 2005), with older adults needing larger quantities of protein (Moore et al., 2015). However, there is an explanation for this phenomenon over acute periods of measurements (i.e., 90–120 minutes) (Wilkinson et al., 2018a). Although such small doses of LEAA or isolated AA (i.e., free-LEU) lead to increased MPS, all EAA are needed for myofibrillar FSR and so must be derived from other pools (i.e., plasma and/or intracellular pools) if they become rate limiting, via whole-body or tissue protein breakdown to contribute to myofibrillar FSR (Wilkinson et al., 2018a; Stokes et al., 2018). This was evident in earlier flooding studies of a single bolus EAA (i.e., LEU, phenylalanine, threonine) without the other EAA, through declines in other EAA such as intramuscular valine (-30% (Smith et al., 1992)) and threonine (-20% (Smith et al., 1998)). Again, upon provision of low-dose WP (6.25 g, ~3 g EAA), blood valine, isoleucine and total EAA concentrations were observed to fall despite increased MPS stimulation (Churchward-Venne et al., 2014). Although having no additional nutritional value, β -hydroxymethylbutyrate (HMB) supplementation increased MPS to comparable extents as LEU supplementation and suppressed MPB, despite no exogenous EAA (Wilkinson et al., 2013). Therefore, it is clear from the literature that in the short term, the absence of additional EAA does not acutely limit MPS.

The delivery profile of protein/AA has been proposed to influence SKM anabolism to nutrition, with rapid aminoacidaemia being associated with large

boluses of fast-absorbing protein (i.e., WP) thought to be key to maximising the muscle anabolic response (Mitchell et al., 2015b). For instance, milk protein concentration is more rapidly digested and absorbed compared with micellar casein, but more slowly digested and absorbed compared with WP (Gorissen et al., 2020). Interestingly, over the entire postprandial period (5 hours), a greater fraction of phenylalanine appearance in circulation was evident after milk protein ingestion ($65 \pm 13\%$) when compared with WP ($57 \pm 10\%$) or casein ($45 \pm 11\%$) ingestion (Gorissen et al., 2020). These findings indicate that despite ingestion of a dietary protein that is rapidly digested and absorbed (e.g., WP), this does not necessarily result in the greatest plasma availability of dietary protein-derived phenylalanine (Gorissen et al., 2020) and perhaps MPS – especially across the whole postprandial period. It has previously been suggested that the more rapid digestion of soy protein when compared with milk protein resulted in more AA being directed towards deamination pathways and liver protein synthesis (Fouillet et al., 2009). In the current study, as expected and previously observed (e.g., Churchward-Venne et al., 2012; Churchward-Venne et al., 2014; Wilkinson et al., 2018a), postprandial availability of plasma EAA, BCAA and LEU increased in circulation with an increase in the protein dose. Unsurprisingly, BLG_10 demonstrated greater peak plasma EAA, BCAA and LEU concentrations compared to BLG_5. Such differences in plasma EAA and LEU concentrations may aid explanation of greater MPS rates following BLG_10 in the FED state, with the potential for greater quantities of EAA (especially LEU) to be incorporated into SKM. Like the previous chapter, plasma LEU concentrations remained elevated above baseline in both experimental conditions throughout the study. As discussed previously, such

elevations in LEU concentrations throughout the duration of the assessment period could be indicative of a reduced whole-body AA flux in older adults (Short et al., 2004; Koopman et al., 2006) and/or reduced distribution volume for specific AA (Puthrasingham et al., 1996), but this requires further investigation.

Disparity between anabolic signalling and MPS rates was evidenced in the current study, with no alterations in phosphorylation status observed in the FED state for examined targets despite increased MPS. We did, however, observe differences in phosphorylation status following FED-EX of p-RPS6 following both supplements, p-p70S6K1 following BLG_5 and p-eEF2 following BLG_10, likely due to the superior nutrition plus exercise stimulatory effects. In older individuals performing RE (60–90% 1RM), although not significant, older muscle seems to be “delayed” in initiating signalling responses to anabolic stimuli compared to younger individuals (Kumar et al., 2009). This may explain why we have observed changes to phosphorylation status to investigated targets in research studies 2 and 3, but not study 1 (young individuals). Further, unlike young males, the authors demonstrated no relationship between myofibrillar FSR and p70S6K1 phosphorylation in older individuals (Kumar et al., 2009), highlighting the blunted response and discordance of MPS signalling in older adults.

To conclude, the current study highlights supplementation of BLG_5 and BLG_10 stimulated MPS in both the FED and FED-EX states of older males. We demonstrate potential evidence for a dose-response to BLG in the FED state

only, with BLG_10 leading to a significantly greater MPS response. In the FED-EX state, similar MPS rates were evident between BLG_5 and BLG_10, highlighting the potency of layering exercise on top of protein nutrition for muscle anabolism, even at low doses of protein. Future research should be directed into determining the optimal BLG dose to maximise muscle anabolism in older adults, in addition to examining whether these acute results translate into improvements in SKM mass in conjunction with RET.

Chapter 7

7. Final discussion and conclusion

Global population demographics shifting towards a more ageing world is one of the most pressing issues facing the western world. The rate of population ageing does not seem to be slowing, with predictions suggesting that by 2050, the global population of those aged 65 and 85 years is set to increase by 188% and 551% respectively (McLeod et al., 2016), with it being projected that one in seven people in the UK will be over 70 years of age in 2040 (Centre for Ageing Better, 2020). There are many social, clinical, and economic implications linked with an ageing world, with many to do with associated declines in independence and quality of life in older adults. Resultingly, interventions which may prevent declines in health and well-being such as allowing individuals to sustain normal activities of daily living and functional independence must be sought, developed, and implemented.

Chronic disease status is influenced by genetic and other pre-disposing factors which cannot be altered. However, multiple lifestyle factors can be influential in minimising risk factors related to the development of chronic disease, especially in older adults. One main consideration of healthy ageing is the number of years an individual can live disability-free (i.e., disability-free life expectancy), with those who present with disability early in life but live to an old age likely have a lesser quality of life compared to an individual who presents with disability later in life (Centre for Ageing Better, 2020). In recent years, disability-free life expectancy has stalled rather than improved, especially

in women, despite significant advances in health care (Centre for Ageing Better, 2020). Of course, lifestyle choices are cumulative throughout life; thus, older people need to adopt positive lifestyle and nutrition practices that will maximise their chances of healthy, active, and independent living with ageing.

Reduced SKM mass and function (i.e., sarcopenia) is one of the most significant and visually apparent changes with ageing. Indeed, sarcopenia is a multifactorial disease which is severely accelerated by physical inactivity (Doherty, 2003) and is a significant predictor of age-related disability and mortality rates (Cruz-Jentoft et al., 2010; Cruz-Jentoft et al., 2019). Large population-based studies have reported sarcopenia prevalence to be anywhere between 8% and 50% in people over 50 years, depending on ethnicity, setting, age and diagnostic methodology (Abellan van Kan, 2009). The loss of SKM mass becomes noticeable after the age of 50 years in most individuals, which further accelerates with ageing, reducing muscle mass from ~45% of body mass in younger adult humans to ~27% in older adults (Bilski et al., 2022). This decline in muscle mass, predominantly at the expense of fast-twitch type II muscle fibres (Mitchell et al., 2012), does not fully explain declines in muscle function as muscle strength losses are $2\text{--}5 \times$ greater than declines in muscle mass (Mitchell et al., 2012; Lauretani et al., 2003). Consistent levels of SKM mass are typically observed during the early years of life until ~50 years (with the exception of disease), where the muscle begins to significantly atrophy at rates of ~1% per year in men and ~0.5% per year in women (Mitchell et al., 2012). Around 30% of an individual's peak muscle mass is lost by the age of 80 years, with this loss drastically exacerbated by physical inactivity and poor nutrition (Janssen et al.,

2000). It is no surprise that age-related muscle wasting and loss of function has both severe physiological and psycho-social problems including the loss of independence, depression, increased risk of frailty and falls, sedentarism and increased risk of all-cause mortality (Wilkinson et al., 2018b).

One metabolic feature contributing to sarcopenia is the presence of SKM anabolic resistance. The premise of anabolic resistance centres around aged muscle displaying a blunted effect to increases in MPS and suppressions in MPB in response to the key environmental stimuli regulating muscle maintenance i.e., protein intake (Cuthbertson et al., 2005; Moore et al., 2015) and exercise (Kumar et al., 2009; Wall et al., 2015). It is well recognised anabolic resistance is a multifaceted condition that presents resulting from older age, but also in response to periods of inactivity and disease. The molecular dysregulation of MPS and MPB with advancing age presents a key role in the development of anabolic resistance, and subsequently, sarcopenia, which can influence the anabolic responses to exercise, nutrition, and the upstream drivers of these processes (Wilkinson et al., 2018b).

Following consumption of dietary protein (or AA), MPS is increased temporally and transiently. Regardless of protein source, SKM is receptive to the EAA component of a protein meal, in particular LEU. The AA-induced stimulation of MPS is purportedly dose-dependent with maximal anabolic stimulation being achieved at 20–40 g protein or 10–20 g EAA (Cuthbertson et al., 2005; Moore et al., 2009a; Moore et al., 2015; Witard et al., 2014) depending on an individual's age, activity levels, and health/disease status. However, questions

have been presented regarding whether there is, in fact, a dose dependent response to AA intake due to the ability of LEU to robustly stimulate MPS in isolation (Smith et al., 1992) and in a LEU-enriched “suboptimal” protein/EAA dose (Atherton et al., 2017; Bukhari et al., 2015; Wilkinson et al., 2018a). It may, therefore, be that the dose-response of MPS to protein/EAA is not driven by protein quantity *per se* but instead by the quality of the protein, and specifically the LEU content. Throughout this thesis, and in line with most existing literature, we have highlighted the research studies performed and in our narrative review, the potency of LEU for enhancing muscle anabolism in both younger and older adults.

Exercise is without a doubt one of, if not the most, important lifestyle factors an individual can integrate into their lives to improve their physical and mental wellbeing. As with the provision of dietary protein, RE can stimulate MPS rates in both younger and older individuals with the magnitude of MPS seemingly dependent on both workload and intensity of the RE; albeit blunted in older adults. It should be noted that not all older adults are able to perform heavy RE or even the action of RE. However, comparable magnitudes in MPS stimulation were evidenced at low- vs. high-intensity RE (30% 1RM vs. 90% 1RM) but only when exercise was performed to failure (Burd et al., 2010). The lifting of heavy weights may, therefore, be escaped by increasing the volume of work at a lower intensity which may overcome the blunted MPS response and represent an alternative approach to stimulate SKM hypertrophy. The provision of dietary protein is, of course, critical for muscle adaption (i.e., maximising muscle hypertrophy) in response to RE. This is exemplified by a greater prolonged

elevation in MPB, which induces a negative protein balance when RE is performed in the absence of protein/EAA nutrition (Biolo et al., 1995; Phillips et al., 1997). RE heightens the anabolic sensitivity of SKM, with both the magnitude and duration of the MPS response enhanced through increasing the availability of dietary EAA post-exercise and delaying the onset of muscle full. Sufficient protein/EAA intake must, therefore, complement exercise to promote positive NPB and subsequent muscle hypertrophy via cumulative muscle protein accrual following each bout of resistance exercise over time (Atherton & Smith, 2012; Pennings et al., 2011b; Stokes et al., 2018).

Our results in each of the experimental chapters of this thesis highlight the potency of BLG (a novel WP rich in EAA, especially LEU) for stimulating MPS in both rested and acute exercise conditions, at a suboptimal dose. We highlight such results in both young and older adult males. It came as no surprise that we observed no differences between BLG and WPI supplementation concerning SKM anabolism in the rested and exercised states of young males. Young SKM is highly receptive to protein nutrition and exercise, thus observing positive responses of MPS and plasma AA concentrations to suboptimal (10 g protein) BLG supplementation allowed for “proof of concept” before applying the study to older adults.

Arguably, despite our results and previous research highlighting positive SKM anabolic response to LEU-enriched protein/EAA, even in young muscle, consumption of low protein doses is not necessarily of benefit to younger compared to older adults. This is due to increased satiety (i.e., feeling full after

a meal) and the presence of clinical care being uncommon in younger populations. Despite this, BLG supplementation may still be of additive benefit for young individuals engaged in sport/exercise to maximise muscle adaption/recovery, or for those with dietary restrictions making it difficult to consume optimal quantities of protein (e.g., vegetarianism).

In comparison, we highlight greater stimulation of MPS following BLG (vs. WPI) supplementation in the rested state of older adult males. Although we still observed a significant stimulation in MPS following WPI supplementation (1.0 g LEU) from basal values at rest, the greater MPS response from BLG (1.5 g LEU) highlights the crucial impact superior LEU content (i.e., protein quality) may play in stimulating MPS at low doses of protein in older muscle. As older adults typically consume lower quantities of dietary protein, BLG supplementation between meals may aid in maximising muscle protein anabolism throughout the day by preventing an excessive increase in MPB between meals – maintaining a positive net protein balance.

The quantity of BLG may also be important in SKM anabolism of older muscle, especially in rested conditions. This is due to our results demonstrating BLG_10 to stimulate MPS to greater extents than BLG_5, suggesting BLG containing 5 g protein (0.75 g LEU) may be too low of a dose to robustly stimulate MPS in older men at rest. Despite this, we demonstrate the additive effects acute RE has on sensitising SKM to EAA, with BLG_5 robustly stimulating MPS in older adult muscle to a comparable extent as BLG_10. Consequently, we recommend RE should be conducted in close proximity to BLG (or dietary protein)

consumption when possible to maximise SKM anabolic effects and promote muscle hypertrophy. Furthermore, despite suboptimal LEU consumption having positive effects on SKM anabolism, we recommend whole foods/protein (e.g. eggs/meat) should be consumed (or increased where possible) over isolated protein sources due to the other nutrients within the whole food promoting whole body health. Isolated protein sources should supplement a diet to optimise protein consumption, not be the main source of protein. This is especially true when supplementing with high in LEU or just free-LEU, as the other EAA are crucial for maximising MPS/SKM hypertrophy, thus limiting the other EAA in protein sources may be counterintuitive.

Alteration to protein digestion kinetics has been suggested as a contributing mechanism to age-related anabolic blunting (Mitchell et al., 2016). In response to both BLG (BLG_5 and BLG_10) and WPI supplementation, plasma LEU concentrations remained elevated above baseline for the duration of the assessment period in older individuals only. This failure to return to basal concentrations following a feed may indicate an inability of older adults to “handle” or utilise larger/certain AA in a metabolically efficient manner (Mitchell et al., 2016). Further, as we highlight a difference of ~17% in peak plasma LEU concentrations following BLG (10 g protein) between young and old participants, this is likely also indicative of a reduced whole-body AA flux in elderly humans (Short et al., 2004; Koopman et al., 2006) and/or reduced distribution volume for specific AA (Puthrasingham et al., 1996). Further research regarding AA kinetics following BLG supplementation should be conducted in older, and perhaps clinical, populations to investigate both age-

related differences but also alteration of AA handling in presence of chronic disease, to optimise protein nutrition for such populations.

There are several limitations necessary to discuss relating to the research studies presented in this thesis. Firstly, all participants who volunteered for the research studies (both young and old) were healthy and recreationally active. Despite the older adults demonstrating evidence of sarcopenia through reduced muscle size and strength, we may have captured results of the “best case scenario” with regards to healthy ageing in our older adult groups, rather than a true representation of older adults in the population. The SKM anabolic response observed in the older males may not have been as receptive if we solely studied sedentary older adults. Secondly, although the researchers provided clear, regular instructions to volunteers regarding preparation for the assessment visits, we cannot guarantee all participants adhered to the abstinence of exercise for 72 hours before the assessment visits. If participants did not adhere to exercise abstinence, it could drastically impact the rates of MPS, skewing both the rested and exercised response. Thirdly, all research studies were performed in male volunteers, thus omitting the development of knowledge on how women may respond to BLG supplementation. Therefore, the results we present throughout this thesis should not be assumed to be the identical in females, especially due to the proposed sexual dimorphism in rates of muscle atrophy with advancing age (Mitchell et al., 2012). Fourthly, we administered a set protein dose (e.g., 10 g protein) rather than administering protein doses relative to body mass. This is considered a limitation due to the inter-variability in body mass between subjects, with older adults tending to weigh more than younger individuals; thus,

potentially receiving less protein per kg of body mass. This is also relevant to conducting research in women, who typically observe lower muscle mass and higher fat mass compared to their age-related male counterparts. Future research should attempt to determine the quantity of BLG needed for optimum muscle anabolism relative to body mass, or in an ideal world SKM muscle mass, especially as the RDA for protein is expressed in g/kg body mass. However, individuals may find a set protein dose per meal (e.g., 20 g protein) easier to understand, as general population knowledge of optimal protein consumption is lacking and in need of improvement/better scientific communication. Finally, although acute interventions are crucial in determining metabolic responses to isolated supplements in controlled conditions, they do not offer comparison to “real world” scenarios, in addition to more chronic protein feeding and the interaction with RET across (e.g.) weeks/months to maximise SKM mass and function. Since protein supplementation has significant positive effects on influencing SKM hypertrophy in response to RET, we speculate that low-dose BLG (10 g protein) may be a similarly effective supplement in older men, without the need for a more satiating higher protein dose. Indeed, caution should be applied in terms of extrapolation of acute metabolic studies to a chronic scenario. However, we contend the acute metabolic responses of protein metabolism recorded in such studies can contribute to allowing for the identification of interventions for testing more chronic supplementation studies.

Future directions of this work would be to initially attempt to establish whether there is an optimum dose of BLG for maximising MPS at rest and following acute exercise. Due to the impact of the COVID-19 pandemic, we had to alter

the direction of the research studies (especially research study 3) discussed throughout this thesis. Originally, we intended to conduct research study 3 using 3 doses of BLG (5 g, 10 g and 20 g protein) to establish a “true” dose-response. This would have enabled us to assess whether low-dose BLG can indeed be utilised to avoid higher more satiating protein doses in older individuals and those in clinical care. Further, with the data we collected from research study 1 in young individuals, we would have been able to compare whether different doses of BLG were needed to optimise the anabolic response in young vs. older males, or whether the additional LEU naturally present in BLG somewhat negates this need for greater protein consumption in older adults. We would then attempt to establish whether optimum BLG supplementation (compared to e.g., a regular WP), alongside RET, would contribute to greater SKM hypertrophy in addition to offsetting losses in SKM mass and function in older individuals. Finally, of vital importance, we would want to establish the effects of BLG supplementation on muscle anabolism in both acute and chronic conditions in women to investigate the presence of sexual dimorphism in protein feeding. Females are vastly underrepresented in physiological research, and we believe it important to address this matter as sarcopenia is not sex-specific and severely impacts women as well as men.

To conclude, BLG stimulates SKM anabolism in both healthy younger and older adult men in rested and acute exercise conditions at different protein doses. Such results should be directed into optimising the type of BLG and other LEAA supplements (i.e., to ice-cream/yoghurt given older patient preference for soft

foods) and further address the effectiveness of chronic BLG/LEAA supplementation with respect to ageing, exercise training, inactivity and with a focus in clinical populations to preserve/increase SKM mass.

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Publications

Peer-reviewed publications

Ely I.A., Phillips B.E., Piasecki M., Smith K., Wilkinson D.J., Breen L., Larsen M.S., Atherton P.J. (2023) A focus on leucine in the nutritional regulation of human skeletal muscle metabolism in ageing, exercise and unloading states. *Clinical Nutrition*, pp. 1849 – 1865. doi: 10.1016/j.clnu.2023.08.010.

Ely, I. A., Jones, E. J., Inns, T. B., Dooley, S., Miller, S. B. J., Stashuk, D. W., Atherton, P. J., Phillips, B. E. and Piasecki, M. (2022) Training induced improvements in knee extensor force accuracy are associated with reduced vastus lateralis motor unit firing variability. *Experimental Physiology*, pp. 1–10. doi: 10.1113/EP090367

Guo, Y., Jones, E. J., Inns, T. B., **Ely, I. A.**, Stashuk, D. W., Wilkinson, D. J., Smith, K., Piasecki, J., Phillips, B. E., Atherton, P. J. and Piasecki, M. (2022) Neuromuscular recruitment strategies of the vastus lateralis according to sex. *Acta Physiologica*. pp. 1–14. doi: 10.1111/apha.1380

Deane, C. S., **Ely, I. A.**, Wilkinson, D. J., Smith, K., Phillips, B. E. and Atherton, P. J. (2020) Dietary protein, exercise, ageing and physical inactivity: interactive influences on skeletal muscle proteostasis. *Proceedings of the Nutrition Society*. pp. 1–12. doi: 10.1017/S0029665120007879.

Conference proceedings

Isabel A Ely, Bethan E Phillips, Kenneth Smith, Daniel J Wilkinson, Leigh Breen, Mads S Larsen, Philip J Atherton. The effects of beta-lactoglobulin (BLG) *versus* whey protein isolate (WPI) on blood aminoacidemia and muscle protein synthesis in older males. Physiology, The Physiological Society, 2023, Harrogate, UK. Proc Physiol Soc 54, PCA044.

Isabel A Ely, Eleanor J Jones, Thomas B Inns, Síobhra Dooley, Sarah B J Miller, Daniel W Stashuk, Philip J Atherton, Bethan E Phillips, Mathew Piasecki. Training induced improvements in knee extensor force accuracy are associated with reduced motor unit firing variability. Biomedical Basis of Elite Performance, The Physiological Society, 2022, The University of Nottingham, UK. Proc Physiol Soc 49, PC13 (oral presentation).

Isabel A Ely, Mads S Larsen, Leigh Breen, Daniel J Wilkinson, Kenneth Smith, Bethan E Phillips, Philip J Atherton. Leucine and older aged postprandial skeletal muscle anabolism: a literature review. National Institute of Health Research and Nottingham Biomedical Research Centre Musculoskeletal Themed Virtual Conference, 2022. East Midlands Research into Ageing Network (EMRAN) Discussion Paper Series, Issue 47, ISSN [2059-3341].

Isabel A Ely, Síobhra Dooley, Sarah B J Miller, Philip J Atherton, Bethan E Phillips, Mathew Piasecki. Neuromuscular function and motor unit firing following 4 weeks of motor control training. National Institute of Health Research and Nottingham Biomedical Research Centre Musculoskeletal

Themed Virtual Conference, 2021. East Midlands Research into Ageing Network (EMRAN) Discussion Paper Series, Issue 37, ISSN [2059-3341].

Isabel A Ely, Daniel McCormick, Philip J Atherton, Bethan E Phillips, Mathew Piasecki. Bilateral differences in quadriceps muscle strength associated with dominance and non-dominance characteristics, are not explained by muscle size or motor unit firing rate. Integrative Physiology of Exercise, American Physiological Society, 2020, virtual conference. APSIPE.109.

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Awards

2020 De Luca Foundation COVID Financial Aid, De Luca Foundation and Delsys Inc.

Appendix 1



**University of
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UK | CHINA | MALAYSIA

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Clinical, Metabolic & Molecular Physiology
MRC Versus Arthritis Centre for Musculoskeletal Ageing Research & NIHR Nottingham BRC
Division of Medical Sciences & Graduate Entry Medicine
School of Medicine
University of Nottingham
Royal Derby Hospital Centre
Uttoxeter Road, Derby
DE22 3DT

Dear Ms Ely

Ethics Reference No: FMHS 207-0221 – please always quote	
Study Title: The efficacy of a leucine-enriched whey protein for improving muscle health in older adults	
Principal Investigator/Supervisor: Professor Philip Atherton, Dr Bethan Phillips, Associate Professor, Clinical Metabolic & Molecular Physiology, Medical Sciences & Graduate Entry Medicine, School of Medicine	
Lead Investigators/student: Isabel Ely, PhD	
Other Key investigators: Professor Kenneth Smith, Dr Daniel Wilkinson, Assistant Professor, Dr John Williams, Associate Professor and Consultant Anaesthetist, CI Clinical Metabolic & Molecular Physiology, Medical Sciences & Graduate Entry Medicine, School of Medicine	
Proposed Start Date: 01/02/2021	Proposed End Date: 31/01/2025

Thank you for submitting the above application which was considered by electronic sub-committee meeting on 22 February 2021 and the following documents were received:

- FMHS REC Application form and supporting documents version 1.0: 19.01.2021

These have been reviewed and are satisfactory and the research study is given a favourable ethics opinion.

A favourable ethics opinion is given on the understanding that:

1. The protocol agreed is followed and the Committee is informed of any changes using a notice of amendment form (please request a form).
2. The Chair is informed of any serious or unexpected event.
3. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

Dr VG Wilson, Associate Professor and Reader in Autonomic Pharmacology

Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences
Acting Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

Appendix 2

Division of Medical Sciences and Graduate Entry Medicine

School of Medicine

Participant Information Sheet

***Assessing the impact of “super-whey” (vs. isonitrogenous whey)
on muscle protein synthesis at rest and in response to acute
exercise across the lifespan.***

Name of Investigators:

Professor Philip Atherton

Dr Daniel Wilkinson

Dr Bethan Phillips

Miss Isabel Ely

Professor Kenneth Smith

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish to. Please take time to decide whether or not you wish to take part. Ask us if there is anything that is not clear or if you would like more information. If you decide to take part, you will be given a copy of your consent form and this leaflet.

Background

Skeletal muscle accounts for approximately 45-55% of total body mass in healthy adults and plays a pivotal role in whole-body metabolic health, locomotion, and physical independence. Undesirable loss of skeletal muscle mass (atrophy) is, however, a common feature of many diseases and scenarios including ageing, bed rest/immobilisation, cancer, and physical inactivity. Despite the exact mechanisms causing muscle atrophy being not yet fully understood, “anabolic resistance” (reduced muscle building in response to

protein feeding and exercise) is thought to be key, especially for age-related skeletal muscle losses (known as sarcopenia). As such, the search for optimal strategies (e.g., exercise and/ or nutritional interventions) to combat this anabolic blunting remains a hot-topic in scientific research.

Leucine, an essential and branched chain amino acid (EAA/BCAA), is thought to be the most potent AA for stimulating muscle protein synthesis (MPS; the muscle building process). Although, as a stand-alone supplement, leucine is unlikely to provoke a robust and prolonged state of MPS, low doses of leucine-enriched mixed-EAAs can elicit similar increases in MPS as compared to a large dose of whey protein. As reduced appetite and increased satiety (feeling fuller) are common with advancing age, supplementation of a low-dose protein (i.e., leucine-enriched) that can adequately stimulate MPS may contribute to muscle health maintenance in older adults and reduce satiation following a meal.

This study aims to examine whether a novel whey protein with greater leucine content (“super-whey”) has superior muscle building properties compared to a regular whey protein, at rest and after a single bout of exercise, in both young and older adults.

Why have I been invited?

We are looking to recruit 15 younger individuals aged 18-35 years and 15 older individuals aged 65-85 years for the current study. This study is cross-over in nature, meaning volunteers will be asked to complete both supplementation trials separated by a washout period (meaning 2 assessment days will occur). In

order for this study to be successful, healthy volunteers have been chosen who fit the inclusion criteria and do not have significant on-going medical problems.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

Screening visit

Prior to being enrolled onto the study you will be asked to attend the Medical School at the Royal Derby Hospital at approximately 9am for a screening visit to assess your suitability for the study, to give you the opportunity to ask any questions you may have about the study, and to ask you to provide written consent for your participation in the study. We ask that you attend this session fasted from midnight, although you may drink water freely. This visit will last approximately 1.5 hours and will include:

1. a small blood sample to measure health parameters, including liver and kidney function
2. a medical history discussion
3. measures of your height and weight
4. an assessment of your heart, including an electrocardiogram (ECG) to monitor your heart and a blood pressure check
5. strength measures of one of your legs

Assessment visits

Both study days will almost be identical, with only one difference between them:

- You will be given either a “super-whey” protein drink or standard whey protein drink in each of these studies and the order of this will be randomised.

Assessment visits 1 and 2

On the basis that all your screening results mean you are eligible to take part, you will be asked to attend the University of Nottingham Medical School at the Royal Derby Hospital at approximately 8am, fasted from midnight (you may drink water freely).

First, two cannulae (a fine plastic tube) will be inserted into a forearm vein for the infusion of an amino acid tracer and into a forearm vein to allow multiple blood samples to be taken throughout the study. Your hand with the cannula will be kept in a warmed box for the duration of the study. Warming the hand allows us to take blood from a vein while it acts like blood from an artery. The amino acid tracer allows us to track how much of the protein we will be giving you has been delivered to and taken up by your muscle (muscle building). Next, ultrasound scans of both legs will occur (only during assessment visit 1) to look at your muscle size and structure. An ultrasound machine uses soundwaves which humans cannot hear and provides information about structure underneath the skin (it does not use x-rays or any other form of radiation).

One and a half hours after the start of the tracer infusion, a small muscle biopsy will be taken from the side of your thigh (both non-exercise and exercise legs, one at a time) under local anaesthetic. Three hours after this, you will be asked to perform some resistance exercise (weight training) with one of your legs. The weight of this exercise will be based on your strength test at the screening session. Immediately after the exercise, another muscle biopsy will be taken from both legs. These first two biopsies will tell us about your fasted and rested rates of muscle building. After this second muscle biopsy, we will ask you to drink one of the protein drinks (either “super-whey” protein drink or standard whey). We will ask you to drink this within 10 minutes. Three hours after the protein drink, we will take two further muscle biopsies, one from each leg, to assess your muscle building at two different time-points in response to feeding alone and feeding-plus-exercise. There will be 6 muscle biopsies in total (3 per leg) on each assessment visit.

Each biopsy is about the size of a small orange pip and will involve a 1 cm cut to the skin following an injection of local anaesthetic to numb the area. With appropriate local anaesthetic you should feel pushing and pulling during this procedure but not pain. 1-2 stitches are placed in the skin after each biopsy to aid healing and a tight bandage will be placed around the thigh after each study (to remain in place until you go to bed that night), again to speed healing. The stitches applied will be self-absorbing and will disappear 1-2 weeks after your biopsies have taken place.

Throughout both assessment days, blood samples will be taken from the cannula every 15-20 minutes; over the course of each assessment visit approximately 150ml (10 tablespoons) of blood will be taken. This is far less than what is given in a blood donation session (1 pint).

Assessment day 2 will take place 21-42 days (~30 days) after assessment day 1 to allow for significant “wash-out” of the first supplement.

5-7 days after each assessment day a member of the research team will contact you via telephone to “check-in” with how you are after the acute study day. You will be asked a short series of questions to ensure your biopsy sites are healing well and showing no signs of infection. You will also be asked (if you are comfortable to do so) to send a photographic image of your biopsy sites so a clinician can observe them also. If there are any issues or you are concerned about your biopsy sites you should inform a member of the research team immediately.

What are the exclusion criteria?

You will be ineligible to participate in this study if you have evidence of:

- Being significantly underweight or obese
- Active cardiovascular disease (including uncontrolled hypertension)
- Cerebrovascular disease
- Severe respiratory disease (not including well-controlled asthma)
- Metabolic disease
- Active bowel disease

- Recent steroid or hormone replacement therapy (within the last 6 months)
- Malignancy
- Clotting dysfunction
- Neurological or musculoskeletal disorders
- Lactose intolerance.

Please speak to a member of our research team if you are interested in participating but are unsure of your eligibility. Eligibility for this study against all these criteria will be determined by a clinician.

Would there be an inconvenience allowance?

There is an inconvenience allowance of £300 (for 2 study days) is associated with this study, which you would receive on completion of the study.

What are the possible disadvantages and risks of taking part?

Healthy volunteers have been chosen for this study; however, it is possible that the routine tests included as part of this study could detect unknown health problems. Examples of these include diabetes and high blood pressure, as they are common and often undiagnosed. Should this be the case, you will be informed and advised to attend your GP practice for further management. Your GP will also be notified.

Research studies often involve some risks, not all of which may be currently known. Infusions carry a risk of infection or irritation of the vein at the administration site when given repeatedly; however, as this research comprises

only short acute studies side-effects are not expected. Studies using labelled amino acids have been performed for over 30 years without side-effects.

In light of our experience, muscle biopsies are well tolerated by all subjects. Scarring is hardly perceptible and fades with time to near invisibility. The procedure is painless with appropriate local analgesia; you will however feel a pushing/pulling sensation. With appropriate application of pressure afterwards, followed by one or two stitches and a pressure bandage to aid healing, there are no long-lasting effects. Some discomfort (muscle tenderness and stiffness) may be felt for 2-3 days afterwards but this is usually effectively abolished by mild painkillers. In all cases there is a slight risk of infection at biopsy sites, however this risk is minimized by the use of sterile procedures. Damage to cutaneous and other soft tissue nerves during the biopsy is possible, but rare. These nerves usually grow back with restoration of normal sensation without difficulty.

The risks involved in cannulation and blood sampling is very small. Occasionally there is bruising due to leakage from a blood vessel on withdrawal of the cannula, but this is rare with good practice. Loss of 150 ml of blood has no ill effects in healthy people.

What are the possible benefits of taking part?

We cannot promise the study will help you, but participation in the study would provide you with information about your muscle function. Participation in the study will also provide you with some information about your muscle structure from the ultrasound scans. Additionally, you may benefit from performing the

resistance exercise training programme by increasing your muscle strength which is known to be beneficial to aid muscle function in day-to-day tasks. Overall, it is hoped that the information we gain from this study will give us a better understanding of the efficacy of this novel whey protein (“super-whey”) to improve muscle health in older adults. This information may help us to identify optimal nutritional and exercise strategies to aid healthy ageing.

What happens when the research study stops?

Once the data has been analysed the results of the study will be presented at national and international academic conferences and published in the relevant academic literature. Data from this study will also be included in PhD theses. These results will be used to guide further research and possibly the future development of medical interventions.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers’ contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting the principal investigator Dr Bethan Phillips. If this achieves no satisfactory outcome, please contact the FMHS Research Ethics Committee Administrator, e-mail: FMHS-ResearchEthics@nottingham.ac.uk.

Will my taking part in the study be kept confidential?

We respect your right to privacy, and we will take measures to safeguard confidentiality. A single form, on which you are asked to sign to give consent

for involvement will carry details of your name and address, but no health-related details. This is kept securely in a locked cabinet within the Medical School. Access to this cabinet is restricted to personnel directly involved in the study and to University staff with direct responsibility for ensuring the study is conducted appropriately. We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, we will use information collected from you during the course of the research. This information will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database at the University of Nottingham. Under UK Data Protection laws, the University is the Data Controller (legally responsible for the data security), and the Principal Investigator of this study (named above) is the Data Custodian (manages access to the data). This means we are responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited as we need to manage your information in specific ways to comply with certain laws and for the research to be reliable and accurate. To safeguard your rights, we will use the minimum personally identifiable information possible.

You can find out more about how we use your information and to read our privacy notice at:

<https://www.nottingham.ac.uk/utilities/privacy.aspx>.

The data collected for the study will be looked at and stored by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people from regulatory organisations to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

Information about you which leaves the site will have your name and address removed and a unique code will be used so that you cannot be recognised from it.

Your contact information will be kept by the University of Nottingham for 5 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies (unless you advise us that you do not wish to be contacted). This information will be kept separately from the research data collected and only those who need to, will have access to it. All other research data will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team given permission by the data custodian will have access to your personal data.

In accordance with the University of Nottingham's, the Government's, and our funders' policies we may share our research data with researchers in other Universities and organisations, including those in other countries, for research

in health and social care. Sharing research data is important to allow peer scrutiny, re-use (and therefore avoiding duplication of research) and to understand the bigger picture in particular areas of research. Data sharing in this way is anonymised (so that you could not be identified).

What will happen if I don't want to carry on with the study?

Your participation is completely voluntary, and you are free to withdraw at any time without giving any reason. If you withdraw, we will no longer collect any information about you or from you, but we will keep the information about you that we have already obtained as we are not allowed to tamper with study records and this information may have already been used in some analyses and may still be used in the final study analyses.

Involvement of the General Practitioner/Family doctor (GP)

Your GP will not be routinely notified that you are taking part in this study. However, if we find anything abnormal as part of the screening investigations, we will inform you in the first instance, but we will also notify your GP.

What will happen to any samples I give?

We will also seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). These samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265). Some of these future studies may be carried out by researchers other than the current team who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway. If you do not

agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

Will any genetic tests be done?

No genetic tests will be conducted as part of this study.

What will happen to the results of the research study?

Data collected during the study will be published in the scientific literature enabling other health professionals to use the information. You will not be identified in any publication. There is usually a delay of up to one year from the study completion before this occurs. Should you wish to be informed of publications resulting from this study, please inform the study team.

Who is funding the research?

This study is funded by the University of Nottingham and Arla Foods Ingredients.

Who has reviewed the study?

All research at the University is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the University of Nottingham Medical School Research Ethics Committee.

Additional important information

COVID-19 statement

Following the recent outbreak of Covid-19 infection in the UK, close contact between people outside of family groupings or 'support bubbles' is being

discouraged at this time to reduce the spread of the virus, and to protect vulnerable individuals from contracting Covid-19.

It is important for you to know that during this research study it will not always be possible to maintain recommended social distancing between researchers and yourself. Research staff will be required to be close to you when performing certain tasks, including (e.g.,) during blood and muscle sampling and resistance exercise.

Government guidelines are constantly evolving in response to changes in the Covid-19 situation, and procedures we employ during this study will therefore be changing in line with these. However, currently we will be implementing the following measures to protect you, and the researchers working on this project:

1. Please do not attend any scheduled study sessions if you, or anyone in your social unit, is displaying (or has displayed within the previous 14 days) signs of COVID-19 infection*, or if you are self-isolating following contact with someone who has displayed signs of infection. We will contact you the day before each time you are due to attend the University to check your symptom and isolation status.
2. Similarly, study researchers will not enter University buildings if they, or anyone in their social unit, is displaying (or has displayed within the previous 14 days) signs of infection or if they are self-isolating following contact with someone who has displayed signs of COVID-19 infection.
3. Social distancing will be observed at all times where possible.
4. To keep the number of people within University buildings to a minimum, please do not bring additional people (i.e., siblings or partners) to any study session.

5. The number of individuals (researchers and participants) in study areas will be kept to a minimum, with all individuals required to maintain good hand hygiene whilst in the units and to wear face coverings when social distancing measures are not possible.
6. Prior to your arrival, the research areas that you may touch (e.g., door handles and work surfaces) will be cleaned using surface sanitiser.
7. On arrival, you will be asked to cleanse your hands thoroughly at a hand-wash station and/or using alcohol gel and will be provided with a face mask to wear during the visit.
8. All utensils and crockery used to prepare and serve any study food products or refreshments during the study visit will be thoroughly cleaned with detergent in hot water immediately prior to use and dried using paper towels which will be discarded following use.
9. At the end of the study visit, you will be asked to wash your hands thoroughly again before leaving.

*Please visit: <https://www.nhs.uk/conditions/coronavirus-covid-19/symptoms/>

Further information and contact details

Thank you for your interest in this study. For further information please contact:
Miss Isabel Ely (Isabel.Ely@nottingham.ac.uk) or Dr Bethan Phillips (Beth.Phillips@nottingham.ac.uk). Alternatively, they are both contactable at The University of Nottingham Division of Medical Sciences & Graduate Entry Medicine, Royal Derby Hospital Centre, Derby, DE22 3DT.

Appendix 3

Division of Medical Sciences and GEM

Healthy Volunteer Consent Form (HVCF)



University of
Nottingham
UK | CHINA | MALAYSIA

Premises covered	MSGEM	This is version	8
Document Created	15.7.2009	Document issued in	June 19
Review Period	Every two years	To be reviewed in	June 21

Project Title: Assessing the impact of “super-whey” (vs. isonitrogenous whey) on muscle protein synthesis at rest and in response to acute exercise across the lifespan – Part A.

Please read this form and sign it once the investigator above-named, or their designated representative, has explained fully the aims and procedures of the study to you.

Please Initial Box

- I voluntarily agree to take part in this study. ☐
- I confirm that I have been given a full explanation of the study and that I have read and understand the information sheet (version number 1.2a dated 08.09.2021) for the above study and have had the opportunity to ask questions. ☐
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing. ☐
- I agree to comply with the reasonable instructions of the research team and will notify them immediately of any unexpected, unusual symptoms or deterioration of health. ☐

- I give permission to collect, store, analyse and publish information obtained from my participation in this study. I understand my personal details will be kept confidential. ☐
- I understand that data collected in the study may be looked at by responsible individuals from the University of Nottingham and regulatory authorities where it is relevant to my taking part in this study. ☐
- I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others, it will be made anonymous. Data will be kept for 7 years after all results of this study have been published. After this time, it will be disposed of in line with University policy. ☐
- I understand that the tests or procedures are carried out for research only and not for clinical diagnostic purposes. However, if the study investigator should feel it necessary to inform my GP of my participation in the study, or of an adverse event or abnormal test result, I understand I am giving my consent to do so. ☐
- I confirm that I have disclosed relevant medical information before the study. ☐
- I have not been a volunteer in any other research study in the *last three months*, which involved: taking a drug, being paid an inconvenience allowance, having an invasive procedure (e.g. venepuncture >50ml, endoscopy) or exposure to ionising radiation. ☐
- I confirm that I have not been exposed to more than 5 mSv of ionising radiation in the *last 12 months* (to clarify, please discuss with a member of the research team). ☐

Healthy Volunteer Consent Form (HVCF)

Human Tissue Act:

- **OPTIONAL** - I agree to my tissue/ blood samples being shared with other researchers including those working outside the University. I understand that I would not gain commercially. ☐

- **OPTIONAL** - Where tissue is removed from my body for the purposes of this study, but not used for this study, and when the ethical approval for this project expires, I agree to the future and continued storage and use of this tissue for future research into functioning of the human body and disease. Such tissue will fall under the University of Nottingham licence of the Human Tissue Act. As specified under the Licence tissue records will be maintained for 25 years. ☐

Personal Data:

- **OPTIONAL** - I agree to my contact details being stored for the purpose of being invited to participate in future research studies. ☐

Volunteer Name:

.....

Signature:.....Date:.....

I confirm that I (investigator) have fully explained the purpose of the study and what is involved to:

.....

I have given the above named a copy of this form together with the information sheet.

Investigator Name:

.....

Signature:.....Date:.....

PI signature:.....Date:.....

Study Volunteer Number:

.....

2 copies: 1 for the participant, 1 for the study file.

Appendix 4

Arla-Whey (Part A)

Assessing the impact of “super-whey” (vs. isonitrogenous whey) on muscle protein synthesis at rest and in response to acute exercise across the lifespan

Food Diary (Household Measures)

Participant:

Pre-intervention

Please complete your diary on the following days:

Day 1.....	Date.....
Day 2.....	Date.....
Day 3.....	Date.....
Day 4.....	Date.....

Please return the diary when you come for your next visit

For office use only

Diary checked by:

Date:

Diary coded by:

Date:

Diary entered by:

Date:

Computer software used:

Version of software:

INSTRUCTIONS FOR FILLING IN YOUR FOOD DIARY

- Please keep a food diary for the 4-days indicated on the front cover. The record should include as much detail as possible. Please start a new page each day (ensure the day and date is recorded at the top of the page).
- Fill in the food diary each time you consume a drink, a meal, or a snack. Please note the time and describe everything you eat or drink. Use a further page if you need more space. (Please remember to record the day and date at the top of the new page).
- When you record a food or drink, please give a thorough description, and include any Brand names. You cannot give us too much detail! For example:-
 - Was the bread white/wholemeal/granary
 - Did you have a baguette/cob/pitta/slice?
 - Did you use butter or margarine, was the margarine made with olive/sunflower oil?
 - Was the product a diet product- e.g. sugar-free lemonade?
 - Was anything added- e.g. sugar to tea, butter to toast, tomato ketchup or mayonnaise etc?
 - Do you use full fat/semi-skimmed/skimmed milk?
 - How did you cook your food – e.g. boiled/fried/baked/raw?
 - How was the food processed – e.g. fresh/tinned/frozen?
 - Was the product Heinz/Tesco's own etc.?
 - If you have given us a weight, does this include any think inedible (e.g. the skin on a banana, the core of an apple, or a bone in a chop?
- The amounts you have should be described as fully as possible using household measures
 - Use kitchen equipment – a measuring jug, a mug (of know volume), a teaspoon (see below) and a tablespoon



- Look on cans and packets to see whether the weight/volume is given and describe how much of the packet you had (e.g. Half a 400g can of Heinz tomato soup)
 - Record the number of sausages/fish fingers/bacon rashers etc. that you ate
 - Describe the size of a piece of food – e.g. egg sized potato, small matchbox sized piece of cheese
- Please record each item of food separately. If you drink white tea with sugar, please record the type and amount of tea, milk, and sugar separately.
- If you had a packed lunch, each part must be recorded in the diary.

- It is important that you complete the 'leftovers' section with anything you did not actually eat. Please give us as much detail as possible. This might include, for example, that you have left an apple core, or not consumed the skin on a piece of chicken
- For homemade recipes, the raw ingredients should be recorded in the back of the diary in the 'Recipes' section. Please tell us what the meal is composed of and how many it served. You can then put an entry in the diary such as "one quarter of the spaghetti Bolognese made with recipe 1". It is helpful if you can also include the weight of the portion that you had.
- Please would you also include details of any supplements that you take. This might, for example, be a multi vitamin tablet, or protein supplement. Please would you bring the packet or bottle so that we can record the details.

**AN EXAMPLE OF HOW TO COMPLETE YOUR FOOD DIARY AND
RECIPE PAGE IS SHOWN ON THE NEXT PAGE**

Subject ID -Example		Day – 1, Wednesday	Date - 01/10/13	
Time	Type of food or drink with brand names and cooking method	Amount	Food or drink left over and amount	Office use only: code and weight
7.30am	Beans on toast:			
	Heinz baked beans (reduced sugar and salt)	3 heaped tablespoons		
	White toast (Warburtons)	2 thick slices		
	Flora spread	2 heaped teaspoons		
	Cup of tea:			
	PG tips tea	300ml		
	Semi-skimmed milk	50ml		
	Sugar	1 teaspoon		
10.30am	Dairy Milk chocolate	40g bar		
	Diet coke	330ml can		
12.45pm	Ham sandwich:			
	Ham (Tesco Honey Roast)	3 thin slices	¼ of a slice	
	White bread (Warburtons)	2 thick slices		
	Flora spread	2 heaped teaspoons		
	Tomato	½ beef tomato		
	Cup of tea (as above)	300ml		
	Walkers Salt & Vinegar crisps	30g bag		
	Granny Smith apple	medium		
7.15pm	1/4 of spaghetti Bolognese made using recipe 1			
	John Smiths beer	2 pints		
	Mr Kipling Bramley Apple pie	2 individual pies		

9.00pm	Hot chocolate made with semi- skim milk	2 teaspoons chocolate/ ½ pint of milk		

Subject ID.....Example.....

Recipe no: 1	Dates used: ...01. 10.13	Serves: 4 Please also indicate on food diary the proportion of recipe you had on each occasion	Name of recipe: Spaghetti Bolognese
Ingredient (with brand names and cooking method)	Amount	Comments	
Garlic	1 clove	Fried in the sunflower oil	
Onion	1 medium	Fried in sunflower oil	
Sunflower oil	2 teaspoons		
Sainsbury's organic lean minced beef (uncooked weight)	500g		
Ragu original smooth Bolognese sauce	454g jar		
Dried spaghetti	250g		

Diet diary contained subsequent blank tables like above for participants to complete the diet diary across 4 days.

Appendix 5

Division of Medical Sciences and Graduate Entry Medicine School of Medicine

Assessing the impact of “super-whey” (vs. isonitrogenous whey) on muscle protein synthesis at rest and in response to acute exercise across the lifespan

Name of Investigators:

Professor Philip Atherton, Dr Bethan Phillips, Professor Kenneth Smith, Dr Daniel Wilkinson, and Miss Isabel Ely

Participant code:

Date of check-up:

Biopsy aftercare:

Questions to ask participant on the telephone to “check in” with how they are doing post-acute study day and regarding biopsy wounds (questions below).

Are you experiencing **any** of the following?

Question	Yes/No
1. Swelling, redness and increasing pain in the affected area	
2. Weeping/discharge from the wound	
3. Feeling generally unwell	
4. A high temperature (fever) or shivers etc	
5. Excessive bleeding (blood-soaked dressing).	

Ask participant if they are happy to send a photographic image of their biopsy wounds so a clinician can visually inspect them. The participant will not be able to be identified by the photographic image.

Reminder for participant: *stitches can take up to a couple of weeks to completely dissolve, so if they haven't gone after a week then that's nothing to worry about.*