MYCOPROTEIN & SKELETAL MUSCLE ANABOLISM

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ABSTRACT

Skeletal muscle is critical for human locomotion, postural control and the regulation of whole-body metabolism. Concomitantly, understanding how the food we eat influences skeletal muscle protein metabolism, and skeletal muscle mass, is vitally important. This is particularly true in those seeking to increase skeletal muscle mass, and for older individuals seeking to mitigate the seemingly inevitable loss of muscle mass. It is exceptionally well evidenced that protein ingestion increases muscle protein synthesis rates, with postprandial elevations in plasma essential amino acids (and leucine in particular) predominately responsible. The foundation of our evidence-base on protein intake and muscle protein synthesis rates in humans has largely been formed by investigating animal-derived protein sources, which are potent stimulators of muscle protein synthesis rates. However, there is relatively little comparative data in non-animal-derived sources. Consequently, given the prevalence of non-animal-derived proteins within the diet, there is a pressing need to develop an evidence base for sustainable alternative non-animal-derived protein sources. Mycoprotein, *Fusarium venenatum*, is a sustainably produced fungal derived whole food protein source. Accordingly, the purpose of this thesis was to thoroughly characterise the effect that mycoprotein ingestion has on muscle protein synthesis rates and muscle mass, with specific attention afforded to the interaction between mycoprotein ingestion and resistance exercise in younger and older adults.

Firstly, I demonstrate the novel finding that the ingestion of a single bolus of mycoprotein (70 g; 31.5 g protein, 2.5 g leucine) stimulates resting and post-exercise muscle protein synthesis rates, and that it does so to a greater extent than a leucine matched bolus of milk protein (Δ 0.040±0.006 vs Δ 0.018±0.005%·h^{-1}, respectively; P<0.01). I followed this by demonstrating that ingesting a smaller, more palatable, dose (35 g) of mycoprotein, enriched with BCAAs, was unable to stimulate resting or post-exercise muscle protein synthesis rates

to the same extent as a BCAA matched larger bolus (70 g) of mycoprotein (Δ 0.040±0.006 vs Δ 0.019±0.005%·h^{-1}, respectively; $P<0.05$). In both cases, greater muscle protein synthesis rates were observed following the ingestion of a 70 g bolus of mycoprotein, despite significantly lower plasma leucine concentrations, perhaps suggesting the presence of a "whole food" potentiating effect. Conjunctively, these data suggested to us that mycoprotein would support skeletal muscle remodelling over a longer period of time.

As such, I subsequently transitioned to investigating daily muscle protein synthesis rates in both young and older individuals, who consumed either an omnivorous (OMNI) or non-animal-derived (VEG) high-protein (1.8 g·kg body $mass^{-1}$·d^{-1}) diet for three days, whilst completing a daily bout of resistance exercise. Omnivorous and non-animal-derived dietary protein sources supported equivalent rested and exercised daily myofibrillar protein synthesis rates in both younger (OMNI 2.19±0.14 vs 2.46±0.11%·d^{-1}, VEG 2.01±0.17 vs 2.39±0.09%·d^{-1}; $P>0.05$), and older individuals (OMNI 1.59±0.12 vs 1.77±0.12%·d^{-1}, VEG 1.76±0.14 vs 1.93±0.12%·d^{-1}; $P>0.05$). As such, obtaining dietary protein from animal-derived sources is not an essential prerequisite to support daily myofibrillar protein synthesis rates in healthy younger and older adults.

I translated this line of work further, demonstrating that a high-protein (~2 g·kg body $mass^{-1}$·d^{-1}), mycoprotein-rich, non-animal-derived diet can support equivalent resistance training-induced skeletal muscle adaptation as a high-protein omnivorous diet. After progressively resistance training 5 d/week for 10 weeks, increases in lean mass (OMNI 2.6±0.3 kg, VEG 3.1±0.8 kg; $P>0.05$), thigh muscle volume (OMNI 8±1%, VEG 8.2±1.4%; $P>0.05$), muscle fibre CSA (OMNI 33±10%, VEG 32±17%; $P>0.05$), and various measures of muscle strength ($P>0.05$) were equivalent, regardless of whether participants consumed an omnivorous or non-animal-derived diet. In turn, this demonstrates that under near-optimal nutritional and

exercise-training conditions, non-animal-derived diets have the capacity to facilitate hypertrophic and strength adaptations in healthy young men and women.

Collectively this thesis demonstrates that mycoprotein is an anabolic non-animal-derived protein source, capable of stimulating acute postprandial muscle protein synthesis rates, supporting daily muscle protein synthesis rates when incorporated into a non-animal-derived diet, in both young and older individuals, and, as a result, facilitative of considerable resistance training-induced skeletal muscle remodelling. Therefore, herein details a unique and novel body of work characterising the effect of mycoprotein on skeletal muscle tissue, translating from the level of molecular and metabolic minutiae, to the level of functional movement.

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I dedicate this thesis to George Downend, who taught me that there is no greater gift one can give, nor legacy one can leave, than the kindness and generosity one shows to others.

DECLARATION

All *in vivo* human data collection was carried out by myself. All of the analysis presented within this thesis were carried out by myself, with the exception of Western blotting for mTOR, which was carried out by Jamie Blackwell, and mass spectrometry, which was carried out by Doaa Abdelrahman, at the University of Texas Medical Branch.

Associate Professor Benjamin Wall, Dr Marlou Dirks, Professor Francis Stephens, and Dr Sarah Jackman conducted the skeletal muscle biopsy procedure described throughout, in that order of prominence. The MRI and DXA scans described in Chapter 6 were conducted by Dr Jonathan Fulford and Associate Professor Benjamin Wall, respectively.

I hereby declare that the present thesis has been composed by myself, and that it is a record of work performed by myself, except where assistance has been acknowledged. No part of this thesis has been submitted in any other application for a higher degree and all sources of information have been appropriately referenced.

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Other Publications

Coelho, M., **Monteyne, A. J.**, Dunlop, M. V., Harris, H. C., Morrison, D. J., Stephens, F. B., & Wall, B. T. (2020). Mycoprotein as a possible alternative source of dietary protein to support muscle and metabolic health. *Nutrition Reviews*

Coelho, M., **Monteyne, A. J.**, Dirks, M. L., Finnigan, T., Stephens, F. B., & Wall, B. T. (2020). Daily mycoprotein consumption for 1 week does not affect insulin sensitivity or glycaemic control but modulates the plasma lipidome in healthy adults: a randomised controlled trial. *The British Journal of Nutrition*

Davenport, A. D., Jameson, T., Kilroe, S. P., **Monteyne, A. J.**, Pavis, G. F., Wall, B. T., Dirks, M. L., Alamdari, N., Mikus, C. R., & Stephens, F. B. (2020). A Randomised, Placebo-Controlled, Crossover Study Investigating the Optimal Timing of a Caffeine-Containing Supplement for Exercise Performance. *Sports Medicine*

Abstracts

Monteyne, A.J., Coelho, M.O., Jameson, T.S.O., Jackman, S.R., Porter, C., Abdelrahman, D.R., Finnigan, T.J.A., Dirks, M.L., Stephens, F.B., & Wall, B.T. Mycoprotein ingestion stimulates protein synthesis rates to a greater extent than milk protein in both rested and exercised skeletal muscle in healthy young men. *European College of Sports Science 2019*

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LIST OF ABBREVIATIONS

1RM one repetition maximum

^{2}H deuterium

aCSA anatomical cross-sectional area

BCAA branch chain amino acid

BMI body mass index

BMR basal metabolic rate

cDNA complementary DNA

CSA cross-sectional area

CT computed tomography scan

D2O deuterated water

DEXA dual-energy x-ray absorptiometry

EAA essential amino acids

ENR lower-dose enriched mycoprotein

EX exercise

FFM fat-free mass

FM fat mass

FSR fractional synthetic rate

GC-C-IRMS gas chromatography combustion isotope ratio mass spectrometry

GC-IRMS gas chromatography-isotope ratio mass spectrometer

IPAQ international physical activity questionnaire

LBM lean body mass

MILK milk protein concentrate

MPB muscle protein breakdown

MPE mole percent excess

MPS muscle protein synthesis

MRI magnetic resonance imaging

mRNA messenger RNA

MVC maximum voluntary contraction

MYCO mycoprotein

OMNI omnivorous diet

PAL physical activity level

PCR polymerase chain reaction

RDA recommended dietary allowance

RE rest

REX resistance exercise

rRNA ribosomal RNA

RT-qPCR real-time quantitative polymerase chain reaction

T tesla

tRNA transfer RNA

VEG non-animal derived diet

CHAPTER 1

GENERAL INTRODUCTION

“*Well. What can we do, except try to do better*?”

REGULATION OF MUSCLE MASS

Overview

The principal role of skeletal muscle tissue is to produce contractile force to facilitate locomotion and postural control. Concurrently, muscle tissue is a major contributor to the demands placed on whole-body metabolism, accounting for around a third of resting energy expenditure (Zurlo et al., 1990), which can increase 20 fold in response to the increased energetic demands of exercise (Gaitanos et al., 1993). Muscle represents the major site of postprandial glucose disposal, where it is oxidised, and stored as muscle glycogen (Thiebaud et al., 1982), the major reservoir of amino acids (~60% of body protein) within the human body, and a significant store of lipids (Baskin et al., 2015). During postabsorptive conditions, amino acids are released from the muscle to meet metabolic demands and preserve glucose homeostasis, via amino acid oxidation and gluconeogenesis, whilst also acting as precursors to be incorporated into other tissues where required. Proteins within skeletal muscle are in a state of constant turnover; with free amino acids continuously synthesised into polypeptide chains and subsequently broken down. In healthy recreationally active individuals this occurs at a rate of 1-2% a day, with the entirety of the muscle tissue being completely broken down and resynthesised every 3 months or so. Muscle is a highly plastic tissue, able to adapt the type and quantity of specific proteins over time in order to meet the demands of various environmental stimuli, providing a phenotypic change within the bounds of genetic responsiveness (Timmons, 2011).

Muscle mass is maintained through dynamic and equilibrated fluctuations in muscle protein synthesis and muscle protein breakdown, such that the net difference between the two is negligible over time and muscle protein is neither accrued nor lost. If this equilibrium is broken, such as through a decrease in protein synthesis, then protein will be lost, and, if continued over time, muscle mass will be diminished (Kilroe et al., 2020b). Concomitantly,

if protein synthesis were to increase, and protein breakdown remain unchanged, there will be a net gain of protein if this pattern remains consistent over time, such as with prolonged resistance exercise (Damas et al., 2016). Muscle protein synthesis is considered to be the more consequential anabolic stimulus in healthy individuals (Biolo et al., 1995b, Biolo et al., 1997), and it is typically through dynamic fluctuations in muscle protein synthesis that tissue is accrued or lost. Consequent to this, populations seeking to increase muscle mass (resistance trainers) or who are at risk of losing muscle mass (older adults) are of particular research interest.

Postprandial Protein Handling

Skeletal muscle protein turnover is highly responsive to feeding in healthy adults. Protein ingestion increases muscle protein synthesis and decreases proteolysis, albeit to a lesser extent in the latter, resulting in a positive net muscle protein balance. Following the ingestion of a meal, proteins are hydrolysed into amino acids and small peptides in the stomach and small intestine under the action of specific proteases. These amino acids and small peptides are then absorbed across the small intestine, before being delivered to the liver by the hepatic portal vein. The tissues of the portal drained viscera extract a proportion of these amino acids (20-75%) for protein turnover (Volpi et al., 1999, Gorissen et al., 2020, Boirie et al., 1997, Koopman et al., 2009b, Fouillet et al., 2001, Dangin et al., 2003), as does the liver, where amino acids are retained for protein synthesis, deamination, transamination, and subsequently gluconeogenesis and liponeogenesis. After extraction by the liver, the remaining amino acids become available in circulation for utilisation by other tissues, including muscle. Resultantly, plasma amino acid concentrations increase, and consequently there is an increase in pancreatic insulin secretion. An increase in circulating insulin concentration, in turn, increases perfusion to peripheral tissues, lowering resistance in terminal arterioles thereby increasing perfusion to capillary beds and available capillary

surface area, thus enhancing the nutritive flow to muscle tissue and the potential for increased delivery of nutrients. Upon delivery to the muscle, amino acids are transported across the sarcolemma, into the mycocyte, and intramyocellular amino acid concentrations increase in response to protein ingestion (Bergström et al., 1990). This increase in intramyocellular amino acids, particularly leucine, initiates a signalling cascade through the mTOR pathway that affects an increase in muscle protein synthesis rates (Bar-Peled and Sabatini, 2014, Dickinson et al., 2011, Anthony et al., 2000). Leucine is sensed in the mycocyte, by sestrin2 (Wolfson et al., 2016), which causes Rag GTPases to recruit mTOR to the lysosome, where it then initiates a kinase signalling cascade that phosphorylates/dephosphorylates the downstream effectors p70S6K and 4EBP-1, activating translation initiation (Betz and Hall, 2013, Bar-Peled and Sabatini, 2014). A continued high rate of muscle protein synthesis is then contingent, at least theoretically, on adequate availability of dietary derived amino acids to act as precursors to continue the formation of polypeptide chains (Fuchs et al., 2019).

Muscle protein synthesis increases quickly in response to dietary protein ingestion, within an hour of ingestion, and can then remain elevated for at least 5 hours when provided in sufficient quantity (Vliet et al., 2019). Insulin secreted in response to nutritional stimuli appears to play a largely permissive role, with only a very modest rise in insulin required to facilitate optimal muscle protein synthesis rates (Greenhaff et al., 2008). Physiological conditions that elicit high plasma insulin concentrations confer no additional stimulation of muscle protein synthesis (Gorissen et al., 2014), although insulin alone does appear to have an anti-catabolic function, suppressing muscle protein breakdown rates (Greenhaff et al., 2008, Wilkes et al., 2009). Insulin modulates muscle protein synthesis at multiple levels of the regulatory cascade, increasing microvascular perfusion through PI3-eNOS signalling, and ensuing nitric oxide production (Dimmeler et al., 1999, Montagnani et al., 2001, Kubota et al., 2013), directly acting upon mTOR phosphorylation via Akt pathway signalling (Saxton

and Sabatini, 2017), and through interactions with amino acid transporters (Kandasamy et al., 2018).

The anabolic response is refractory in nature, such that even under conditions of continued high concentrations of circulating amino acids muscle protein synthesis does not remain elevated indefinitely (Bohé et al., 2001, Atherton et al., 2010). This is biological frugality, as a continued elevation in muscle protein synthesis, assuming breakdown remained constant, would allow for a continued accrual of muscle protein, and the continuation of an energetically expensive process. Muscle protein synthesis is, ostensibly, optimally stimulated with a dose of ~20g protein (Witard et al., 2013, Moore et al., 2008), and above this level there is a sharp rise in amino acid oxidation (Moore et al., 2008, Witard et al., 2013), as either the muscle becomes saturated with amino acids, or counter-regulatory mechanisms desensitise the muscle to the effects of amino acids. This amount of ~20g high-quality protein appears adequate to overcome any stimulatory threshold required to increase translation initiation, whilst providing an adequacy of amino acids for incorporation into polypeptide chains.

The extent of the postprandial anabolic response to food ingestion is modulated by multiple converging factors, and regulated across multiple compartments, including, and potentially not limited to, amino acid digestion and appearance in circulation (Koopman et al., 2009a, Pennings et al., 2013), insulin secretion (Greenhaff et al., 2008, Groen et al., 2016, Trommelen et al., 2015, Fujita et al., 2006), vascular perfusion (Timmerman et al., 2010a, Timmerman et al., 2010b, Zhang et al., 2011), amino acid uptake into the muscle (Moro et al., 2019, Dickinson et al., 2013), intracellular signalling events (Fry et al., 2011b, Cuthbertson et al., 2005), and the continued availability of substrate to support muscle protein synthesis (Fuchs et al., 2019, Witard et al., 2013, Moore et al., 2008) (Figure 1.1). The relative importance of each of these factors, and the extent to which each of these

contributes to the potency of the anabolic response, is difficult to determine and seems highly context-specific. Moreover, these factors are inextricably linked, such that an effect upstream exerts influence downstream, and *vice-versa*. As such, in order to properly address the matter, we must anchor ourselves to the investigation of robust physiological responses.

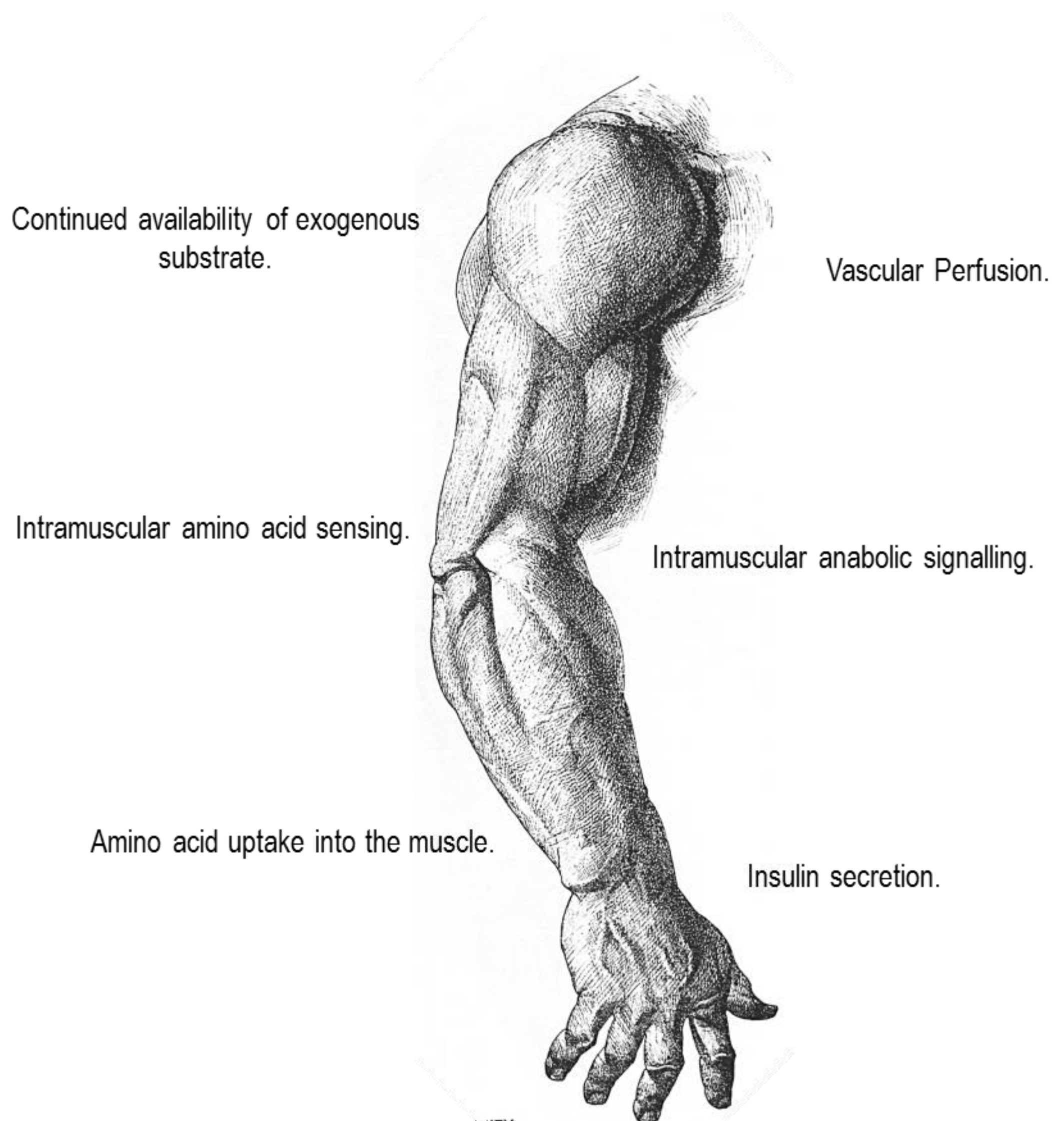


Figure 1.1. Factors regulating postprandial muscle protein synthesis**.**

The Role of Leucine

It has been strongly suggested that the rapidity and magnitude (within ~ 60–90 min) of postprandial leucinemia is perhaps the single most important factor determining the magnitude of the subsequent muscle protein synthetic response (Phillips, 2016, Devries et al., 2018). This has led to what has been variably termed the “leucine hypothesis”, “leucine threshold”, or “leucine trigger”, whereby a certain peak plasma leucine concentration, and presumably intracellular concentration, is required to optimally stimulate postprandial muscle protein synthesis, with a degree of proportionality existing between the two (Phillips, 2016). Whilst there is significant evidence to support this hypothesis (Tang et al., 2009, Pennings et al., 2011b, Koopman et al., 2009a, Burd et al., 2012, Churchward-Venne et al., 2013), the evidence is not overwhelming and appears to vacillate depending upon the specific context in which protein is provided. Indeed there are sufficient instances of disagreement with this hypothesis to make us think that it is more than a case of *the exception proving the rule* (Staples et al., 2011, Gorissen et al., 2014, Hamer et al., 2013, Gorissen et al., 2017, Churchward-Venne et al., 2015, Chan et al., 2019, Reidy et al., 2013, Mitchell et al., 2015, Borack et al., 2016, Koopman et al., 2007). Whilst undoubtedly suggestive of the subsequent muscle protein synthetic response when considering isolated protein sources, this hypothesis becomes problematic when considering the coingestion of macronutrients with protein, whole foods, or meal ingestion. Under such conditions, the postprandial rise in plasma leucine concentrations is significantly attenuated, due to a slower rate of gastric emptying, digestion, and absorption, which, if we were to subscribe to the leucine hypothesis, would beget a suboptimal muscle protein synthetic response. Yet this is not the case, and evidence exists to suggest that the rapidity and magnitude of leucine availability in the plasma is not wholly predictive of the muscle protein synthetic response. Specifically, coingesting protein with an additional macronutrient significantly curtails the rise in plasma leucine concentrations, without having a deleterious effect on muscle protein

synthesis rates (Gorissen et al., 2017, Gorissen et al., 2014, Hamer et al., 2013, Staples et al., 2011, Koopman et al., 2007). Gorissen and colleagues (2014) showed equivalent muscle protein synthesis rates between the ingestion of casein alone (20g), and casein coingested with carbohydrate (60g); despite more than two-fold higher plasma leucine concentrations following the ingestion of casein alone (Gorissen et al., 2014). This divergence between leucinemia and muscle protein synthesis rates is likely to be amplified when considering protein ingestion within the context of a mixed meal, although to date very few studies have investigated protein ingested within a mixed meal (Kim et al., 2016). Moreover, important recent work demonstrates that, at least when considering dairy protein, the degree of postprandial aminoacidemia does not modulate the post-exercise anabolic response (Chan et al., 2019). The cited study elegantly manipulated the digestibility of dairy protein, utilising a process of mineral modification to increase the speed of digestibility of milk protein concentrate without altering amino acid composition or hydrolysing protein structures. This allowed a comparison to a more typical milk protein concentrate, whilst clamping amino acid content and total protein content (25 g protein). Despite a significant divergence in leucinemia between conditions, there was no difference in the myofibrillar protein synthetic response. This is an important finding, as it demonstrates that even under conditions that are isonitrogenous, amino acid equivalent, and almost energy matched, that a larger degree of leucinemia neither appears to modulate the time-course nor magnitude of postprandial myofibrillar protein synthesis. Considering this weight of evidence, it would appear that postprandial plasma leucine concentrations are only partially predictive of postprandial muscle protein synthesis rates. Indeed, patterns of food ingestion that introduce more complex nutrient interactions may in turn introduce further intricacies into the regulation of postprandial muscle protein synthesis (Burd et al., 2019). Our knowledge of the regulation of postprandial muscle protein synthesis is essential in our ability to critically evaluate, and optimise, dietary strategies, and thus understanding the nuanced role that leucine plays

within this is imperatively important. This thesis will provide further insight into the role of dietary leucine, using mycoprotein as a methodological tool to investigate the importance of the rapidity and magnitude of leucinemia (Dunlop et al., 2017) upon muscle protein synthesis, under iso-leucinemic conditions.

Whole Foods

It has been suggested that consuming protein within a whole food matrix may convey additional stimulation of muscle protein synthesis when compared to consuming protein in isolation (Burd et al., 2019). There is a long-held belief that amino acids alone are almost solely responsible for the stimulation of muscle protein synthesis, and this supposition is supported by an abundance of evidence collected through a period spanning half a century, and across multiple different laboratories. Whilst this body of evidence is ostensibly overwhelming, it is worthy of note that the vast majority of this work has considered ingested protein and amino acids in isolation, and that from a historical perspective the vast majority of our knowledge has developed with little appreciation of, and in isolation of, protein consumed as part of foods, and even less so as a component of a meal. Just so, as far as the author is aware only a single study has considered genuine meal ingestion and protein synthesis (Kim et al., 2016). Further, a proportion of the supporting evidence is obtained from models involving the direct infusion of substrates, which further complicates interpretation, as it obviates the indirect effects of nutrient ingestion, such as insulinemia, bypasses gastric effects, such as GLP-1 secretion, and smooths out the peaks associated with the postabsorptive-postprandial transition. Accordingly, we have little to no evidence considering whole foods, or meals, which is alarming when one considers that the vast majority of human caloric intake occurs within meals and within complex food matrices, and always has done. Significantly, the current guidelines advising on dietary protein ingestion are largely composed of evidence obtained utilising isolated protein sources. Only in recent

years has there been a significant shift in focus towards whole foods (van Vliet et al., 2017, Burd et al., 2019, Kim et al., 2016, Trommelen et al., 2019, Gorissen et al., 2015), and a suggestion that there may be non-amino acid components within foods, or an interactive effect, capable of potentiating the muscle protein synthetic response.

Van Vliet and colleagues recently reported that the ingestion of whole egg stimulated greater muscle protein synthesis rates compared with the ingestion of an isonitrogenous dose of egg white alone, despite a delayed and lower peak magnitude of postprandial leucinemia (van Vliet et al., 2017). The authors ascribed this potentiating effect to consuming protein within a more complete "whole food" matrix, where there is a wider array of macronutrients and micronutrients, and the potential for myriad nutrient interactions. Whether this is an effect of the food matrix *per se* (Churchward-Venne et al., 2015, Elliot et al., 2006), a combined effect of the presence of additional macronutrients (and lipid subfractions (Castellano et al., 2017, Sawan et al., 2018, Yasuda et al., 2014)), or an effect of other micronutrient factors (Salles et al., 2013, Zhao et al., 2016, Marzani et al., 2008), is unclear. The authors have subsequently demonstrated that this potentiation may in part be modulated by greater mTOR colocalisation with the lysosome following the ingestion of whole egg, potentially as a result of a provocational factor within the egg yolk. Similarly, a study pre-dating van Vliet and colleagues by a decade illustrated that net muscle protein balance was greater following the ingestion of whole milk than skimmed milk, even when matched on caloric content and providing slightly less protein (Elliot et al., 2006), adding credence to the idea of the importance of a more complete food matrix. Churchward-Venne and colleagues also investigated the effect of the food matrix, comparing casein to casein dissolved in diluted milk serum (Churchward-Venne et al., 2015). They demonstrated that whilst ingesting casein in a milk matrix delayed protein digestion and absorption, it did not modulate muscle protein synthesis rates. Whilst this finding may appear contrasting, it might reflect that the study was investigating the idea of a food "matrix", as opposed to a nutrient

rich food. The formulation of casein suspended in milk serum perhaps lacks the nutritional complexity of the other two whole foods cited, lower in fat as it is, which could in part explain the differences. Taken in concert, these studies present the intriguing suggestion for something other than amino acids alone exerting an effect on postprandial muscle protein synthesis.

The categorisation of what is a "whole food" is inherently problematic, as it is difficult to state what represents a natural food matrix, and at what point the processing of food renders a food something else; of a lesser complexity, or vice-versa. For example, is homogenised milk with a portion of its fat removed, or beef that has been hung, trimmed of excess fat, and then minced a *whole food*? As such, it is probably more useful to consider protein-rich whole foods on a spectrum, as opposed to bisected diametric categories, from hydrolysed amino acids and isolated proteins at one end, through coingestion and then whole foods in the middle, to a nutrient-rich balanced meal at the other end.

The theoretical mechanistic framework as to why whole foods *may* be more anabolic remains largely in its infancy, and we have only just begun to adequately unravel the complexities or nuance it may encompass. Nevertheless, mycoprotein, the primary object of this thesis, is a "whole food", possessing a complex food matrix and a wide array of composite nutrients, which at least in theory could have a potentiating effect of muscle protein synthesis rates. Furthermore, mycoprotein is a very different food to those that have been considered thus far (egg and milk), and this thesis offers the opportunity to explore the whole food hypothesis in a unique whole food.

Animal & Non-Animal Protein Sources

It is generally assumed that animal-derived proteins are superior with respect to their capacity to stimulate muscle protein synthesis compared to non-animal-derived sources

(van Vliet et al., 2015b). Comparatively, animal proteins typically have a higher essential amino acid content (van Vliet et al., 2015b, Gorissen et al., 2018), and are rapidly and easily digested, leading to a substantial increase in plasma essential amino acid concentrations (Tang et al., 2009, Gorissen et al., 2016b). Indeed, when the muscle protein synthetic response to animal and non-animal-derived sources has been compared, the animal-derived source has stimulated a significantly greater muscle protein synthetic response. Tang and colleagues (2009) demonstrated that whey protein isolate stimulated a greater muscle protein synthetic response than the ingestion of soy protein isolate; a result that they attributed to the more rapid and greater peak magnitude of aminoacidemia and leucinemia in the whey protein condition. Still, it is worthy of note that soy protein isolate stimulated a greater muscle protein synthetic response than the ingestion of casein, a point rarely discussed, and confounding the simplicity of the "animal vs plant" narrative. Similarly, Gorrissen and colleagues (2016b) demonstrated that the myofibrillar protein synthetic response to the ingestion of 35 g casein is greater than after an equal amount of wheat protein in older men. Conversely, 35 g whey protein isolate stimulated muscle protein synthesis rates to a lesser extent than an equal amount of casein, somewhat obfuscating the interpretation of these findings. Whilst the ingestion of a larger amount of wheat protein (60 g) substantially increased myofibrillar protein synthesis rates, plasma amino acid availability still remained well below that of a leucine matched bolus of whey protein (35 g). As previously alluded to, and as clearly evidenced here, lower essential amino acid availability does not preclude a robust stimulation of muscle protein synthesis. That being the case, if, due to lower amino acid bioavailability, such a large amount of protein is required to stimulate a muscle protein synthetic response, then it makes questionable the practicality of such a strategy and the sustainability credentials of such a source. This is likely to be a consistent predicament when considering many alternative protein sources, where one must judge the extent to which the protein provides value in terms of sustainability, against the

extent to which it provides metabolic/anabolic value. As it is impractical, and potentially unsustainable, to simply consume more protein, we might wish to consider ways in which certain less anabolically promising non-animal-derived proteins can be enriched to enhance their anabolic capacity.

Patently, there is a paucity of evidence relating to non-animal-derived sources, and many of the axioms surrounding the anabolic properties of non-animal proteins are gleaned from underlying assumption rather than direct evidence. Besides, as noted, many of these underlying assumptions are equivocal in their interpretation. This thesis seeks to build significantly upon the dearth of data on non-animal-derived protein sources, taking the research in the new direction of fungal derived protein. Further to this, this thesis will also more broadly explore a non-animal-derived diet, as opposed to a single protein source and a single meal, thereby offering proof-of-concept as to the ability of a non-animal diet as a whole to stimulate muscle tissue anabolism.

Ageing Muscle

The human ageing process is associated with a progressive loss of skeletal muscle mass, termed sarcopenia (Evans, 1995b). Sarcopenia is recognised as a disease by the World Health Organisation (WHO), and is defined as the loss of muscle mass *combined* with alterations in physical function (dynapenia) and muscle quality. The latter two characteristics are important within this definition, as they are strongly associated with morbidity and mortality (Newman et al., 2006, Fabbri et al., 2017, Bischoff-Ferrari et al., 2015, Cheung et al., 2015). Indeed, the loss of skeletal muscle mass is associated with an increased incidence of falls, and subsequently fractures, alongside an increased risk of metabolic disease (Beaudart et al., 2017, Biolo et al., 2014). With this comes a concomitant burden on the healthcare system (Janssen et al., 2004, Sousa et al., 2016), which is exacerbated, and is likely to be exacerbated further, by our ageing society.

Muscle is an organ that applies force to bone via the tendinous unit, thereby facilitating movement. The stark counterpart to this is, therefore, that a deterioration in the ability of a muscle to contract forcefully will impinge upon movement. Moreover, as discussed earlier in the chapter, muscle is essential to metabolic homeostasis, and a loss of muscle mass is concomitant to a deterioration in metabolic capacity. This is in turn parallel to a loss of cells that indirectly play a role in proper immune function. Accordingly, and simply stated, a significant loss of muscle mass in later life means poorer movement, a poorer and less flexible metabolism, and a less competent immune system. Clearly, muscle mass plays a critical role in what can be termed "healthy ageing". This is not just the mere absence of infirmity, but also the ability to live an active lifestyle largely unfettered by a severe, and at least partially irreversible, deterioration in one's physical capabilities. Ergo, muscle mass plays what is probably an underappreciated role in facilitating a high quality of life during our later years, which in turn has a myriad of consequences for us as a society.

The age at which a decline in muscle mass begins is not entirely clear, dependent as it is on a host of modifying factors, and the discrepancy between chronological age and biological age. Nevertheless, there appears to be a 0.5-1% yearly decrease in muscle mass from the age of 70 onwards, with the trend appearing more aggressive in males (Mitchell et al., 2012), yet considered more debilitating in women due to lower critical thresholds. A loss of Type II myofibrillar protein content accounts for a disproportionality high fraction of this loss (Larsson, 1978, Coggan et al., 1992, Larsson et al., 1997, Cristea et al., 2010), which again may be more aggressive in males (Yu et al., 2007). Further, the loss of muscle mass is exacerbated by a general lack of physical activity (Oikawa et al., 2019), or periods of severe reduction in physical activity (Dirks et al., 2014, Kouw et al., 2019), both of which are more likely with advancing age due to lifestyle factors and the presence of comorbidities. The loss of muscle strength occurs at a faster rate than the loss of muscle mass, up to 5 fold greater in fact (Mitchell et al., 2012), which may in part be due to the proportionally high

loss of type II fibres, but is also the result of numerous converging deleterious neuromuscular modifications (Larsson et al., 2019).

The loss of protein is the result of a net imbalance between protein synthesis and protein breakdown. Sarcopenia appears to have a basis in both a decline in the synthesis of the contractile apparatus, myofibrillar and mitochondrial proteins, and an impaired regulation of proteolytic function. The exact extent to which each of these two factors contributes to a loss of protein is unclear, and is likely to vary considerably across a population. Nutritionally at least, the aetiology of sarcopenia is due to a postprandial resistance to the anabolic effects of nutrient intake (Wall et al., 2015a). There is considerable evidence available to suggest that older adults have a blunted muscle protein synthetic response to the anabolic effects of amino acids; both intravenous (Volpi et al., 1999, Guillet et al., 2004) and oral (Cuthbertson et al., 2005, Katsanos et al., 2005b, Moore et al., 2014b). As we age, we become less sensitive to the anabolic effects of protein (Wall et al., 2015a, Moore et al., 2014b); either the ceiling for the maximal stimulation of muscle protein synthesis drops, or we simply require more protein to stimulate an equivalent response to that of our younger selves. Further, there are data to suggest that the postprandial inhibition of muscle proteolysis may be diminished in older men (Wilkes et al., 2009). This reduced responsiveness to the anabolic effects of nutrients has been termed “anabolic resistance”.

The mechanistic crux that is responsible for this insensitivity to nutrients remains unclear. Muscle protein synthesis is regulated across multiple levels and compartments; consequently, deficiencies may be present at the level of protein digestion and amino acid absorption (Pennings et al., 2011b, Ferrando et al., 1996, Pennings et al., 2010), post-prandial microvascular perfusion (Rasmussen et al., 2006, Timmerman et al., 2010a), amino acid extraction by skeletal muscle tissue (Drummond et al., 2010), intramuscular signalling events (Cuthbertson et al., 2005, Fry et al., 2011b), and/or the accretion of myofibrillar

proteins (Cuthbertson et al., 2005). Impairments in the digestion of protein, and greater splanchnic retention of amino acids, has been shown in older compared with younger individuals, suggesting a lower presentation of plasma essential amino acids to muscle tissue in older individuals (Volpi et al., 1999, Boirie et al., 1997, Milan et al., 2015, Gorissen et al., 2020). Although these findings remain equivocal, with evidence existing to the contrary (Gorissen et al., 2014, Koopman et al., 2009b), the study by Gorissen and colleagues is particularly cogent in suggesting at least some level of impairment in the appearance of amino acids in circulation (Gorissen et al., 2020). It may be that deficits in amino acid absorption are relatively small, and therefore do not always present themselves clearly empirically, although that is not to say that such deficits are not insidiously consequential over a long period of time. Any decrement in the ability to absorb amino acids may be exacerbated by the suggestion that insulin mediated capillary perfusion following meal ingestion might be compromised (Rasmussen et al., 2006, Timmerman et al., 2010b, Timmerman et al., 2010a), alongside potential decrements in the expression of amino acid transporters (Dickinson et al., 2013). Further, nutrient stimulated intracellular anabolic signalling events integrated by mTOR have been shown to be impaired by ageing, and downstream effectors of mTOR that control translation initiation activated to a lesser degree (Cuthbertson et al., 2005, Guillet et al., 2004). Although, whether this is itself a mechanism, or a consequence of the decrements listed above is difficult to ascertain (the proverbial chicken and egg). All told, ageing muscle may receive fewer amino acids from the bloodstream, be able to extract fewer amino acids from the interstitium, and may be less able to utilise those amino acids due to an impaired ability to initiate the translation of proteins. Recent research has shifted focus to also consider daily muscle protein synthesis rates in older individuals, so as to understand in more depth the presentation of postprandial anabolic resistance over a lengthier period of time, and develop strategies to manage the process. This evidence has further underlined the importance of protein quality (Oikawa et

al., 2020b, Murphy et al., 2016b, Devries et al., 2018), and allowed us to start considering the diet as a whole. Nevertheless, we lack a point of comparison to younger anabolically healthy individuals when discussing anabolic resistance from the perspective of daily muscle protein synthesis rates.

The net result of these research efforts has been a call for an increase in the RDA for protein in older individuals, with a focus on high-quality proteins consumed in an even distribution pattern (as opposed to the more typical protein-heavy evening meal) (Traylor et al., 2018, Wall et al., 2014b, Phillips, 2017b). Regardless of whether a change in government recommendations occurs or not, the scientific community is now advocating that those who want to forestall the insidious loss of muscle mass that occurs with ageing should consume an amount of protein at or above 1.2 g·kg bm^{-1}·day^{-1} (Traylor et al., 2018). With the advocacy to consume *more* protein, and protein of high quality, where such protein should come from is particularly pertinent in this population, as is the cogency of the parallel consideration as to how an increase in protein should match sustainability values. Consequently, this thesis will consider the ability of non-animal-derived protein sources, with a specific focus on mycoprotein, can support daily muscle protein synthesis rates in older individuals.

RESISTANCE EXERCISE

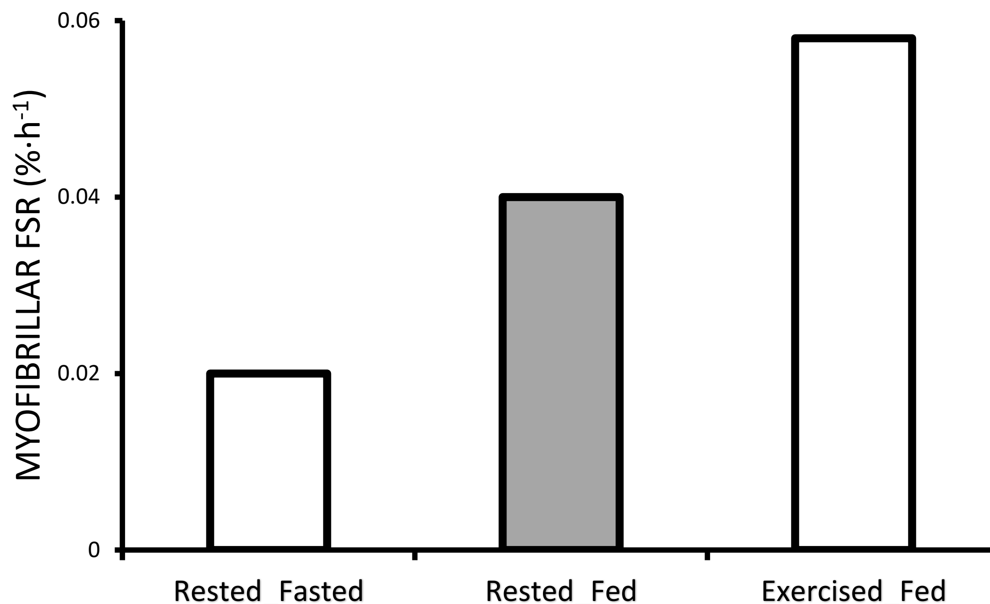
Acute Regulation

Resistance exercise represents a fundamentally anabolic stimulus. As alluded to, skeletal muscle mass remains relatively constant without the presence of a stimulus that either up or downregulates muscle protein synthesis or breakdown, usually in the form of disease, malnutrition, physical activity, or the ageing process. A bout of resistance exercise, or more precisely the sensing of mechanical tension within the muscle, stimulates muscle protein synthesis, and to a lesser extent muscle proteolysis (Phillips et al., 1997). Elevations in

muscle protein synthesis persist for up to 48 h in untrained individuals, but are shorter-lived in trained individuals (Tang et al., 2008, Damas et al., 2015); which may be a reflection of a greater requirement to rectify muscle damage in untrained individuals. It would seem logical that the extent to which muscle protein synthesis and protein breakdown are upregulated is modulated in part by the extent of muscle damage. Such that where there is a high degree of muscle damage, there is likely to be greater muscle protein breakdown to remove damaged contractile proteins, and greater muscle protein synthesis directed towards regeneration of that tissue, Damas and colleagues (2016) provided empirical evidence to support this, demonstrating that resistance training-induced changes in integrated myofibrillar protein synthesis are related to hypertrophy only after attenuation of muscle damage. Whilst this was an important study in its own right, it might also make us consider the perspective from which we view the resistance exercise mediated stimulation of muscle protein synthesis. Where there has been a large eccentric loading stimulus, as is the case with traditional resistance exercise, and a degree of muscle damage, a considerable portion of the increase in muscle protein synthesis rates may be directed towards the remodelling of damaged tissue. Concomitantly, only a portion of the increase in muscle protein synthesis rates is truly anabolic in nature, that is to say that only a portion of this response is directed at the accretion of *de novo* contractile proteins. Therefore, much of the transient increase in muscle protein synthesis following exercise is largely reflective of an increase in the amplitude of protein turnover. In line with the conclusions of Damas and colleagues, this provides reasoning as to why gains in muscle protein do not match that suggested from acute measurements of muscle protein synthesis (Mitchell et al., 2014, Mayhew et al., 2009), why the accrual of muscle protein is a gradual process, and why further accretion of protein is exceedingly difficult once a high degree of training volume/experience has been attained. Furthermore, it may explain why greater total muscle protein synthesis is exhibited in untrained compared with trained individuals following a bout of resistance exercise, where

one is likely to experience a greater degree of damage and turnover due to the novel nature of the stimulus (Damas et al., 2015).

Figure 1.2. The muscle protein synthetic response to protein ingestion, and protein ingestion following a bout of resistance exercise (Wall et al., 2016b, Damas et al., 2016, Moore et al., 2005).



Resistance exercise, like nutrition, also stimulates muscle protein synthesis via the activation of mTORC1 (Drummond et al., 2009), with muscle fibres detecting mechanical tension through cell membrane receptors, which then trigger a mechanotransductive signalling cascade converging on mTORC1. Concomitantly, the TGF-B and MAPK signalling pathways have also been shown to respond to muscle contraction (Kollias and McDermott, 2008, Wretman et al., 2001), with myostatin expression (a negative regulator of muscle protein synthesis) reduced and MAPK1 expression increased following contractile stimulus. Whilst mechanical-tension induced mechanisms are considered the predominant mechanism through which resistance exercise stimulates muscle protein synthesis, it would be inappropriate to discount the role of metabolite accumulation and substrate depletion entirely, given that significant shortcomings still remain in our understanding of how resistance exercise induces muscle protein synthesis.

Whilst the application of external mechanical load, and the intrinsic perception of mechanical tension, increases muscle protein synthesis, net balance will remain negative without the provision of dietary protein, as the rate of breakdown will exceed that of synthesis (Biolo et al., 1995b, Phillips et al., 1997). Consuming dietary protein following resistance exercise further augments the muscle protein synthetic response, and inhibits muscle proteolysis (Biolo et al., 1997); permitting a positive net protein balance above that seen with nutrition alone (Wall et al., 2016b). In young men, resistance exercise confers an enhanced sensitivity of muscle protein synthesis to plasma amino acids for up to 24h following exercise (Burd et al., 2011b); permitting a prolonged period of positive net protein balance if adequate protein is ingested. The extent of the muscle protein synthetic response to exercise would appear to hinge on three inter-related factors; volume of contraction (sets × reps), the degree of muscle fibre recruitment, particularly type II fibres (Burd et al., 2010), and the ability to load muscle fibres through their full contractile range. If the combined stimuli of resistance exercise-induced intermittent increases in muscle protein synthesis and adequate protein intake is repeated over time, there is persistent positive net protein balance and an accrual of muscle proteins, orchestrating muscle fibre hypertrophy (Damas et al., 2016).

Resistance Exercise Training

In humans, an increase in muscle size arises predominantly from an increase in muscle fibre volume. Muscle fibres increase in volume primarily due to increase in their diameter, secondarily due to an increase in their length. An increase in diameter occurs as a result of an increase in the number of sarcomeres in parallel (Farup et al., 2012, Johnson and Klueber, 1991). An increase in length occurs as a result of an increase in the number of sarcomeres in series (Seynnes et al., 2007). Either way, both require an increase in protein content through an upregulation of muscle protein synthesis, and subsequently a positive net protein balance. An increase in myocyte size is accompanied by a greater number of

myonuclear domains (the domain a specific nuclei controls) and/or myonuclear domain size. Either the area a nucleus controls must increase in size, or the number of domains must increase, although the exact contribution and relationship between these two factors remains somewhat unclear (Murach et al., 2018, Petrella et al., 2006). An increase in the number of myonuclear domains necessitates an increase in the number of myonuclei, which occurs via the fusing and donation of nuclei from satellite cells. Satellite cells being the undifferentiated quiescent progenitor stem cells committed to a skeletal muscle lineage (Dumont et al., 2015, Hawke, 2005). As, theoretically, each myonuclei controls mRNA transcription, and therefore protein synthesis, over a limited volume of sarcoplasm (Hall and Ralston, 1989, Cheek, 1985), an enlargement in myocyte size, at a given point, is contingent on an addition of new myonuclei to increase gross transcriptional capacity (Allen et al., 1995, Kadi and Thornell, 2000, Bellamy et al., 2014, Petrella et al., 2006). The point at which cell size, and myonuclear domain size, increase to an extent that requires additional myonuclei is poorly defined, although there is general consensus that a threshold for myonuclear addition exists; a point at which a domain can no longer increase in volume without additional DNA and mRNA (Petrella et al., 2006, Mackey et al., 2007, Kadi et al., 2004, Snijders et al., 2016). Increases in myonuclei number have been observed with ~10% myocellular hypertrophy, but appears more pronounced with myocellular hypertrophy greater than ~20% (Conceição et al., 2018). This appears to be accompanied by an increase in ribosomal biogenesis, permitting greater ribosomal capacity and ribosomal efficiency, which facilitates a greater capability for polypeptide formation (Figueiredo et al., 2015, Figueiredo et al., 2016, Hammarström et al., 2020, Mobley et al., 2018, Figueiredo and McCarthy, 2019, Stec et al., 2015, Fyfe et al., 2018). However, a clear relationship between translational capacity and hypertrophy is somewhat tenuous at this moment in time. It has been suggested that an increase in ribosomal density may represent an early phase response to training, to meet the demands of greater protein turnover (Damas et al., 2016), after which point there is a

stabilisation in hypertrophied fibres (Joanisse et al., 2020). Theoretically at least, this increase in myonuclear and ribosomal content provides the framework for greater protein turnover, and the ability to meet the escalated demands of a larger cell in response to overload stimulus.

In order to facilitate continued muscle hypertrophy, and concomitantly continued elevations in muscle protein synthesis, the loading stimulus has to increase in challenge over time, thereby surpassing the challenge to cellular capability and integrity that was previously experienced. This concept of progressive overload has been appreciated since antiquity, illustrated by the anecdote of Milo of Croton carrying a bull up a hill each day as he, and it, simultaneously grew. More verifiably, English Longbowmen progressively increased the draw weight on their bows as they matured through adolescence, facilitating greater draw weights (up to ~90 kg), more forceful projectiles, and an increasingly deadly weapon. Such was the difficulty in physically mastering such force, the necessity for training, and the importance to the militarised state, that multiple monarchs enshrined the practice of archery in law (Bartlett, 1995). This tangent represents a comprehensible example of the facilitative action of progressive overload, and the extent to which this principle exploits the awesome potential for muscle plasticity. Contractile protein content increases in response to a loading stimulus, meaning that more actin-myosin cross-bridges can be formed. This subsequently facilitates the same amount of force to be produced with fewer motor units recruited, and therefore a lesser number of fibres, as a compensatory mechanism. Concomitantly, it facilitates greater force production than was previously possible. To continually accommodate adaptation, either the number of contractions at that given force, or the force itself must be progressively increased over time in order to maintain motor unit and fibre recruitment. In practicality, this means more repetitions at a given load, a greater load, a refinement in technique that permits more tension to be placed upon a specific muscle at the expense of surrounding musculature, or potentially a more focused approach to

contraction (Neumann, 2019, Schoenfeld et al., 2018). Progressive resistance exercise training appears to provoke greater increase in the size of Type II fibres, around 50% greater (Adams and Bamman, 2012), than Type I fibres (Aagaard et al., 2001, Charette et al., 1991, Kosek et al., 2006, Schuenke et al., 2012, Staron et al., 1991, Snijders et al., 2015), although significant variability exists in the extent of fibre specific hypertrophy. In agreement, there is evidence that muscle protein synthesis is elevated to a greater degree following mechanical loading in the Type II dominant vastus lateralis muscle, than the Type I predominant soleus muscle (Trappe et al., 2004). In contrast, when individual muscle fibres are isolated from the same muscle, this does not appear to hold true, with Koopman and colleagues showing higher post-exercise muscle protein synthesis rates in type I than type II fibres (Koopman et al., 2011). This, in turn, suggests a more complex picture than a greater Type II hypertrophy purely being the result of a greater ability to acutely upregulate post-exercise muscle protein synthesis rates. Mechanical loading also results in adaptive responses in several other non-myofibrillar tissues including the muscle extracellular matrix (Hyldahl et al., 2015), tendon (Miller et al., 2005, Dideriksen et al., 2013) and bone (Zehnacker and Bemis-Dougherty, 2007, Shenoy et al., 2013), in order to increase the capacity of the musculotendinous unit to produce greater subsequent force.

The link between a progressive overload stimulus and muscle protein synthesis is incompletely elucidated. Presumably, after a period of acclimation to a resistance exercise training programme, a greater level of mechanical tension is then required to stimulate muscle protein synthesis to a level that maintains net protein balance, and the newly attained level of muscle mass. A new set-point is established. Furthermore, if resistance exercise stimulates muscle protein synthesis above basal levels for as long as the available evidence suggests (Tang et al., 2008, Damas et al., 2015, Burd et al., 2011b), then those following a structured resistance exercise programme likely spend more time in a "post-exercise" state than in a "rested" one. Further to this, "rested" and "exercised" states exist upon a spectrum,

contingent on contractile volume and proximity to prior contractile activity, and we only diametrically demarcate them as so for experimental reasons. Ergo, a truly basal level of muscle protein synthesis might be an irregular occurrence in resistance-trained individuals, and some degree of post-resistance exercise stimulation of muscle protein synthesis probably represents the predominant state in such individuals. Logically, when progressive overload is achieved, there is a modest increase in muscle protein synthesis above the previous ceiling stimulated with training, permitting tissue accretion. As training progresses, and as a greater level of muscle mass is attained, it follows that progressive increases in muscle protein synthesis become vanishingly small as incremental increases in mechanical tension diminish in extent. This would explain why further increases in muscle mass become increasingly difficult, and yet maintaining muscle mass is relatively easy.

The hypertrophic response appears highly variably from one individual to another. A consistent finding from training studies is a substantial degree of variability in the magnitude of muscle hypertrophy in individuals completing identical resistance training programmes (Hubal et al., 2005, Kosek et al., 2006, West and Phillips, 2012, Volek et al., 2013). Whilst the extent of the hypertrophic response appears normally distributed (Hubal et al., 2005), like many biological processes, this may not be truly reflective of a genetic propensity to increase muscle protein content. Indeed, if this is the case it would be posited on the assumption that all individuals were able to create the same stimulus, and thus the same level of mechanical tension. This would in turn assume that all individuals were able to train through a full range of motion (Schoenfeld and Grgic, 2020), execute a high level of effort, tolerate fatigue, and apply the same level of intent to their training (Schoenfeld et al., 2018). This would appear doubtful. In actuality, assuming that nutrition was optimal for each individual, the extent of hypertrophy is likely determinant on the interplay between one's ability to create a large mechanical stimulus, progressively so, and the genetic heritability that imbues the individual with biology to facilitate a high degree on anabolism. Accordingly,

whilst there *might* be non-responders to resistance exercise training, this is likely to be contingent on multiple factors other than purely biological factors.

Nutritionally, consuming ~1.6 g·kg $bm^{-1}·day^{-1}$ of protein appears sufficient to support the optimisation of the hypertrophic process (Morton et al., 2018), although a greater amount may be advisable during periods of energy deficit (Longland et al., 2016). Consuming this protein in an evenly distributed pattern (Areta et al., 2013b), including a serving of protein prior to sleep (Snijders et al., 2019), may confer additional benefits. Further to this, to truly optimise the amount of lean tissue accretion (regardless of fat tissue accretion), it may be a profitable strategy pursue a small caloric surplus (Slater et al., 2019), a strategy based upon limited experimental evidence but substantial anecdotal evidence.

THE ENVIRONMENTAL CONTEXT OF PROTEIN FOODS

Environmental Concerns

The Gordian Knot of feeding an increasing world population, whilst also mitigating climate change and environmental catastrophe, is an incredibly complex contemporary issue. The world population is projected to increase to 9 billion by the middle of the century, and is inextricably coupled with global trends for a rise in urbanisation, wealth creation, consumerism, and social mobility. A change in world population alone will drive an increase in the gross caloric requirements, and the demand for food will undoubtedly be exacerbated by the factors listed above. In parallel, increasing affluence is leading to increased consumption of animal proteins (Wellesley et al., 2015). Intensively factory-farmed livestock typically require more resources in their production than arable crops, are a major contributor of greenhouse gas emissions, and are a principal driver of habitat and species loss through deforestation (Bailey et al., 2014). This creates the uncomfortable juxtaposition of an increasing demand for food, and an increasing demand for animal-protein. This is not to say

that pastoral agriculture is inherently bad, or that arable agriculture is inherently good; this would be a gross misrepresentation and the picture remains enormously complex. Indeed, whilst livestock farming receives the Lion's the share of media attention, in the UK over-intensive arable farming is associated with deteriorations in soil quality, reducing carbon sequestration, and monoculture that is concomitant with a loss of biodiversity and species decline (Burns et al., 2016, Robinson and Sutherland, 2002, Hayhow et al., 2019). Nevertheless, increasing demand and competitive markets do not lend themselves to a framework of environmental responsibility and good farming practice, which is acutely true when it comes to livestock production. There is also the uncomfortable wider point that yield, efficiency, and low production costs make poor bedfellows with animal ethics, biodiversity, soil quality, and land management. There comes a point, at least with "traditional" agriculture, where optimising yield comes at the expense of environmental concerns, regardless of the type of agriculture. As such, we may be able to optimise yield, we *may be able* to optimise environmental sustainability, but it is perhaps naïve to think that we can universally optimise both simultaneously. Only by producing and purchasing food with this balance in mind are we likely to find the path towards a sustainable nutritional future.

To add a further element of complexity, and further compound an already precipitous situation, there is mounting scientific evidence that supports protein consumption at levels greater than the currently accepted Recommended Dietary Allowances (RDA) in various populations. Specifically, there is evidence to suggest that the maintenance of muscle mass in older adults (Wall et al., 2014b, Traylor et al., 2018, Phillips, 2017b, Paddon-Jones et al., 2015b, Phillips et al., 2016), the promotion of muscle tissue remodelling in athletes (Morton et al., 2018, Phillips, 2012), retention of muscle mass in athletes (Wall et al., 2015b), and successful weight management (Westerterp-Plantenga et al., 2012), all benefit from modest increases in dietary protein intake above the currently accepted RDA. The foods we eat and climate change are clearly inextricably linked, and it is essential that we view developments

in the understanding of human nutrition alongside those associated with developing a sustainable, and resilient, food production system. Consequently, developing and investigating alternative sustainable protein sources is a key aspect of securing a nutritionally sustainable future.

Mycoprotein

During the 1960s, the chair of the Rank Hovis McDougall (RHM) group of companies, the late Lord Rank, initiated a search for an alternative means to produce protein to serve an increasing world population. RHM was a major producer of cereals, and produced an abundancy of starch as a by-product. The ability to convert this abundant carbohydrate by-product into valuable protein, therefore, would represent a lucrative commercial and epidemiological solution. The concept of converting carbohydrate into a protein source is not especially novel, differing little theoretically from grazing or raising livestock, although the directness of the approach was novel indeed. For much of human history, certainly since *Homo sapiens* transitioned to agricultural settled communities, protein, or more properly animal protein, has been relatively scarce in its availability due to the energy and time associated with its production. It requires an intermediate step, converting cultivated or managed pasture into a resultant product, with energy loss and waste inherent in the process. When protein was consumed, it was more likely to be as a product from a live animal; a poor family did not eat the cow when it could be milked or bled. Consequentially, the removal of this intermediate step to produce a protein product, whilst utilising a by-product, was radically innovative. It was reasoned that the method to engender this would be to utilise an aerobic microfungus to convert carbohydrate into protein. Whilst microorganisms have been used throughout human history to flavour, develop and preserve foods, the development of a microorganism as a whole food in its own right was a step into the unknown. Eventually, after testing over 3000 soil samples, a suitable microorganism

(*Fusarium venenatum)* was identified, in a garden in Marlow, Buckinghamshire, and selected for development. *Fusarium venenatum* is a filamentous member of the *Ascomycota* branch of the fungi family, alongside truffles. Mycoprotein is the food derived from the mycelium of this fusarium. After the development of the currently used air lift fermentation technology, in partnership with ICI, and with clearance from the UK Ministry of Agriculture, Fisheries, and Food in 1985, mycoprotein was ready for commercial production under the Quorn^{TM} brand.

Fusarium venenatum for mycoprotein production is grown via continuous aerobic flow fermentation, with the addition of carbohydrate and nutrient substrates, under tightly controlled conditions in three 150,000 L fermenters, each capable of producing ~ 2 tonnes per hour of mycoprotein (Finnigan, 2011). The mycelium of the fungus is heat-treated to reduce the ribonucleic acid content to levels deemed safe for human consumption, before the suspended hyphae are centrifuged with the resultant solid mass further concentrated by vacuum chilling. The resultant product resembles the appearance of inelastic bread dough. In the production of QuornTM the mycoprotein product is processed and subsequently frozen to form the fibrous hyphael bundles that give Quorn a meat-like texture.

Mycoprotein is a high-protein, high-fibre, and relatively low-energy complete food source (Table 1.1). The dietary fibre profile of mycoprotein is relatively unique, composed of two-thirds b-glucan and one-third chitin, which together form a fibrous insoluble matrix that is uncommon in Western diets. It has been suggested that the digestive and metabolic properties of chitin and b-glucan may, in turn, mediate a number of positive cardiometabolic effects. Incorporating modest amounts of mycoprotein into the diet positively influences specific circulating lipid subfractions (Turnbull et al., 1990, Turnbull et al., 1993), and the acute ingestion of mycoprotein attenuates insulinemia, and potentially glycaemia, even under conditions of energy balance (Coelho et al., 2019, Cherta-Murillo et al., 2020). More

recent research has demonstrated that substituting meat/fish for mycoprotein at lunch and dinner for a week considerably impacts the plasma lipidome, demonstrating modulation of whole-body lipid metabolism (Coelho et al., 2020). Concomitantly, mycoprotein has been demonstrated to induce satiety (Bottin et al., 2016, Williamson et al., 2006, Turnbull et al., 1993, Turnbull and Ward, 1995), and may be a useful tool in the management of an energy deficit. Mycoprotein is likely to derive much of its appetite suppressing effect from its high protein content, as the link between protein and satiation is well established (Astrup, 2005, Westerterp-Plantenga et al., 1999, Astbury et al., 2010, Bertenshaw et al., 2008, Lejeune et al., 2006). However, the high fibre content of mycoprotein may also mediate appetite through both sensory and metabolic properties. Metabolites associated with partial fermentation of dietary fibre may partially explain any fibre-derived appetite-suppressing effect of mycoprotein (Bottin et al., 2016). Mycoprotein promotes the production of propionate, a short-chain fatty acid, which has been shown to induce the secretion of the anorexigenic gut hormones PYY and GLP-1, and may, in part, explain the short-term appetite-regulating effects of some dietary fibres (Chambers et al., 2015, Harris et al., 2019).

Mycoprotein has a protein content of around 45% dry-weight, 11.5% wet-weight, and has protein digestibility corrected amino acid score (PDCAAS) of 0.996. One hundred grams of mycoprotein provides 21 g of EAA (46% of total amino acids), and 3.9 g leucine (8% of total amino acids). Our laboratory has previously reported that total postprandial essential amino acid (and leucine) concentrations following the ingestion of mycoprotein are similar to that following the ingestion of a comparable bolus of milk protein (Dunlop et al., 2017). This is a finding atypical when comparing animal and non-animal dietary protein sources (Tang et al., 2009, Gorissen et al., 2016b), as the amino acids derived from the ingestion of non-animal proteins typically appear less bioavailable (van Vliet et al., 2015b, Tang et al., 2009, Gorissen et al., 2016b). Further, essential amino acid concentrations following mycoprotein ingestion increased in a dose-response fashion ≤60–80 g mycoprotein consumption (27–

36 g protein, 2.1–2.9 g leucine) (Dunlop et al., 2017). Mycoprotein, as we would expect, also robustly stimulated pancreatic insulin secretion. In short, this suggests mycoprotein would be capable of robustly stimulating muscle protein synthesis rates, and may represent a viable alternate protein source. An overarching intention of this thesis is to characterise the effect that the ingestion of dietary mycoprotein has on muscle tissue anabolism, both in rested and exercised tissue. This includes the determination of acute postprandial muscle protein synthesis rates in response to mycoprotein ingestion, whether mycoprotein can be incorporated into the diet to support daily muscle protein synthesis, and whether mycoprotein can support resistance training adaptation when consumed as a component of a vegan dietary pattern

Table 1.1. – Nutritional and amino acid composition of mycoprotein (dry-weight)

Nutrient Composition / 100 g	
Protein (g)	45
Fat (g)	13
Carbohydrate (g)	10
Fibre (g)	25
Energy (kcal)	340
Energy (Kj)	1423
Amino Acid Composition / 100 g	
Alanine	2.8
Arginine	3.3
Aspartic acid	4.6
Cystine	0.4
Glutamic Acid	5.6
Glycine	2.0
Histidine	1.6
Isoleucine	2.4
Leucine	3.9
Lysine	3.8
Methionine	1.0
Phenylalanine	2.3
Proline	2.0
Serine	2.3
Threonine	2.5
Tryptophan	0.8
Tyrosine	1.8
Valine	2.8
EAA	20.9
NEAA	24.6
BCAA	9.0

EAA, total essential amino acids; NEAA, total non-essential amino acids; BCAA, total branched chain amino acids. Data adapted from internal analyses published in part previously (Dunlop et al., 2017).

METHODOLOGIES

Stable Isotope Tracers

An isotope is a species of an element that is chemically identical but differs in mass, because it possesses more neutrons in the atomic nucleus. Although naturally occurring, such isotopes are far less abundant than their lighter isotopes; for example, carbon-13 makes up only ~1.1% of the earth's natural carbon, compared to carbon-12 which accounts for 98.9%. A stable isotope tracer is a molecule that is chemically and functionally identical to the naturally occurring molecule of interest (the molecule to be traced, the tracee), and following the metabolic path of the tracer allows for information to be garnered on the metabolism of the tracee. By utilising stable isotope-labelled amino acid tracers we are able to characterise muscle protein synthetic responses during acute postabsorptive and postprandial periods of time. Most commonly, muscle protein synthesis rates are measured using the continuous infusion of stable, isotopically-labelled amino acid tracers, alongside blood samples and, in most cases, muscle biopsy samples. Amino acids act as precursors for *de novo* muscle protein synthesis, thus using labelled amino acids allows for the tracing of these amino acids from the free amino acid pool (plasma and intracellular fluid) to their incorporation into tissue (protein-bound amino acid pool). This is the precursor:product method; the rate of incorporation of precursor into a product. Although labelled amino acids are not the true precursors for muscle protein synthesis, the true precursor pool (tRNA charged with amino acids) is infinitesimally small and difficult to sample, and consequently intracellular or plasma amino acid enrichment is used as a surrogate. After a steady-state in free amino acid pool enrichment is achieved, isotopically-labelled amino acids are incorporated into tissue in a relatively linear and constant pattern. By quantifying the incremental change in muscle protein-bound labelled amino acid enrichment between incremental biopsy samples, and the average enrichment in plasma or intracellular free amino acids, we are in turn able to

calculate the fractional synthetic rate of muscle protein synthesis; the rate of synthesis of that specific amino acid into muscle protein (Wolfe and Chinkes, 2005). These techniques allow us to investigate metabolism from a dynamic perspective, as opposed to a static one.

Measuring muscle protein synthesis during the transition from postabsorptive to postprandial conditions presents additional challenges for the researcher. Ingesting dietary protein causes an increase in exogenously-derived amino acids in the free amino acid pool, which causes a dilution in isotopically-labelled amino acid(s) in the free amino acid pool, and a disturbance to the steady-state during continuous intravenous infusion. To negate this issue, the dietary protein ingested can be enriched with the isotopically-labelled amino acid that is being infused, so that there is an increase in both labelled and unlabelled amino acids in the free amino acid pool. If the enrichment of the protein ingested can be matched to that of the target enrichment of the precursor pool, then any disturbance to the steady-state can largely be obviated. To this ends, intrinsically labelled proteins have been utilised to great effect (Pennings et al., 2012b, Wall et al., 2016b), however, practicality and expediency are often prohibitory in the production and utilisation of intrinsically labelled proteins.

Whilst the use of the infusion of intravenous stable isotope tracers allows for the accurate measurement of muscle protein synthesis rates, it does also possess limitations. Namely, the method dictates usage for a limited period of time, less than ~<24h, and under very controlled laboratory conditions. Such controlled conditions remove much of the nuance associated with typical patterns of human-living, discounting aspects of the complex interplay of environmental stimuli present under free-living conditions. This reductionism has, in turn, led to the applicability of these short term measures to predict long term outcomes to be questioned, with acute measures of muscle protein synthesis not accurately reflecting long term changes in protein content (Mitchell et al., 2014).

An alternative method for determining muscle protein synthesis rates is via the use of the stable isotope tracer Deuterium oxide (D2O) or “heavy water”. This method introduces a tracer *per se* (^{2}H) into circulation, rather than a labelled substrate/precursor, which, at least in theory, allows this method to be applied for any metabolic process that requires H atoms. The use of deuterium oxide to investigate metabolism is not new, it was first described in 1941 by Hans Ussing (Ussing, 1941), but it has undergone a resurgence in recent years due to advances in analytic capabilities (Dufner and Previs, 2003), and a necessity to seek alternative methods to redress gaps in our current understanding of muscle protein synthesis. Deuterium oxide is orally consumed, thereby obviating the need for sterile intravenous infusions under clinical laboratory conditions. Similarly to intravenous precursor:product labelling models, a labelled tracer is provided and its incorporation into tissue is measured. Ingested deuterium oxide is rapidly equilibrated throughout the body water pool (~ 2h), labelling amino acids intracellularly via transamination, which can then charge tRNA and be incorporated into peptide chains on the ribosome. Analogously to intravenous amino acid tracers, a constant enrichment of ^{2}H-labeling in body water is attained and then maintained, which provides an indication that the true precursors are sufficiently labelled, and at a steady-state, for incorporation into tissue. This is aided by the fact that feeding does not appear to significantly dilute the precursor pool, with dilutions to the alanine pool equilibrating rapidly (Belloto et al., 2007). As alanine is relatively abundant, rapidly turned-over, and possesses four potential sites (α- and β-hydrogens) for ^{2}H-labeling to occur, it is almost exclusively the amino acid utilised for the measurement of deuterium incorporation into tissue. As with other tracer techniques, it is assumed that tracer recycling is minimal, particularly within pools that turnover slowly, such as in skeletal muscle, and any dilution through recycling may be aided by the rapid exchange of deuterium into amino acids. Where there are exceptionally long labelling periods, beyond the half-life of most proteins, recycling may be of concern. As deuterium oxide has a relatively long half-life within the

body (7-10 days (Gasier et al., 2010)), and can be provided orally, it permits more prolonged periods of measurement. Resultantly, the use of heavy water to measure muscle protein synthesis is accorded a number of distinct advantages, namely that it can measure the incorporation of tracer into tissue under free-living conditions over a number of days and weeks. This enables researchers to more holistically investigate muscle protein synthetic responses, whereby modulating factors such as the effect of multiple feeding-fasting cycles, diurnal variation and habitual activity are captured and accounted for in the measurement. Furthermore, it allows us to investigate the diet in its entirety, rather than just the response to a single meal, or rather, more usually, a single dose of protein. Given the relative lack of research concerning the effect of the diet as a whole on muscle protein anabolism, the use of this free-living approach represents an invaluable tool to investigate the effect that dietary complexities and interactions have on muscle protein anabolism. Indeed, there is a growing body of research that has utilised cumulative measures of muscle protein synthesis to disentangle some of the intricacies of nutrient and exercise interactions (Bell et al., 2015, Murphy et al., 2016b, Davies et al., 2019, Oikawa et al., 2020b, Kilroe et al., 2020c, Kilroe et al., 2020a, Shad et al., 2019, Holwerda et al., 2018b).

Western Blotting

One technique that is commonly used in complement to measures of muscle protein synthesis is the Western Blot. The Western Blot technique permits the qualitative detection of individual proteins, and protein-modifications, such as post-translational modifications. In muscle, it can be utilised as a method to identify the presence of a specific protein within the myriad of proteins present within the tissue, and the molecular weight of that protein. A semi-quantitative estimation of the desired protein can then be derived from the size and colour intensity of the protein band on the blot membrane. Using this line of investigation key signalling proteins can be identified, for example, mTOR and its downstream effectors, and

the relative extent to which they are expressed determined. Moreover, the researcher can determine the relative quantity of that protein after undergoing post-translational modifications that change the functional qualities of that protein, such as phosphorylation, which represent key regulatory events in the anabolic signalling cascade. This has, in turn, has allowed researchers to investigate the effect that specific stimuli, such as nutrition and mechanical loading, have on key anabolic signalling proteins. By doing so we can potentially glean a more detailed understanding of the regulation of muscle protein synthesis, and potentially explain why certain stimuli prove more or less effective than others. That being said, anabolic signalling events are often observed to be somewhat dissociated from measures of protein turnover (Greenhaff et al., 2008), and a greater quantity of phosphorylated signalling proteins does not inevitably beget higher muscle protein synthesis rates. Moreover, Western Blots can be difficult to interpret, as they remain only *semi*-quantitative, and are reliant upon the correct timing of tissue sampling to coincide with the time course of an upregulation in anabolic signalling. Nor does the Western Blot account for the subcellular location of the protein of interest, which may be an important factor in determining its behaviour and interactions (Abou Sawan et al., 2018a).

Transcriptional Responses

Investigating the transcriptional response to the combination of nutrition and exercise allows further characterisation of the epigenetic response. Measuring acute mRNA expression provides “a snapshot of the intent of the cell”, meaning that it provides an indication of the initial response of the cell to stimuli, to then change or maintain the level of specific proteins in order to maintain, or shift, homeostasis. Whilst it can be argued whether cells have “intents” or not, measuring the expression of specific genes can provide us with a direction of the initial cellular response. This can be particularly valuable when investigating metabolic processes that are difficult to measure, or require specific quirks of experimental design

(Drummond et al., 2010, Mitchell et al., 2017, Drummond et al., 2011, Graber et al., 2017). For example, by measuring the expression of genes associated with muscle protein breakdown we can investigate the effect of nutrient-contractile interactions on proteolysis when experimental considerations preclude direct measurement (Wall et al., 2016c, Wall et al., 2016b). Further, by combining measures of gene expression with known physiological outputs, e.g. muscle hypertrophy, we can speculate as to the importance of changes in specific genes relative to the strength of change in physiological outcome. Although transcriptional changes precede physiological changes, the identification of early transcriptional changes could elucidate mechanisms responsible for phenotypic changes that occur later on in response to diet, exercise and disease. This should, of course, be applied with the caveat that measuring mRNA expression does not necessarily lead to a change in active protein content, as a host of (post) translation modifications can occur that render an increase in mRNA content largely impotent. Further, alterations in muscle metabolism may precede related transcriptional responses, which appear to act as a molecular compensatory response rather than a causative molecular mechanism (Dirks et al., 2018). When measuring transcriptional responses, the researcher has the choice of either taking a targeted approach, a single gene for instance, or a much broader one, up to the level of characterising whole pathways; an “omics” approach. A satisfying balance between the two can be struck by applying a targeted genecard approach, whereby a moderate number of genes of interest are selected for analysis. This permits a relatively large number of genes to be analysed, whilst negating the risk of false positives but still providing a thorough characterisation, and also maintaining a degree of focus. An intention of this thesis is to present data investigating acute transcriptional responses to divergent nutritional signals, alongside exercise, in parallel to the acute measurement of muscle protein synthesis.

Measures of Muscle Hypertrophy

Whilst the acute measurement of skeletal muscle protein turnover is a valuable quantification of the postprandial response, and multiple postprandial responses, it does have limitations in its scope and the extent to which it can be extrapolated. Whilst acute muscle protein synthesis does correlate with muscle hypertrophy, after attenuation for muscle damage, there comes a point at which phenotypic changes in the muscle need to be studied directly in order to inform upon the adaptive response. There are multiple ways in which changes in muscle mass can be measured, all the way from a macro-level, the whole body, through diminishing scope to the micro-level of an individual cell.

A common approach to measuring body composition is the use of Dual-energy X-ray absorptiometry (DXA), which allows the researcher to estimate the relative abundance of different tissues. This allows the researcher to determine lean mass, and subsequently changes in lean mass over time (muscle as well as other protein-containing organs), alongside changes in fat mass and bone mineral density. Additionally, one is able to determine the abundance of lean tissue in different regions of the body, and thus determine lean mass in individual limbs. Whilst undoubtedly useful, this method is insufficient to provide data on individual muscles, and therefore precludes any investigation that does not intervene at the level of the whole limb or bodily.

In order to examine a limb and the constituent muscles in more detail, researchers turn to other scanning methods, such as magnetic resonance imaging (MRI), computed tomography (CT), and ultrasonography. Images are taken as coronal sections, or slices, of a limb and then processed such that the cross-sectional area of that limb and the individual muscles contained within can be calculated (CSA). Multiple cross-sectional images can be combined to calculate muscle volume which gives a more complete description of changes in muscle size with resistance training, accounting more so for potential regional

hypertrophy. MRI offers the higher resolution of the two methods and is therefore considered the gold standard for measures of whole muscle hypertrophy. When investigating an intervention with the intention to hypertrophy a muscle, MRI provides a detailed insight into how individual muscles change in size in each of their dimensions. This provides the researcher with a visual and volumetric measurement representing the terminus of a period of cumulative muscle protein synthesis, net muscle protein balance, and tissue accretion.

Measures at the level of the whole muscle can be complemented with measures of changes in the characteristics of individual muscle fibres using histochemical techniques. Histology makes use of selective identification of proteins by exploiting the principle of specific antibody to antigen binding, thus allowing for the staining of specific proteins within skeletal muscle tissue that can then be imaged under a microscope. This allows for the isolation of fibre types, and subsequently changes in fibre cross-sectional area, alongside the imaging of myonuclei content, satellite cell number, and location. Whilst this technique allows for the direct quantification of muscle fibre cross-sectional area it is not without issue. As it uses what is essentially random sampling of only a small proportion of total muscle tissue, it can be confounded by samples with a low number of fibres and fibres that are not truly transverse in orientation. The combination of the measures listed above provides for a comprehensive characterisation of the hypertrophic response.

SUMMARY

To summarise, it is essential that we develop a robust evidence base to characterise the anabolic response of the muscle to the ingestion of non-animal-derived protein sources. Much of the evidence that we have at our disposal has been collected from investigations of the ingestion of animal-derived proteins, and this has left a significant gap in our knowledge when it comes to the plethora of non-animal proteins. This is pertinent as there

is increasing impetus to reduce the amount of meat that is consumed, as part of developing a more environmentally sustainable food system.

In Chapter 3 I utilised stable isotope labelled amino acid tracers to quantify the muscle protein synthetic response to the ingestion of mycoprotein, in comparison to a leucine matched bolus of milk protein, in young healthy resistance-trained men. Alongside this I applied a targeted genecard approach, utilising real-time PCR, to characterise the transcriptional response to two divergent protein sources, and resistance exercise. I hypothesised that mycoprotein would robustly stimulate muscle protein synthesis, albeit to a lesser extent than a leucine matched bolus of milk protein due to the predicted lower peak magnitude and rapidity of leucinemia following mycoprotein ingestion.

In Chapter 4 I again utilised stable isotope labelled amino acid tracers to quantify the muscle protein synthetic response to the ingestion of a smaller bolus of mycoprotein (35 g), enriched with branched chain amino acids to match that of a larger 70 g bolus of mycoprotein. I hypothesised that the ingestion of the lower branched chain amino acid enriched mycoprotein beverage would stimulate an equivalent muscle protein synthetic response to that of the larger mycoprotein beverage.

In Chapter 5 I applied an oral deuterated water approach (Kilroe et al., 2020a, Kilroe et al., 2020c) to determine whether a mycoprotein-based high-protein vegan diet could support rested and exercised daily muscle protein synthesis in older adults to the same extent as an isonitrogenous omnivorous diet. I hypothesised daily muscle protein synthesis rates would be equivalent irrespective of whether dietary protein was primarily obtained from animal or non-animal sources, and that exercise would increase daily muscle protein synthesis rates compared with rested muscle.

In Chapter 6 I applied a two-fold approach to investigate the adaptive response to non-animal-derived protein diets alongside resistance exercise. Firstly, I applied an oral deuterated water approach to determine whether a mycoprotein-based high-protein vegan diet could support rested and exercised daily muscle protein synthesis rates in younger adults to the same extent as an isonitrogenous omnivorous diet. Secondly, I investigated the adaptive response to a majority non-animal-derived diet, compared to a high-protein omnivorous diet, alongside 10 weeks of structured progressive resistance exercise. To do so I applied DXA scans, MRI scans, muscle biopsy samples, blood samples, measures of muscle strength, and measures of muscle function. In light of the data obtained from the first two studies of this thesis, my hypothesis was two-fold. Firstly, I hypothesised daily muscle protein synthesis rates would be equivalent irrespective of whether dietary protein was primarily obtained from animal or non-animal sources, and that exercise would increase daily muscle protein synthesis rates compared with rested muscle. Secondly, I hypothesised that resistance exercise adaptation would be equivalent irrespective of whether dietary protein was primarily obtained from animal or non-animal sources.

Intentions of Thesis

- To determine whether the ingestion of a single bolus of mycoprotein would stimulate postprandial mixed muscle protein synthesis rates in both rested and exercised skeletal muscle healthy young men.

- To investigate the acute transcriptional response to mycoprotein and milk protein ingestion, and how this is modulated by resistance exercise.

- To determine whether a smaller bolus of mycoprotein could be enriched with branched chain amino acids to beget a similar muscle protein synthetic response to a larger bolus of mycoprotein.

- To determine whether a mycoprotein based high-protein vegan diet supports equivalent daily myofibrillar protein synthesis rates compared with an isonitrogenous omnivorous diet in younger and older adults.
- To determine whether a majority non-animal-derived diet can support an equivalent skeletal muscle adaptive response to 10 weeks of intense whole-body progressive resistance exercise training as consuming a high-protein omnivorous diet, in young adults.

In summation, this thesis will characterise and translate the response to mycoprotein ingestion from the level of a single meal to the level of a change in muscle mass and physical function. Laconically put; from molecule, to mass and movement,

CHAPTER 2

GENERAL METHODS

Overview

The vast majority of the methods described within this thesis are embedded within the experimental chapters, and as such this chapter is brief. Below I have detailed a comparison between Branched Chain Amino Acid (BCAA) concentrations derived from a spectrophotometric assay, to BCAA concentrations derived from the more typical GC-MS method.

BCAA Assay

In order to obtain pilot data on BCAA concentrations prior to receiving them at the end of the lengthy mass spectrometry process, I piloted a spectrophotometric assay for measuring BCAA concentrations. This method was adapted from the work of Beckett and colleagues (Beckett et al., 1996).

Leucine dehydrogenase, in the presence of excess NAD+, catalyses the following reactions:

Leucine + $NAD^{+} \rightleftharpoons$ a-ketoisocaproate + NADH + H^{+}

Isoeucine + $NAD^{+} \rightleftharpoons$ a- ketomethylvalerate + NADH + H^{+}

Valine + $NAD^{+} \rightleftharpoons$ a- ketovalerate + NADH + H^{+}

The rate of appearance of NADH can then be then be determined from the change in absorbance at 340 nm.

Briefly, NAD (120 mM)(Sigma N7004-1G) was diluted in Glycine-KCl-KOH buffer. The buffer was 0.2 M glycine and 0.2 M KCl, which was adjusted to pH 10.5 by using 2M KOH, before 2 mM EDTA was added. Lyophilised leucine dehydrogenase powder (25U) (Sigma L5135-25UN) was suspended in 1200 µL sodium phosphate buffer (25 mM; pH 7.2). A total volume of 300 µL was pippetted into each well on the plate; 5 µL standard or plasma, 10 µL NAD in glycine-KCl-KOH buffer, 285 µL glycine-KCl-KOH buffer. Blank absorbance was measured

at 340 nm, before adding 2 µL of leucine dehydrogenase (in sodium phosphate buffer), and measuring absorbance again at 60 min. Serial dilutions of 2 mM leucine standard (Sigma PHR1105) were used to give standard concentrations of 0-2 mM. A standard curve was plotted, with the slope and intercept used to calculate concentration from delta absorbance.

When comparing 20 participants who ingested 26.2 g milk protein concentrate there is a strong correlation between the GCMS method and the assay method (r=0.9956; P<0.0001). The assay method produced concentrations significantly higher than the GC-MS method (method effect; P<0.0001), on average 46±5% higher. A Blant-Altman plot showed limited agreement, and significant bias.

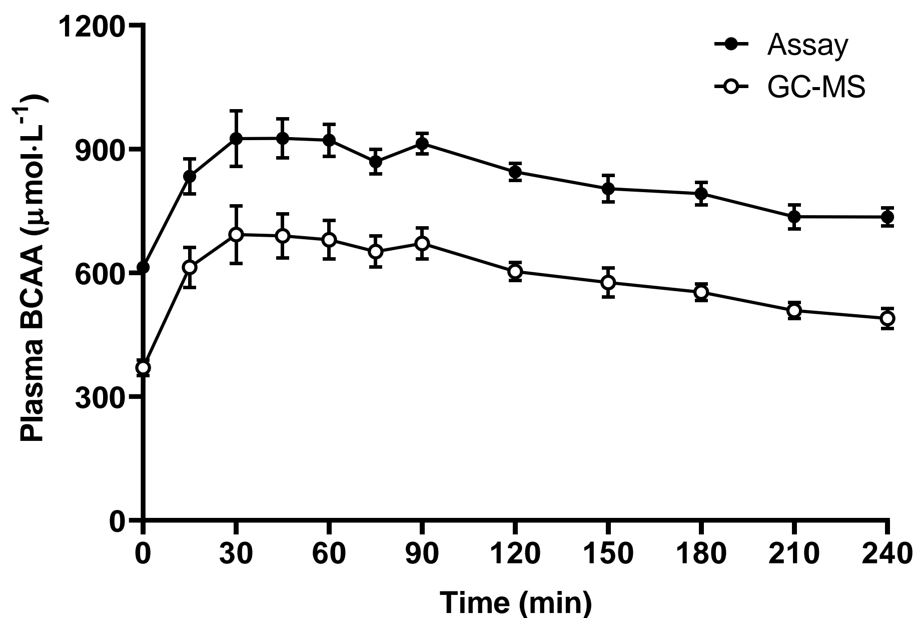


Figure 2.1. The time course of plasma branched-chain amino acid concentrations during a 4-h postprandial period following the ingestion of 26.2 g milk protein in healthy young men. Data were analysed with a repeated measures 2-factor ANOVA. Values are means, with their SEs represented by vertical bars.

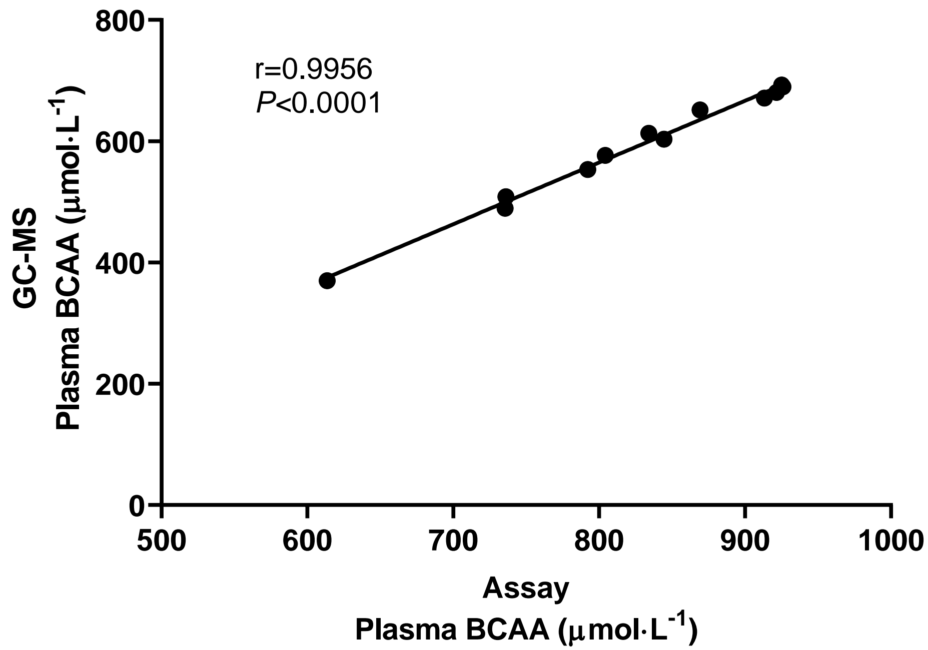


Figure 2.2. Correlation between the GC-MS and the spectrophotometric assay method for quantifying plasma branched-chain amino acid concentrations. Plasma values are taken from a 4-h postprandial period following the ingestion of 26.2 g milk protein in 10 healthy young men. r and P values are displayed on each graph.

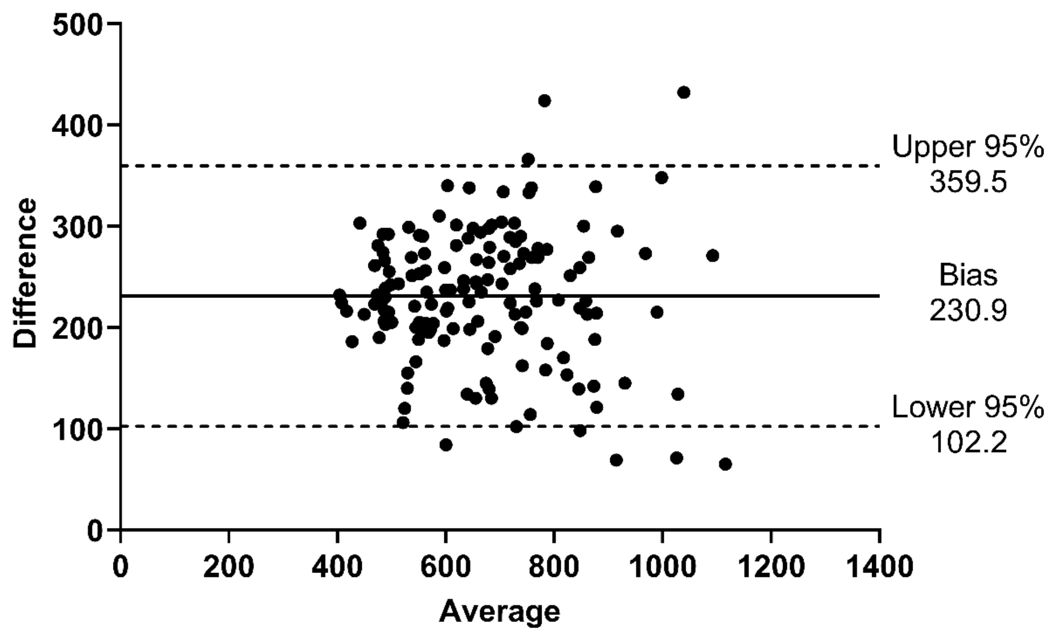


Figure 2.3. A comparison between the GC-MS and the spectrophotometric assay method for quantifying plasma branched-chain amino acid concentrations using a Bland-Altman plot. limits of agreement (dashed lines) and bias (solid line).

CHAPTER 3

MYCOPROTEIN INGESTION STIMULATES PROTEIN SYNTHESIS RATES TO A GREATER EXTENT THAN MILK PROTEIN IN RESTED AND EXERCISED SKELETAL MUSCLE OF HEALTHY YOUNG MEN

The work contained within this chapter is published:

Monteyne, A.J., Coelho, M.O., Porter, C., Abdelrahman, D.R., Jameson, T.S., Jackman, S.R., Blackwell, J.R., Finnigan, T.J., Stephens, F.B., Dirks, M.L. and Wall, B.T., 2020. Mycoprotein ingestion stimulates protein synthesis rates to a greater extent than milk protein in rested and exercised skeletal muscle of healthy young men: a randomized controlled trial. *The American Journal of Clinical Nutrition*.

Abstract

Background: Mycoprotein is a fungal-derived sustainable protein-rich food source, and its ingestion results in systemic amino acid and leucine concentrations similar to that following milk protein ingestion.

Objective: We assessed the mixed skeletal muscle protein synthetic response to the ingestion of a single bolus of mycoprotein compared with a leucine matched bolus of milk protein, in rested and exercised muscle of resistance-trained young men.

Design: Twenty resistance-trained healthy young males (age: 22±1 y, body mass: 82±2 kg, BMI: 25±1 $kg \cdot m^{-2}$) took part in a randomised, double-blind, parallel-group study. Participants received primed, continuous infusions of L-[ring-$^{2}H_{5}$]phenylalanine and ingested either 31 g (26.2 g protein: 2.5 g leucine) milk protein (MILK) or 70 g (31.5 g protein: 2.5 g leucine) mycoprotein (MYCO) following a bout of unilateral resistance-type exercise (contralateral leg acting as resting control). Blood and *m. vastus lateralis* muscle samples were collected before exercise and protein ingestion, and following a 4 h postprandial period to assess mixed muscle fractional protein synthetic rates (FSR) and myocellular signalling in response to the protein beverages in resting and exercised muscle.

Results: Mixed muscle FSR increased following MILK ingestion (from 0.036±0.008 to 0.052±0.006$\% \cdot h^{-1}$ in rested, and 0.035±0.008 to 0.056±0.005$\% \cdot h^{-1}$ in exercised muscle; $P<0.01$) but to a greater extent following MYCO ingestion (from 0.025±0.006 to 0.057±0.004$\% \cdot h^{-1}$ in rested, and 0.024±0.007 to 0.072±0.005$\% \cdot h^{-1}$ in exercised muscle; $P<0.0001$) (treatment × time interaction effect; $P<0.05$). Postprandial FSRs trended to be greater in MYCO compared with MILK (0.065±0.004 vs 0.054±0.004$\% \cdot h^{-1}$, respectively; $P=0.093$) and the postprandial rise in FSR was greater in MYCO compared with MILK (Δ 0.040±0.006 vs Δ 0.018±0.005$\% \cdot h^{-1}$, respectively; $P<0.01$).

Conclusions: The ingestion of a single bolus of mycoprotein stimulates resting and post-exercise muscle protein synthesis rates, and to a greater extent compared with a leucine matched bolus of milk protein, in resistance-trained young men.

INTRODUCTION

Adequate dietary protein intake is required to maintain skeletal muscle mass and to facilitate the remodelling and/or hypertrophy of muscle tissue in response to exercise training. Mechanistically, this is largely achieved by dietary protein ingestion transiently (2–5 h) stimulating muscle protein synthesis rates (Tang et al., 2009, Pennings et al., 2011b, Moore et al., 2008), primarily due to a postprandial elevation of plasma essential amino acid concentrations (Tipton et al., 1999), particularly leucine (Wall et al., 2013b, Norton et al., 2009). A single bout of resistance exercise also stimulates muscle protein synthesis rates for ≤ (and ≥) 48 h (Phillips et al., 1997, Biolo et al., 1997), whilst sensitising muscle tissue to the anabolic effects of dietary protein for ≥24 h (Burd et al., 2011b). Consequently, research has sought to identify aspects of protein nutrition (e.g., amount and timing) that can be manipulated to optimally support post-exercise muscle protein synthesis rates (Tang et al., 2009, Moore et al., 2008, Areta et al., 2013b, Wall et al., 2016b, Witard et al., 2013, Rasmussen et al., 2000). However, information relating to the anabolic properties of non animal-derived dietary protein sources is lacking, which is concerning given the increasing emphasis on dietary sustainability. Animal-derived dietary protein sources, such as whey (Tang et al., 2009, Pennings et al., 2011b, Witard et al., 2013, Gorissen et al., 2016b, Yang et al., 2012b), casein (Gorissen et al., 2016b, Groen et al., 2015), milk (Mitchell et al., 2015, Burd et al., 2015), beef (Burd et al., 2015, Symons et al., 2009, Robinson et al., 2013, Pennings et al., 2013), and egg (Moore et al., 2008, van Vliet et al., 2017) have all been shown to stimulate post-exercise muscle protein synthesis rates. It is assumed that plant-based dietary protein sources are inferior in their capacity to stimulate muscle protein synthesis rates, due to their typically slower digestibility, lower bioavailability, and lower essential amino acid and leucine content (van Vliet et al., 2015b). Indeed, whey protein stimulates muscle protein synthesis rates to a greater extent than soy in young men (Tang et al., 2009, Yang et al., 2012b) and compared to wheat protein in older men (Gorissen et

al., 2016b). To date, however, these are the only non-animal-derived protein sources to be studied with respect to their impact on muscle protein synthesis.

Mycoprotein is a sustainably produced food source rich in protein (~45% of total mass) and essential amino acids (~44% of total protein) derived from the cultivation of the fungus Fusarium venenatum. We have reported that total postprandial essential amino acid (and leucine) concentrations following mycoprotein ingestion are comparable to that seen following milk protein ingestion (Dunlop et al., 2017), a finding atypical of animal versus non-animal dietary protein comparisons (Tang et al., 2009, Gorissen et al., 2016b). Further, essential amino acid concentrations following mycoprotein ingestion increased in a dose-response fashion ≤60–80 g mycoprotein consumption (27– 36 g protein, 2.1–2.9 g leucine) (Dunlop et al., 2017), suggesting mycoprotein would be capable of robustly stimulating muscle protein synthesis rates.

In the present work, we tested the hypothesis that the ingestion of a 70 g bolus of mycoprotein (31.5 g protein, 2.5 g leucine) would stimulate mixed muscle protein synthesis rates over a 4-h postprandial period in both rested and exercised skeletal muscle of resistance-trained, healthy young men. We compared the muscle protein synthetic response of mycoprotein ingestion to a leucine-matched bolus of milk protein. Here, we hypothesised that despite equivalent leucine contents, due to slower aminoacidemia (Dunlop et al., 2017), muscle protein synthesis rates would increase to a lesser extent following mycoprotein ingestion. We chose to match the 2 beverages on leucine content as various lines of enquiry suggest leucine content, rather than total protein, is the primary factor determining the postprandial muscle protein synthetic response when sufficient protein is consumed (Norton et al., 2009, Churchward-Venne et al., 2013, Stokes et al., 2018).

METHODS

Participants

Twenty young, healthy (age: 22±1 y, body mass: 82±2 kg, BMI: 25±1 $kg·m^{-2}$) men volunteered to take part in the present study. Participants' characteristics are displayed in **Table 3.1**. Participants were recreationally active and experienced with resistance training (at least 3 times per week for at least 3 months prior to participation). Participants were deemed healthy based on their blood pressure (≤140/90mmHg), BMI (18-30 $kg·m^{-2}$) and responses to a routine medical screening questionnaire (absence of any diagnosed metabolic impairment, cardiovascular disease, or motor disorders), and were informed of the experimental procedures, potential risks, and the purpose of the study prior to providing full written consent. Participants were all 'tracer naïve' having not undergone any previous stable isotope amino acid infusion protocols. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (REF NO. 161026/B/05) in accordance with standards for human research as outlined in the declaration of Helsinki. Recruitment and data collection were completed between January 2017 and August 2017 at The University of Exeter.

Table 3.1. Participant characteristics.

	MILK (*n = 10*)	MYCO (*n = 10*)
Age (y)	22 ± 1	22 ± 1
Body mass (kg)	84 ± 3	81 ± 3
Height (cm)	178 ± 2	182 ± 3
BMI ($kg \cdot m^{-2}$)	26 ± 1	25 ± 1
Fat (% body mass)	12 ± 2	9 ± 2
Lean mass (kg)	73 ± 3	73 ± 2
Total work done (J)	29540 ± 1782	30722 ± 2059
Energy ($MJ \cdot day^{-1}$)	9.5 ± 0.8	11.6 ± 0.9
Protein ($g \cdot day^{-1}$)	150 ± 20	162 ± 17
Protein ($g \cdot kg\ bm^{-1} \cdot day^{-1}$)	1.8 ± 0.2	2.1 ± 0.2

Values represent mean ± SEM. MILK, Milk protein ingestion condition; MYCO, mycoprotein ingestion condition, BMI, body mass index. Total work represents the amount of work done (in J) during the experimental exercise protocol. No statistically significant differences were observed between conditions ($P>0.05$).

Pre-testing

Following screening and acceptance onto the study, all participants underwent a single pre-testing session, which took place at least 5 days prior to the experimental trial. Participants were familiarised with the exercise equipment and exercise protocol, and body fat and lean mass were determined by Air Displacement Plethysmography (BodPod, Life Measurement, Inc. Concord, CA, USA). Participants were familiarised with the unilateral resistance-type exercise that was employed in the experimental protocol. This consisted of 5 sets of 30 repetitions of maximal concentric isokinetic leg extension and leg flexion contractions on a Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY, USA) at a speed of 60° per second over a central 80° range of motion using their self-reported dominant leg. Verbal encouragement was provided throughout the familiarisation and experimental testing to engender maximal effort through every repetition. Work done (J) was

recorded for each completed set, and fatigue was calculated as the percentage decrement in work done between the first and last set. Participants were instructed to report their habitual dietary intake by recording a weighted food diary for two weekdays, and one weekend day prior to partaking in the study (Table 3.1) (Nutritics LTD, Dublin, Ireland).

Experimental protocol

Participants were randomly assigned to two parallel-groups, A or B, by the lead investigator and completed a single trial in a double-blind fashion. An overview of the experimental protocol is shown in **Figure 3.1**. Participants were directed to abstain from vigorous physical activity and alcohol consumption in the 48 h preceding the trial. All participants were provided with and consumed a standardised meal ~10.5 h prior to the start of the experimental trial (744 kcal [3.1 MJ], 29% energy (%En) fat, 20%En protein, 51%En carbohydrate). On the day of the trial, participants arrived at the laboratory between 07:00 - 08:00 after a 10 h overnight fast. A Teflon™ cannula was inserted into an antecubital vein of one arm in preparation for stable isotope infusion, a baseline venous blood sample was taken from this site to measure background isotope enrichments prior to infusion. Following baseline blood sampling (t = -210 min) the phenylalanine and tyrosine pools were primed with a single intravenous dose of L-[ring-$^{2}H_{5}$]phenylalanine (2.12 µmol/kg) and L-[3,3-$^{2}H_{2}$]tyrosine (0.75 µmol/kg). Thereafter, continuous tracer infusion was initiated and maintained at a rate of 0.035 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for L-[ring-$^{2}H_{5}$]phenylalanine and 0.012 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for L-[3,3-$^{2}H_{2}$]tyrosine for the duration of the protocol. Once the infusion had begun, a second Teflon™ cannula was inserted into a dorsal hand vein of the contralateral arm and placed in a warmed air hand unit (55°C) for arterialised venous blood sampling (Abumrad et al., 1981). Arterialised venous blood samples were collected throughout the experimental protocol at the following time points: t = -180, -120, -60, 0 (drink consumption), 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 min. A baseline muscle sample was

collected after 90 min of the infusion (t -120 min) from the non-dominant leg (designated as the ‘resting leg’). Muscle biopsies were collected from the mid-region of the *m. vastus lateralis* (approx. 15 cm above the patella) with a modified Bergström suction needle under local anaesthesia (2% lidocaine). All biopsy samples were immediately freed from any visible blood, adipose and connective tissue, frozen in liquid nitrogen (within 30 s), and stored at –80°C until subsequent analysis. Eighty-five min after the initial biopsy (t = -35 min), participants undertook the unilateral resistance-type exercise protocol, as previously described, which took 30 min. Immediately following exercise bilateral muscle biopsies were collected (i.e. from both the rested and exercised leg). Immediately post-biopsy (t = 0 min) participants consumed either a milk protein (MILK) or mycoprotein (MYCO) beverage, within an allotted 5 min period, with the experimental drinks administered in a double-blind manner. Thereafter, participants rested in a semi-supine position for 4 h, after which further bilateral biopsies were collected 1–2 cm proximal to the previous incisions (t = 240 min).

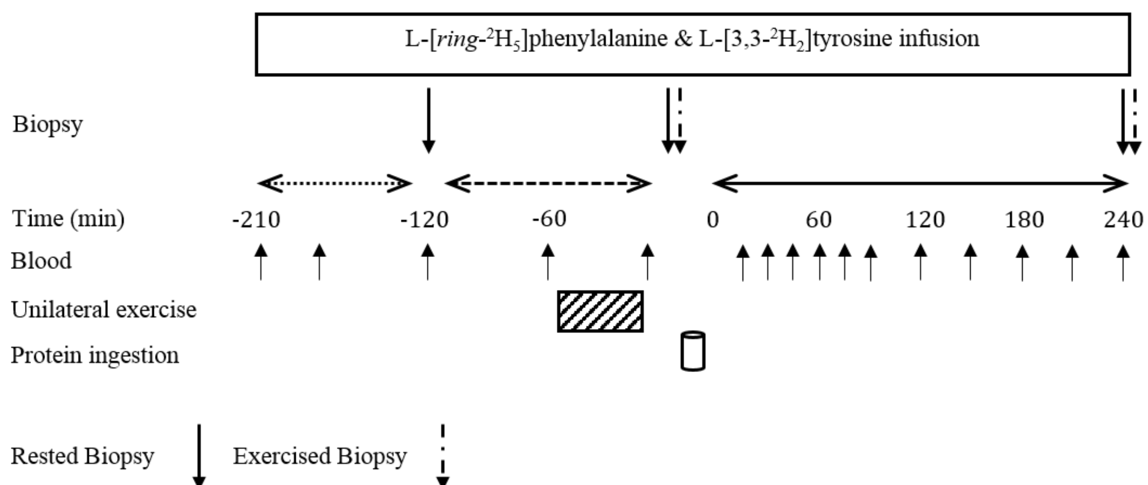


Figure 3.1. Schematic representation of the experimental protocol.

Experimental beverage preparations

Freeze-dried isolated milk protein concentrate was obtained from a commercial supplier (Bulk Powders, Colchester, UK) and freeze-dried mycoprotein was produced by and obtained from Marlow Foods Ltd, Quorn Foods, Stokesley, UK. Both protein sources were independently analysed by a third party company for energy, macronutrient content and amino acid composition (Premier Analytical Services, High Wycombe, UK). The powdered protein sources were prepared the evening before the experimental trial. The protein sources were assimilated with 400 mL water and 10 g of artificial energy-free flavouring (Myprotein, Manchester, UK), blended for approximately 2 min, topped up with water to make a total final beverage volume of 600 mL and refrigerated overnight. Drinks were enriched (2.5%) with L-[ring-$^2H_5$]phenylalanine to account for postprandial tracer dilution by non-labelled phenylalanine and to maintain a systemic isotopic steady-state following protein ingestion (Tang et al., 2009, Moore et al., 2008). Following drink consumption by the participant, an additional 50 mL of water was then added to 'wash' the bottle and ensure

that all protein had been consumed, making a total volume of 650 mL consumed by participants. All drinks were well tolerated, consumed within the allotted time (i.e. 5 min) and resulted in no adverse effects during or after the test day. Double blinding of the drinks was achieved by having a different researcher from the individual running the infusion trial prepare the drinks in an opaque bottle ready for consumption. Despite careful blinding, we cannot discount the possibility that participants allocated to MYCO may have perceived the unusual texture of mycoprotein. The milk protein beverage contained 31 g of milk protein powder which contained 26.2 g total protein (providing 2.5 g of leucine). The mycoprotein beverage contained 70 g of mycoprotein which contained 31.5 g total protein (providing 2.5 g of leucine). The detailed nutritional content and amino acid composition of the drinks are displayed in **Table 3.2.**

Table 3.2. The nutritional content of the experimental drinks.

	MILK	MYCO
Macronutrients		
Protein (g)	26.2	31.5
Fat (g)	0.3	9
Carbohydrate (g)	1.7	7
Fibre (g)	<0.1	17.5
Energy (kcal)	108	238
Energy (kJ)	458	996
Amino acid content (g)		
Alanine	0.8	2.0
Arginine	0.9	2.2
Aspartic acid	1.9	3.3
Glutamic acid	5.8	3.9
Glycine	0.5	1.5
Histidine	0.8	0.8
Isoleucine	1.3	1.5
Leucine	2.5	2.5
Lysine	2.1	2.6
Phenylalanine	1.3	1.5
Proline	2.7	1.6
Serine	1.5	1.6
Threonine	1.1	1.7
Tryptophan	-	1.2
Tyrosine	1.3	1.2
Valine	1.7	1.9

Protein content (g) is calculated from the sum of amino acids measured after protein hydrolysis. The experimental drinks contained 31 g and 70 g of total product for MILK and MYCO, respectively.

Blood sample collection and analyses

Ten mL of arterialised venous blood was collected into a syringe at each sampling point. For each blood sample, six mL was aliquoted into liquid heparin containing tubes (BD vacutainer LH; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged immediately (3000 *g*, 4°C, 10 min). Blood plasma was aliquoted and frozen at –80°C for subsequent analysis. The remaining 4 mL of blood was aliquoted into additional vacutainers (BD vacutainers SST II, Becton, Dickinson and Company) which were left to clot at room temperature for at least 30 min and then centrifuged (3000 *g*, 4°C, 10 min) to obtain blood serum. Serum was aliquoted before freezing at –80°C for subsequent analyses. Serum insulin concentrations were analysed using a commercially available kit (DRG Insulin ELISA, EIA-2935, DRG International Inc, Springfield, IL, USA). Plasma branched chain amino acid (BCAA) (leucine, isoleucine and valine), phenylalanine and tyrosine concentrations, and L-[ring-$^{2}H_{5}$]-phenylalanine, L-[ring-3,5-$^{2}H_{2}$]-tyrosine, and L-[ring-$^{2}H_{4}$]-tyrosine enrichments were determined by gas chromatography-mass spectrometry (GC-MS) as described previously (Wolfe and Chinkes, 2005). Briefly, 10 µL internal standards of leucine, valine, phenylalanine and tyrosine were added to the samples. The plasma was deproteinised on ice with 500 µL of 15% 5-sulfosalicylic acid. Free amino acids were purified using acid-washed cation exchange columns (AG 50W-X8 resin; Bio-Rad Laboratories, Inc., CA, USA), with the amino acids being eluted from the column with 8 mL of 2N ammonium hydroxide. The eluate was then dried under vacuum with a Speed-Vac rotary dryer (Savant Instruments, Farmingdale, NY, USA). In order to derivatize the plasma sample, 40 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 40 µl acetonitrile were added to the dry samples, vortexed and heated at 95 °C for 40 min (Bornø et al., 2014). The samples were analysed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies, Santa Clara, CA, USA) in duplicates using electron impact ionisation and selected ion monitoring for measurement of isotope ratios (Zabielski et al., 2013). One microliter of the sample was

injected in splitless mode (injector temp. 280°C). Peaks were resolved using an HP5-MS 30m × 0.25mm ID × 0.25µm capillary column (Agilent). Helium was used as carrier gas at 1.2ml/min constant flow rate. The temperature ramp was set from 80 – 245 °C at 11°C/min, then to 280 °C at 40 °C/min (Zabielski et al., 2013). Selected ion recording conditions were used to monitor fragments m/z 336, 341 and 346 for phenylalanine, m/z 288 and 296 for valine, m/z 274 and 280 for leucine and isoleucine, and m/z 466 and 475 for tyrosine.

Skeletal muscle tissue analyses

Muscle biopsy tissue samples were analysed for protein-bound and free intracellular L-[ring-$^{2}H_{5}$]phenylalanine, as previously described (Wolfe and Chinkes, 2005). Briefly, 20-30 mg of frozen muscle tissue was weighed and precipitated in 600 µL 10% perchloric acid. The tissue was homogenised by a mechanical tissue grinder. The supernatant, for determination of intracellular L-[ring-$^{2}H_{5}$]phenylalanine enrichment, was subsequently transferred following centrifugation (4000 rpm, 20 min, 4 °C) and stored at −80 °C. This procedure was repeated with an additional 800 µL wash with 10% perchloric acid. The remaining pellet of muscle tissue was washed three times in 2% perchloric acid, twice in ethanol, and then once in ethyl ether, before being oven-dried overnight at 50 °C. The following day, the dried muscle pellet was hydrolysed in 6N hydrochloric acid at 110 °C for 24 h. The hydrolysate, representing the bound protein pool of amino acids, was subsequently used to determine the enrichment of bound L-[ring-$^{2}H_{5}$]phenylalanine. The protein hydrolysate was deionised using ion-exchange columns as described for blood analyses. The supernatant, for determination of intracellular labelled phenylalanine enrichment, was prepared in the same manner as the protein-bound acid hydrolysates. In order to derivatise the muscle sample, 50 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 50 µl acetonitrile were added to the dry samples, vortex mixed and heated at 95 °C for 45 min (Bornø et al., 2014). The samples were analysed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies) in

duplicates using electron impact ionisation and selected ion monitoring for measurement of isotope ratios (Zabielski et al., 2013). One microliter of the sample was injected in splitless mode (injector temp. 280 °C). Peaks were resolved using an HP5-MS 30m × 0.25mm ID × 0.25µm capillary column (Agilent). Helium was used as carrier gas at 1.2ml/min constant flow rate. The temperature ramp was set from 80 – 245 °C at 11 °C/min, then to 280 °C at 40 °C/min (Zabielski et al., 2013). Selected ion recording conditions were used to monitor fragments m/z 237 and 239 for the m+3 and m+5 fragments of phenylalanine bound protein and m/z 336 and 341 for phenylalanine free fraction.

Muscle biopsy tissue samples were analysed for total and phosphorylated forms of mechanistic target of rapamycin (mTORSer^{2448} and pmTOR Ser^{2448}). Briefly, ~10 mg of whole frozen muscle was mechanically homogenised using steel beads (Qiagen, Hilden, Germany) in 20 volumes of buffer (Tris-HCl 50 mM, Triton X-100 1%, EDTA 1 mM, EGTA 1 mM, NaF 50 mM, β-glycerophosphate 10 mM, sodium pyrophosphate 5 mM, 2-mercaptoethanol 0.1%, sodium orthovanadate 0.5 mM, okadaic acid 100 nM and complete Mini protease inhibitor cocktail (Roche Holding AG, Basel, Switzerland). Following centrifugation (10000 *g*, 4°C, 10 min) the supernatant protein content was determined by colorimetric assay (DC protein assay, Bio-Rad Laboratories, Inc.). Proteins were unfolded by incubating for 5 minutes at 95 °C in XT sample buffer (Bio-Rad Laboratories, Inc.). Twenty µg protein per lane were loaded onto 3-8% tris acetate polyacrylamide gels, and separated by electrophoresis in XT tricine running buffer for 65 min at 150 V. Proteins were transferred to 0.2 µM nitrocellulose membranes using a Trans-blot turbo transfer system (Bio-Rad Laboratories, Inc.), at 2.5 A and 25 V for 10 min. Membranes were blocked in 5% BSA in TBST (pH 7.6) for 1 h, before overnight incubation at 4 °C with rabbit anti-phospho-mTOR Ser^{2448} monoclonal antibody (5536, Cell Signaling Technology, Inc., Danvers, Mass, USA; 1:1000 in TBST) and rabbit anti-α-tubulin (11H10, Cell Signaling Technology, Inc.; 1:20000 in TBST) loading control. Following 3 × 10 min washes in TBST, membranes were incubated

for 1 h at room temperature in secondary HRP conjugated anti-rabbit IgG antibody (ab6721, Abcam PLC, Cambridge, UK; 1:3000 in TBST). Following 3 × 10 min washes in TBST, membranes were then exposed for 5 min in Clarity Western chemiluminescent detector solution (Bio-Rad Laboratories, Inc.), visualised using a Chemidoc scanner (Bio-Rad Laboratories, Inc.), and band density quantified using Image Lab software (Bio-Rad Laboratories, Inc.). The expected migration of phospho-mTOR (~289 kDa) and α-tubulin (~52 kDa) was confirmed using a kaleidoscope protein ladder (Bio-Rad Laboratories, Inc.). For total mTOR, membranes were incubated for 15 min in Restore stripping buffer (Thermo Fisher Scientific, Waltham, MA, USA), blocked for 1 h in 5% BSA in TBST and re-probed overnight with an anti-mTOR monoclonal primary antibody (2972, Cell Signaling Technology, Inc.; 1:1000 in TBST) plus anti-α-tubulin, and the above steps were repeated to obtain corresponding bands for total mTOR. The band density for phospho-mTOR was calculated as a ratio against the band density for α-tubulin, within each lane. This was divided by the ratio of mTOR against α-tubulin to give an overall ratio for 'mTOR phosphorylation status', which was finally expressed as a fold change from the rested, fasted, baseline.

Skeletal muscle mRNA expression of 48 genes was analysed as previously described (Dirks et al., 2018). In brief, total RNA was extracted from ~20 mg frozen muscle tissue using TRIzol® Reagent (Thermo Fisher Scientific) (Chomczynski and Sacchi, 1987), according to the manufacturer's protocol. Total RNA quantification was carried out spectrophotometrically at 260 nm (NanoDrop ND-2000 Spectrophotometer; Thermo Fisher Scientific) and RNA purity was determined as the ratio of readings at 260/280 nm. Reverse transcription of RNA was carried out using a commercially available kit (SuperScript™ III First-Strand Synthesis SuperMix, Thermo Fischer Scientific) (Tsintzas et al., 2006). Taqman low-density custom-designed array cards (Thermo Fisher Scientific) were used for the relative quantification of the expression of genes involved in the regulation of cellular amino acid transport, protein

synthesis and protein breakdown. Each card allowed for eight samples to be run in parallel against 48 Taqman gene expression assay targets that had been preloaded into each well on the card (**Table 3.3**). In short, 50 μL Taqman Universal Master Mix II (Thermo Fisher Scientific) was added to 150 ng of RNA equivalent cDNA into an RNAse-free Eppendorf tube, and RNAse-free water was added to make the total reaction volume up to 100 μl. The reaction mixture was vortexed, centrifuged and loaded into one of the fill reservoirs of the Micro Fluidic card, after which the cards were centrifuged (Hereaus 3 S-RMicrofuge, Thermo Fisher Scientific) and run on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). Relative quantification of the genes of interest was performed using the delta-delta Ct method, with the fold change in mRNA amplification expressed relative to the rested fasted leg and the geometric mean of the two housekeeping genes, for which purpose GAPDH and B2M were selected.

Table 3.3 Names and symbols of gene expression assay targets that were preloaded on to the microfluidic cards.

Gene name (human skeletal muscle)	Symbol
Insulin Receptor substrate 1	IRS1
AKT serine/threonine kinase 1	AKT1
AKT serine/threonine kinase 2	AKT2
phosphoinositide-3-kinase regulatory subunit 1	PIK3R1
mechanistic target of rapamycin	MTOR
ribosomal protein S6 kinase B1	RPS6KB1
eukaryotic translation initiation factor 4E binding protein 1	EIF4EBP1
eukaryotic translation initiation factor 4 gamma 1	EIF4G1
eukaryotic translation initiation factor 4E	EIF4E
sestrin 2	SESN2
GATS protein like 3	GATSL3
leucyl-tRNA synthetase	LARS
MAP kinase interacting serine/threonine kinase 2	MKNK2
transcription factor EB	TFEB
tuberous sclerosis 1	TSC1

tuberous sclerosis 2	TSC2
DNA damage inducible transcript 4	DDIT4
DNA damage inducible transcript 4 like	DDIT4L
regulatory associated protein of MTOR complex 1	RPTOR
NPR2-like, GATOR1 complex subunit	NPRL2
AKT1 substrate 1	AKT1S1
forkhead box O1	FOXO1
forkhead box O3	FOXO3
forkhead box O4	FOXO4
calpain 1	CAPN1
calpain 3	CAPN3
caspase 3	CASP3
nuclear factor kappa B subunit 1	NFKB1
F-box protein 32	FBXO32
tripartite motif containing 63	TRIM63/MuRF1
tripartite motif containing 32	TRIM32
activating transcription factor 4	ATF4
Myostatin	MSTN
growth differentiation factor 11	GDF11
SMAD family member 2	SMAD2
SMAD family member 3	SMAD3
transforming growth factor beta 1	TGFB1
inhibin beta A subunit	INHBA
solute carrier family 7 member 5	SLC7A5
solute carrier family 7 member 8	SLC7A8/LAT2
solute carrier family 36 member 1	SLC36A1
solute carrier family 7 member 1	SLC7A1
solute carrier family 38 member 2	SLC38A2
solute carrier family 38 member 9	SLC38A9/SNAT9
solute carrier family 38 member 10	SLC38A10
serum response factor	SRF
glyceraldehyde-3-phosphate dehydrogenase	GAPDH
beta-2-microglobulin	B2M

Calculations

The fractional synthetic rates (FSR) of mixed muscle proteins were calculated using the standard precursor-product equation (28):

$$FSR\ (\% \cdot h^{-1}) = \left[\frac{\Delta E\text{p}}{E_{\text{precursor}} \times t}\right] \times 100$$

Where ΔEp is the increment in L-[ring-$^2H_5$]phenylalanine enrichment in mixed muscle protein between two biopsies, $E_{\text{precursor}}$ is the average L-[ring-$^2H_5$]phenylalanine enrichment in the plasma or intracellular precursor pool over time, and t indicates the tracer incorporation time (h) between two muscle biopsies.

L-[3,3-$^2H_2$]tyrosine and arterialised venous blood sampling were used to assess phenylalanine hydroxylation rates (the initial step in phenylalanine oxidation), which were calculated using modified Steele's equations, as previously described (Gorissen et al., 2017).

Statistical analyses

A two-sided power analysis based on previous research (Tang et al., 2009) showed that n= 9 per condition was sufficient to detect expected differences in postprandial muscle protein synthesis rates between protein conditions (MILK vs MYCO) when using a repeated measures ANOVA ($P < 0.05$, 90% power, $f = 0.67$; G*power version 3.1.9.2). Our primary measure was postprandial muscle protein synthesis rates. The delta change in muscle protein synthesis rates from fasted to fed, plasma amino acid concentrations, serum insulin concentrations, phenylalanine hydroyxlation, intracellular and mixed muscle protein-bound L-[ring-$^2H_5$]phenylalanine represent secondary measures, and skeletal muscle cell signalling responses represent exploratory variables. Differences in participant characteristics and background mixed muscle protein-bound L-[ring-$^2H_5$]phenylalanine were analysed using

independent t-tests. Plasma amino acid and serum insulin concentrations, plasma L-[ring-$^2H_5$]-phenylalanine, L-[ring-3,5-$^2H_2$]-tyrosine, and L-[ring-$^2H_4$]-tyrosine enrichments, and muscle mTOR phosphorylation were tested by two-factor (treatment [milk protein vs mycoprotein] × time) repeated-measures analysis of variance (ANOVA). Mixed muscle FSRs, mixed muscle protein-bound L-[ring-$^2H_5$]phenylalanine enrichments, and muscle gene expression were analysed using a three-factor (treatment × time × exercise/rest) ANOVA. Data were tested for sphericity, and where violations occurred the Greenhouse-Geisser correction was automatically applied. Violations of normality were tested for using the Shapiro-Wilk test, and no considerable violations were found (P>0.05). When significant interaction effects were observed Sidak post hoc tests were performed to correct for multiple comparisons and locate individual differences. Total postprandial amino acid concentrations were calculated as incremental area under curve (iAUC), with baseline set as t = 0, and analysed using independent t-tests. Where plasma time point data were absent, missing data analyses was performed using regression imputation. Statistical significance was set at P<0.05. Calculations were performed using GraphPad Prism version 7.1 (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± SEM.

RESULTS

Participant characteristics

No differences in age, weight, height, BMI, body composition, or habitual nutritional intake were detected between groups (all P>0.05; **Table 3.1**). No differences in total work performed during the experimental resistance exercise bout (29540±1782 J in MILK vs 30722±2059 J in MYCO; P>0.05) or in fatigue (19±3% in MILK vs 26±4% in MYCO; P>0.05) were detected between groups.

Plasma amino acid and serum insulin concentrations

Plasma total and individual BCAA concentrations during the experimental period are shown in **Figure 3.2**. Plasma total BCAA concentrations and each of the individual BCAAs all showed similar kinetic responses. Specifically, from similar fasting values across conditions, all parameters increased with protein ingestion (time effect; P<0.0001) but to differing degrees between conditions (treatment × time interaction effect; P<0.0001). Plasma BCAA concentrations (**A**) peaked more rapidly and to a greater peak magnitude following the ingestion of MILK compared with MYCO. Plasma leucine concentrations (**C** and **D**) were different between conditions (treatment effect; P<0.05), and were greater at 15, 30, and 45 min in MILK compared with MYCO (P<0.01). Plasma leucine concentrations peaked at 30 min in MILK at 299±36 $\mu mol \cdot L^{-1}$ and at 75 min in MYCO at 243±11 $\mu mol \cdot L^{-1}$. Total postprandial plasma leucine concentrations were 19±8% greater in MILK compared with MYCO (24420±2333 in MILK vs 20831±1279 $\mu mol \cdot L^{-1} \times$ 4h in MYCO; P>0.05) (**D**), whereas isoleucine and valine postprandial concentrations did not differ between conditions (P>0.05) (**F** & **H**). Plasma phenylalanine and tyrosine concentrations increased with protein ingestion (time effect; P<0.0001) but also to differing degrees between conditions (treatment × time interaction effect; P<0.0001). Plasma phenylalanine concentrations (**K** and **L**) were greater

in MILK compared with MYCO at 15 and 30 min (*P*<0.01). Plasma tyrosine concentrations (**I** and **J**) were different between conditions (treatment effect; *P*<0.001), and were greater in MILK compared with MYCO from 15-120 min (*P*<0.05).

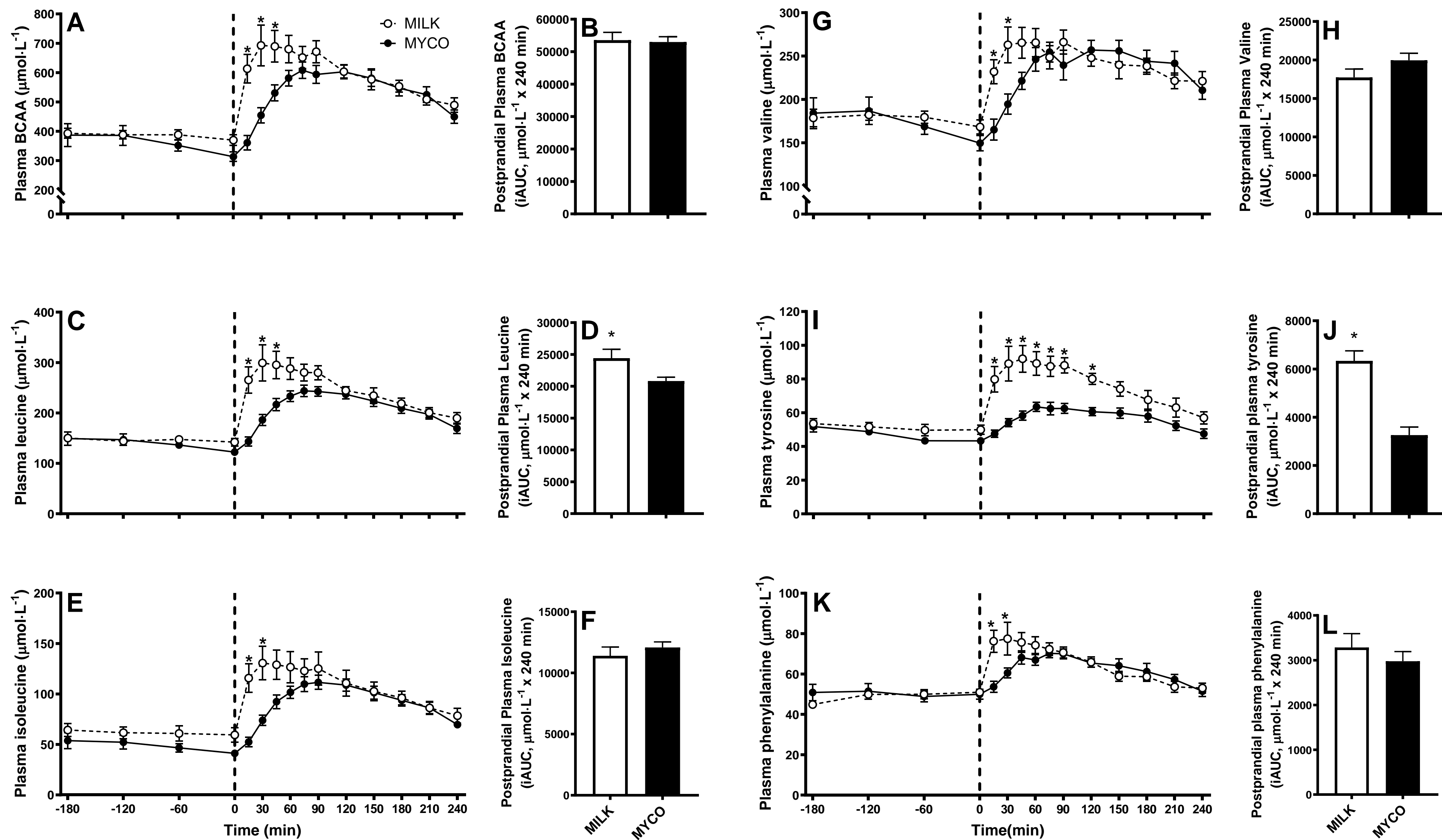


Figure 3.2. The timecourse and incremental AUC (iAUC) of plasma total branched chain amino acid (A and B), leucine (C and D), isoleucine (E and F),

valine (G and H), tyrosine (I and J) and phenylalanine (K and L) concentrations during a 3 h postabsorptive period (time-course graphs only) and a 4 h postprandial period in healthy young men. iAUC graphs represent total 4 h postprandial plasma concentrations above postabsorptive values. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; n=10) or 31.5 g mycoprotein (MYCO; n=10), where a single bout of unilateral leg extension exercise was also performed. Time-course and iAUC data were analysed with a repeated measures two-way ANOVA and independent t-tests, respectively, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. * indicates individual differences between conditions at these time points, and a difference between conditions on the bar graphs. Treatment × time interaction effect; all $P<0.0001$.

Serum insulin concentrations during the experimental period are displayed in **Figure 3.3**. From similar fasting concentrations (15±3 and 16±2 $mU \cdot L^{-1}$ in MILK and MYCO, respectively) serum insulin concentrations increased with protein ingestion (time effect; $P<0.0001$) and to differing degrees between conditions (treatment × time interaction effect; $P<0.0001$). Milk protein ingestion resulted in a more rapid and transient increase in serum insulin concentrations that peaked at 15 min (42±7 $mU \cdot L^{-1}$) and returned to fasting levels by 45 min ($P<0.01$). Mycoprotein ingestion induced a less rapid but more sustained increase in serum insulin concentrations that peaked at 30 min post-ingestion (36±4 $mU \cdot L^{-1}$) and returned to baseline more slowly (60 min; $P<0.0001$). Serum insulin concentrations in MILK were only greater than MYCO at 15 min post-ingestion ($P<0.05$) and postprandial serum insulin AUC was not different between conditions ($P>0.05$).

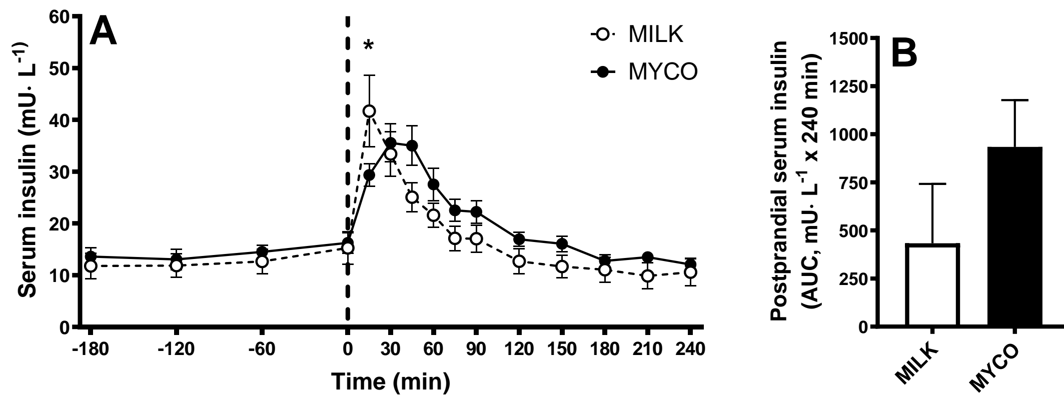


Figure 3.3. The timecourse and incremental AUC (iAUC) of serum insulin concentrations during a 3 h postabsorptive period (time-course graph only) and a 4 h postprandial period in healthy young men, with iAUCs representing total 4 h postprandial plasma concentrations above postabsorptive values. The vertical line indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; n=10) or 31.5g mycoprotein (MYCO; n=10), where a single bout of unilateral leg extension exercise was also performed. Data were analysed with a repeated measures two-way ANOVA and independent t-tests, respectively, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. * indicates individual differences between conditions at these time points, and a difference between conditions on the bar graph. Treatment × time interaction effect; $P<0.0001$.

Whole-body phenylalanine kinetics

The time-course of plasma L-[ring-$^2H_5$]phenylalanine, L-[3,3-$^2H_2$]tyrosine, and L-[ring-$^2H_4$]-tyrosine enrichments are illustrated in **Figure 3.4**. During the postabsorptive period, plasma L-[ring-$^2H_5$]phenylalanine remained in a steady state at ~4–5 MPE (mole percent excess) in both conditions. L-[ring-$^2H_5$] phenylalanine enrichments increased transiently after protein ingestion (time effect; $P<0.001$), with a greater increase in MYCO (treatment × time interaction effect, $P<0.01$). Specifically, plasma L-[ring-$^2H_5$]phenylalanine enrichments increased above postabsorptive levels for 30 min in MYCO only ($P<0.01$) before returning

to baseline enrichments. This was presumably due to either the slower digestion of MYCO, or quicker entry of labelled L-[ring-2H5]phenylalanine into the circulation in the MYCO condition (Figure 4A). Two participants were excluded from the whole body kinetics analysis (final analysis therefore; MILK=9, MYCO=9) due to technical issues with the tyrosine tracer. L-[3,3-$^{2}H_{2}$]tyrosine decreased equivalently after the ingestion of protein (time effect; P<0.0001, treatment × time interaction effect; P>0.05) and remained below postabsorptive levels for 150 min following protein ingestion (P<0.05) (Figure 3.4B). Plasma L-[ring-$^{2}H_{4}$]-tyrosine increased following protein ingestion (time effect; P<0.0001), with a greater increase in MYCO (treatment × time interaction effect; P<0.01) which was elevated above postabsorptive enrichments for 30 min (P<0.0001) (Figure 3.4C).

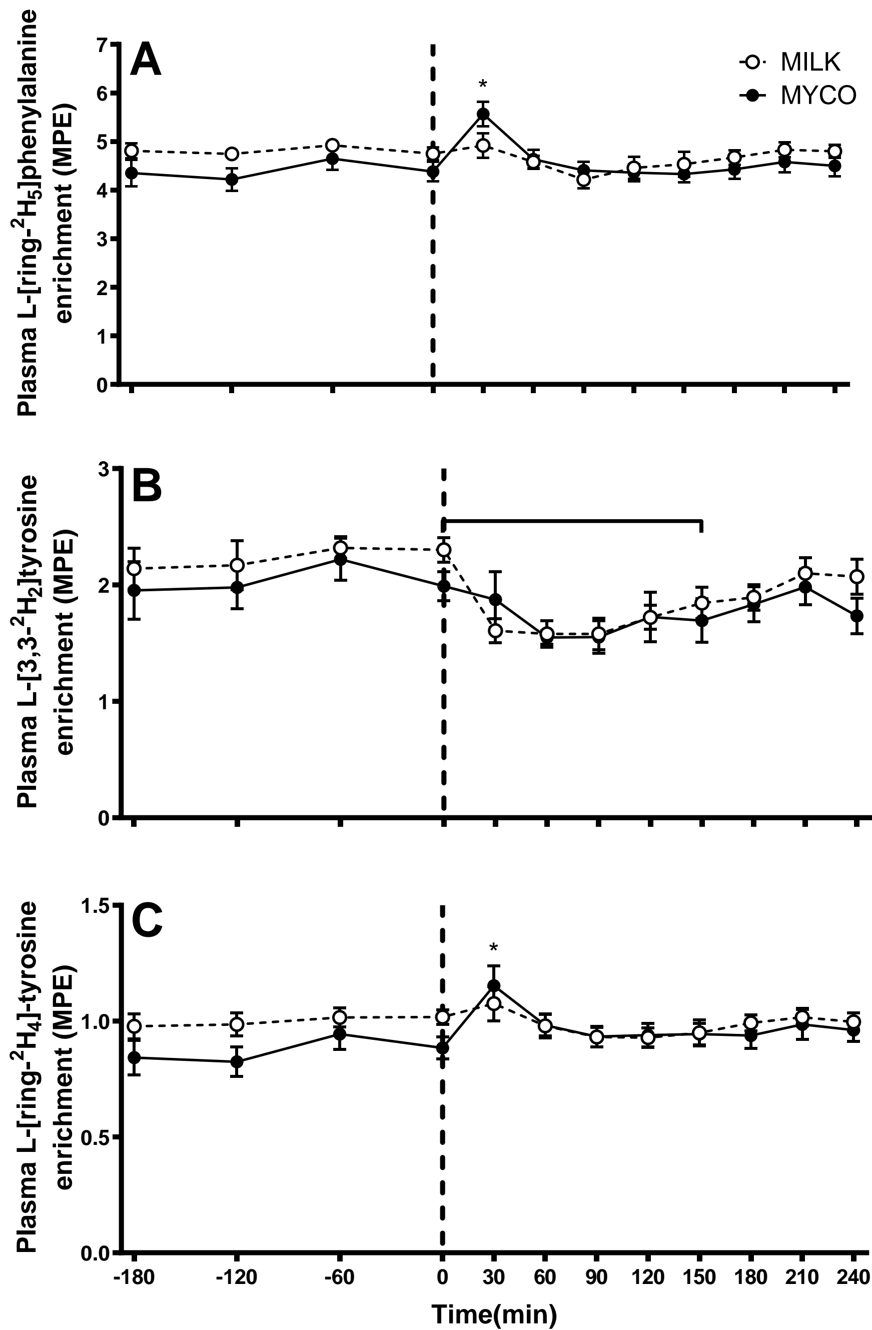


Figure 3.4. L-[ring-$^2H_5$]phenylalanine (A), L-[3,3-$^2H_2$]tyrosine (B), and L-[ring-$^2H_4$]-tyrosine (C) enrichments during a stable isotope experimental test day in healthy young men. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; n=10) or 31.5g mycoprotein (MYCO; n=10), where a single bout of unilateral leg extension exercise was also performed. Data were analysed with a repeated measures two-way ANOVA, with Sidak's post hoc tests applied to locate individual differences (P≤0.05). Values are means, with their standard errors represented by

vertical bars. * indicates different from fasting (t = 0 min) for MYCO. Horizontal bar indicates a change from t=0 across conditions. Treatment × time interaction effect; A, P=0.0018; B, P=0.5357; C, P=0.0026.

Phenylalanine hydroxylation increased following protein ingestion (time effect; P<0.0001), and remained elevated throughout the postprandial period, with no differences between conditions (treatment × time effects; P>0.05) (Figure 3.5).

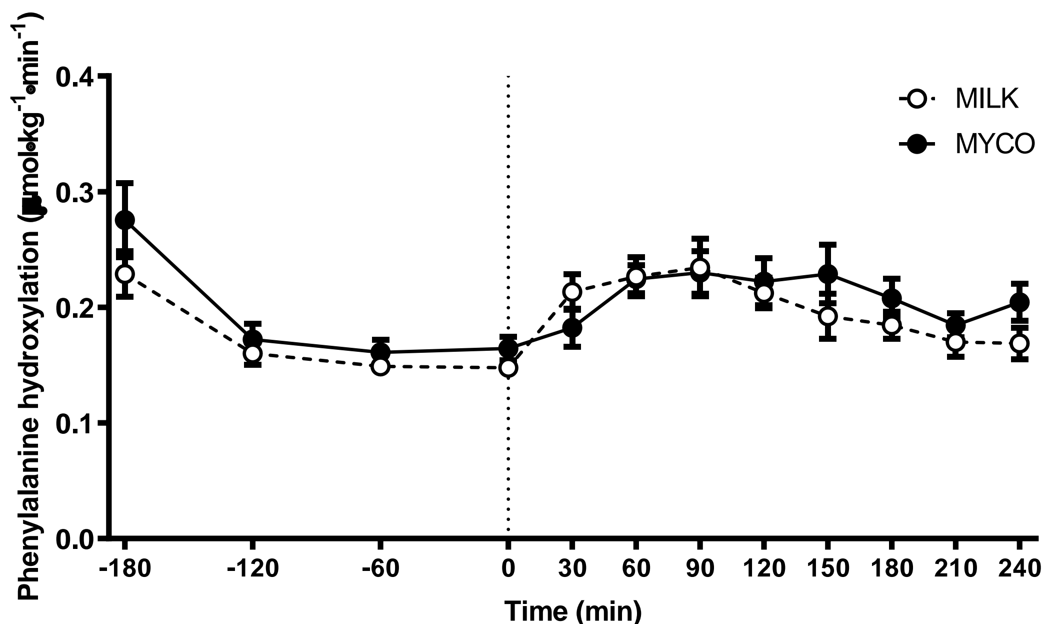


Figure 3.5. Phenylalanine hydroxylation during a 3 h postabsorptive period and a 4 h postprandial period in healthy young men. The vertical line indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; n=9) or 31.5g mycoprotein (MYCO; n=9), where a single bout of unilateral leg extension exercise was also performed. Data were analysed with a repeated measures two-way ANOVA, with Sidak's post hoc tests applied to locate individual differences (P≤0.05). Values are means, with their standard errors represented by vertical bars. Treatment × time interaction effect; P=0.2659.

Skeletal muscle tracer analyses

One participants' samples were excluded from the MYCO condition due to insufficient tissue. Intracellular L-[ring-$^{2}H_{5}$]phenylalanine enrichments increased over time (time effect; P<0.01) with no differences between conditions or interaction effects (both P>0.05). Mixed muscle protein-bound L-[ring-$^{2}H_{5}$]phenylalanine enrichments did not differ between conditions at baseline (0.0030±0.0007 and 0.0034±0.0006 MPE in MYCO and MILK conditions, respectively; P>0.05). Mixed muscle protein L-[ring-$^{2}H_{5}$]phenylalanine enrichments increased during the fasting period in the rested leg (from 0.0030±0.0007 to 0.0065±0.0007 in MILK and 0.0034±0.0006 to 0.0058±0.0006 MPE in MYCO; time effect; P<0.0001) to the same extent in each condition (treatment and treatment × time effects; both P>0.05). Mixed muscle protein L-[ring-$^{2}H_{5}$]phenylalanine enrichments increased with protein ingestion (from 0.0065±0.0007 to 0.0161±0.001 MPE in rested, and 0.0061±0.0008 to 0.0164±0.0012 MPE in exercised muscle in MILK, and from 0.0058±0.0006 to 0.0167±0.0012 MPE in rested, and 0.0055±0.0006 to 0.0187±0.0011 MPE in exercised muscle in MYCO; P<0.0001) and to a greater extent in MYCO compared with MILK (treatment × time interaction effect; P<0.05).

Mixed muscle FSRs calculated using the average plasma L-[ring-$^{2}H_{5}$]phenylalanine plasma enrichment as the precursor pool are displayed in **Figure 3.6**. Exercise did not affect mixed muscle FSR (exercise effect; P=0.0640), nor did exercise interact with protein ingestion (P=0.1251) or condition (P=0.2223). Protein ingestion increased mixed muscle FSRs in rested and exercised muscle in both conditions (time effect; P<0.0001). Mixed muscle FSR increased from 0.036±0.008 to 0.052±0.006%·h^{-1} and 0.035±0.008 to 0.056±0.005%·h^{-1} in rested and exercised muscle, respectively, in MILK, and from 0.025±0.006 to 0.057±0.004%·h^{-1} and 0.024±0.007 to 0.072±0.005%·h^{-1} in rested and exercised muscle, respectively, in MYCO. The increase in mixed muscle FSR was greater in the MYCO condition compared with MILK condition (treatment × time interaction effect; P=0.0199), with the divergence located as trends for a difference between conditions in the postabsorptive

(P=0.0890) and postprandial FSRs (0.065±0.004 in MYCO, and 0.054±0.004%·h^{-1} in MILK, respectively; P=0.0930) (**A**). These trends resulted in the delta postabsorptive to postprandial rise in mixed muscle FSR being greater in MYCO compared with MILK (Δ 0.040±0.006 vs Δ 0.018±0.005%·h^{-1}, respectively; treatment effect; P=0.0084) **(B)**. The group differences became more pronounced when comparing muscle FSRs derived from the L-[ring-$^{2}H_5$]phenylalanine intracellular precursor pool. While main effects of exercise or any exercise interactions were still absent (all P>0.05), protein ingestion increased FSR (time effect; P=0.0003) and to a greater extent in MYCO compared with MILK (treatment × time interaction effect; P=0.0225). Specifically, MYCO ingestion stimulated mixed muscle FSR (from to 0.031±0.007 to 0.070±0.006%·h^{-1} in rested, and 0.028±0.008 to 0.082±0.008%·h^{-1} in exercised muscle; P<0.0001), whereas MILK ingestion only trended to stimulate FSR (from 0.040±0.010 to 0.060±0.007%·h^{-1} in rested, and 0.050±0.012 to 0.058±0.007%·h^{-1} in exercised muscle; P=0.060).

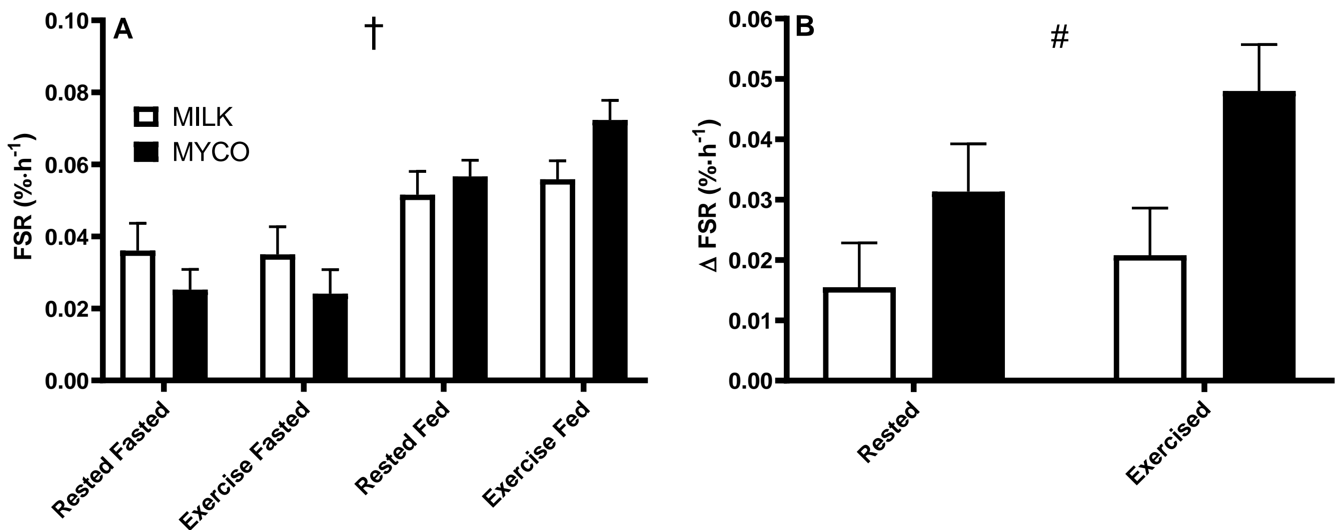


Figure 3.6. Mixed muscle protein fractional synthesis rates (FSRs; A) calculated from the plasma L-[ring-$^{2}H_{5}$]phenylalanine precursor pool in the postabsorptive (fasted) and postprandial (fed) state, in rested and exercised (single bout of unilateral concentric leg extensions) muscle in healthy young men. Postprandial state represents a 4 h period following the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=9). Data were analysed with three-way ANOVA, with Sidak post hoc tests applied to locate individual differences. The delta change in FSR in response to protein ingestion (B), representing the transition from postabsorptive to postprandial conditions in both groups is also presented. Data were analysed with two-way ANOVA, with Sidak's post hoc tests applied to locate individual differences (P≤0.05). Values are means, with their standard errors represented by vertical bars. † indicates a main effect of protein ingestion. There was a trend for a difference in postprandial muscle protein synthesis rates between protein conditions (*P*=0.093). # represents a main effect of condition.

Skeletal muscle cell signalling responses

Skeletal muscle mTOR phosphorylation status was determined in n=15 due to restrictions on remaining muscle tissue (final analysis therefore; MILK=7, MYCO=8) (**Figure 3.7**). Fold change with protein ingestion in muscle mTOR phosphorylation status did not differ between conditions (treatment effect; *P*>0.05), was unaffected by exercise (*P*>0.05), and did not show an interaction effect (*P*>0.05).

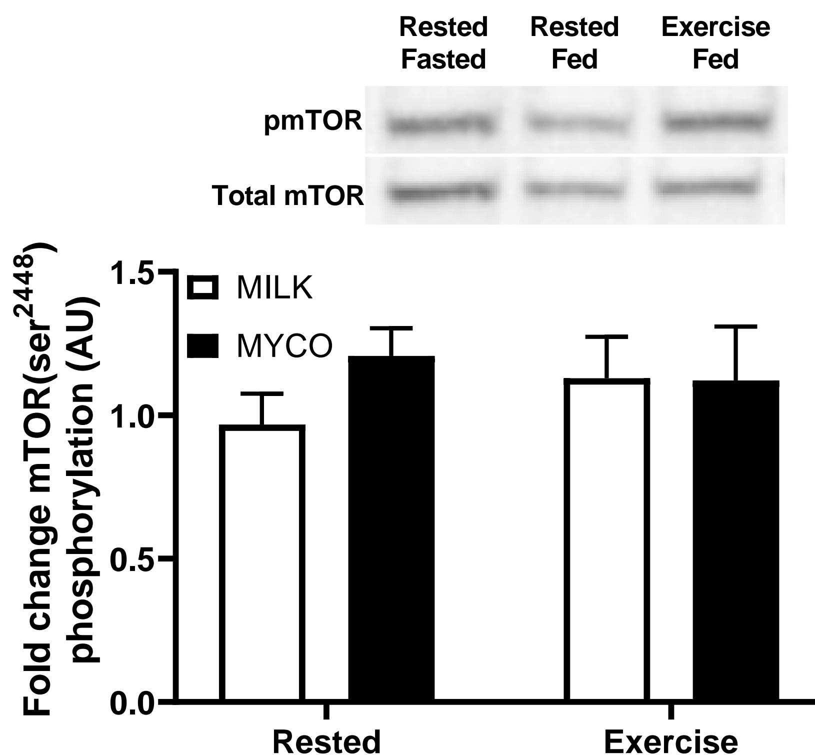
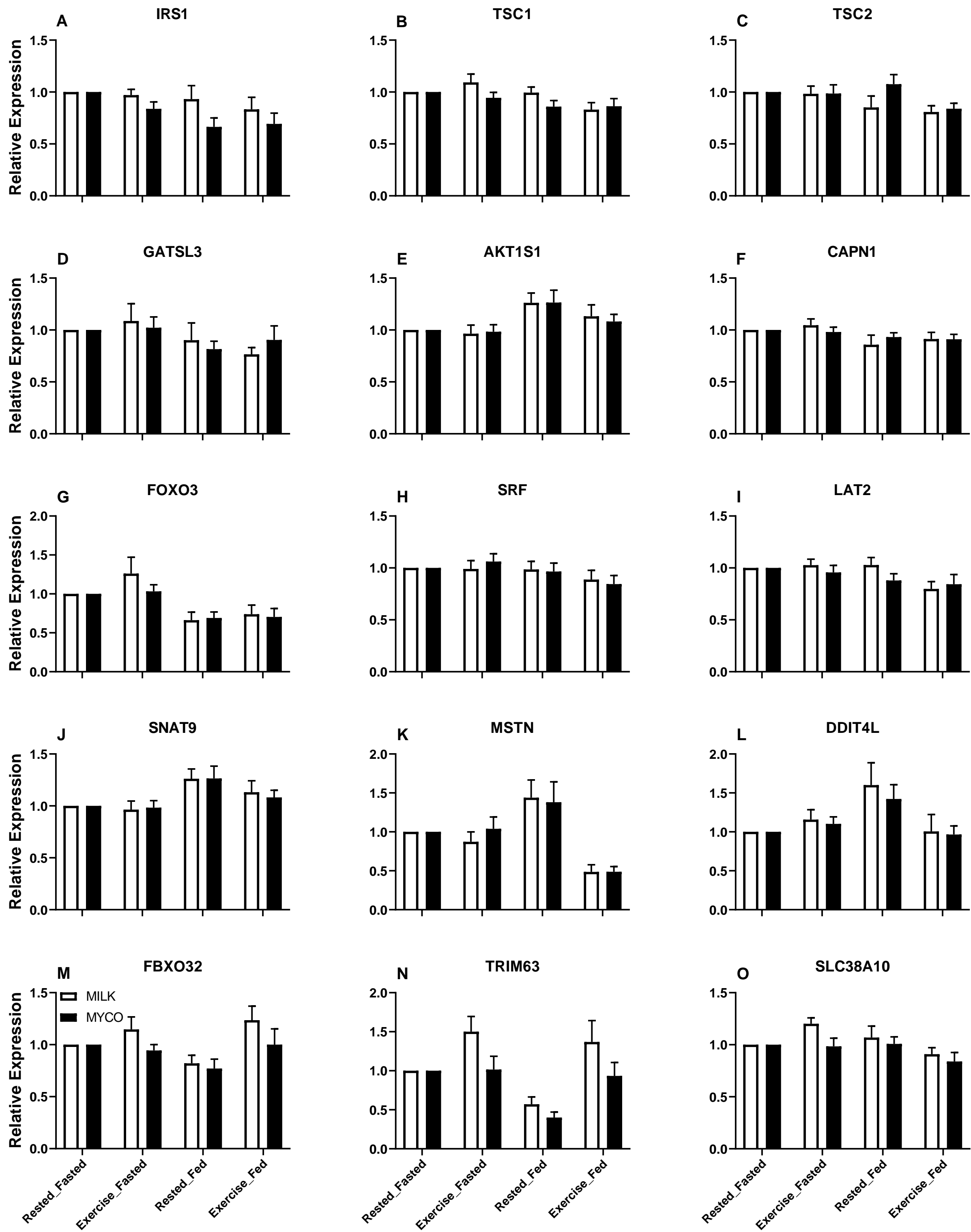


Figure 3.7. Skeletal muscle mechanistic target of rapamycin (mTOR) phosphorylation status, presented as a ratio of phosphorylated (p) to total protein, in the postabsorptive and postprandial state, in rested and exercised legs, after the ingestion of 26.2g milk protein (MILK; n=7) or 31.5g mycoprotein (MYCO; n=8), in young men. Values are means, with their standard errors represented by vertical bars. Data were analysed with two-way ANOVA. No significant effects were detected.

Of the 46 genes analysed for their muscle mRNA expression (see **Table 3.3**), 19 genes showed no changes with exercise, protein ingestion, protein condition, or any interactions (all P>0.05; data not shown). Twenty seven genes responded to protein ingestion and/or exercise, and the muscle mRNA expression of these genes are displayed in **Figure 3.8**. Specifically, protein ingestion either decreased (IRS1, TSC1, TSC2, CASTOR1, FOXO3, CAPN1, SRF, SLC7A8, SLC38A10, DDIT4, TRIM63 and SLC38A2) or increased (AKT1S1 and SLC38A9) muscle mRNA expression of some genes. Similarly, exercise decreased (SMAD2, PIK3R1, RPS6KB1, TFEB, MSTN, SLC38A2 and TRIM32) or increased (FBXO32,

SLC7A1, and TRIM63) the muscle mRNA expression of some genes. Fifteen genes exhibited a time × exercise interaction (*P*<0.05) such that EIF4E and TGFb1 mRNA expression increased in exercised muscle only, and DDIT4L and MSTN mRNA expression increased in rested muscle only (*P*<0.05). FBXO32, MSTN, TRIM63 mRNA expression decreased in rested muscle only, and SLC38A10, DDIT4, SLC38A2, and TRIM32 mRNA expression decreased in exercised muscle only (*P*<0.05). Only a single gene, TRIM32, showed a differential response between nutritional conditions, with its muscle mRNA expression greater in MILK compared with MYCO (treatment effect; *P*<0.05), which was driven by a greater expression in MILK compared with MYCO in the postprandial state (*P*<0.01).


A
IRS1
B
TSC1
C
TSC2
D
GATSL3
E
AKT1S1
F
CAPN1
G
FOXO3
H
SRF
I
LAT2
J
SNAT9
K
MSTN
L
DDIT4L
M
FBXO32
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MYCO
Rested_Fasted
Exercise_Fasted
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Exercise_Fed

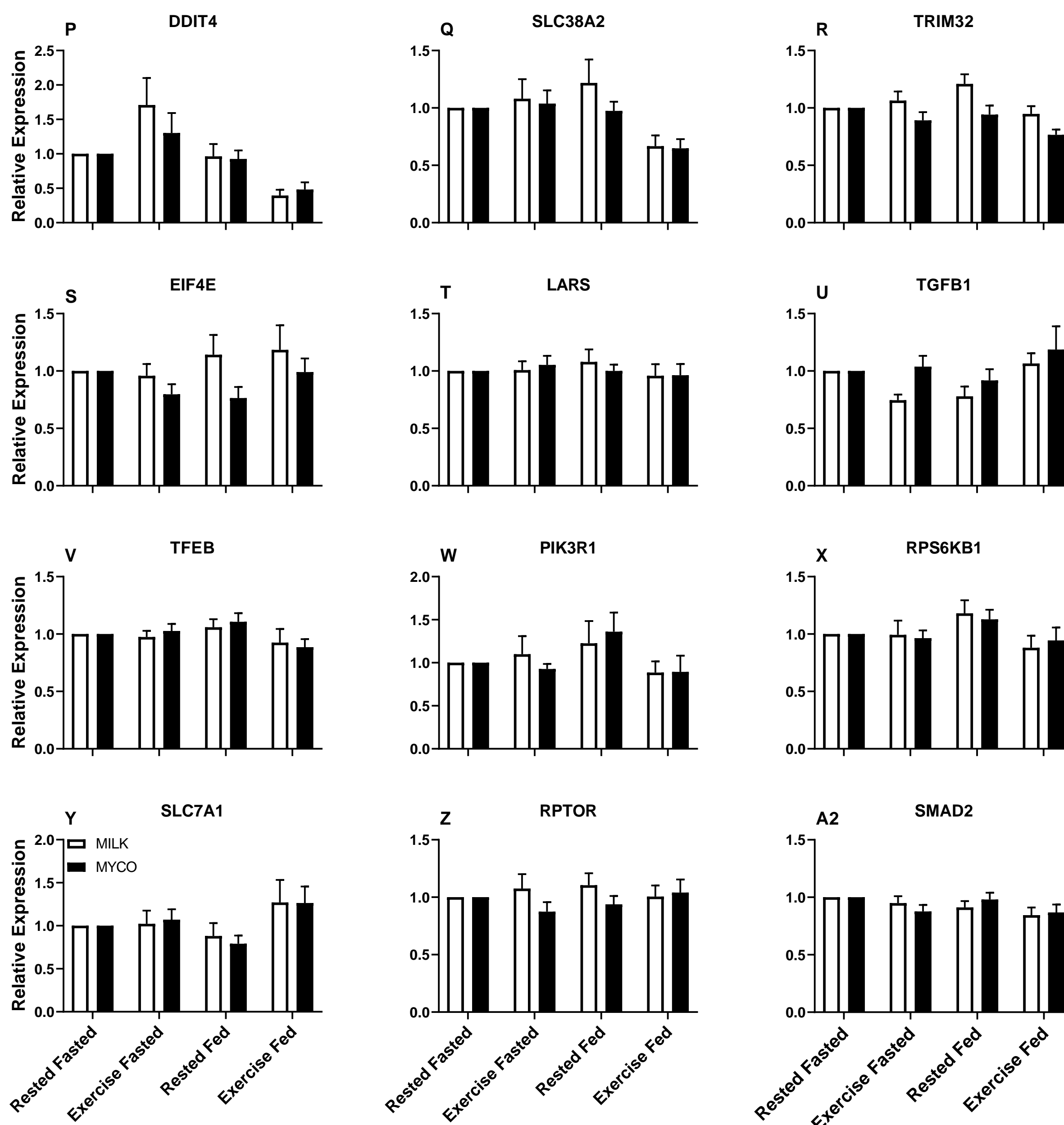

Figure 3.8. Skeletal muscle mRNA expression of genes involved in muscle protein synthesis, muscle protein breakdown, and amino acid transport in the postabsorptive and postprandial state, in rested and exercised legs, after the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=10), in young men. Data were analysed using three-way ANOVA, with Sidak's post hoc tests used to detect differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. There was a main effect of protein ingestion for IRS1, TSC1, TSC2, GATSL3, AKT1S1, CAPN1, FOXO3, SRF, LAT2, SNAT9, SLC38A10, DDIT4, TRIM63, and SLC38A2 ($P<0.05$). There was a time × exercise interaction effect for MSTN, DDIT4L, FBXO32, TRIM63, SLC38A10, DDIT4, SLC38A2, TRIM32, EIF4E, LARS, TGFB1, TFEB, PIK3R1, RPS6KB1, and

SLC7A1 ($P<0.05$). SMAD2 showed a main effect of exercise ($P<0.05$). RPTOR and TSC1 showed a three way interaction (time × condition × exercise; $P<0.05$). The full names of all genes are stated in Table 3.3.

DISCUSSION

We assessed *in vivo* protein synthetic responses to the ingestion of leucine matched boluses of milk protein and mycoprotein within resting and exercised skeletal muscle, in healthy and trained, young men. In support of our initial hypothesis, mycoprotein ingestion robustly stimulated protein synthesis rates in resting and exercised muscle. Mycoprotein ingestion resulted in slower and lower rises in plasma amino acid (and leucine) concentrations compared with the ingestion of milk protein. Despite this, and contrary to our secondary hypothesis, we report that mycoprotein ingestion stimulated muscle protein synthesis rates to a greater extent when compared with milk protein.

Previous work has suggested that the rate and/or magnitude of plasma essential aminoacidemia and leucinemia following dietary protein ingestion are the key determinants that modulate postprandial muscle protein synthesis rates (Pennings et al., 2011b, Norton et al., 2009, Churchward-Venne et al., 2013, Burd et al., 2012, Phillips, 2016). As expected (Dunlop et al., 2017), we observed more rapid protein digestion and intestinal amino acid absorption rates following milk compared with mycoprotein ingestion, as evidenced by larger and more rapid postprandial aminoacidemia and leucinemia (Figures 3.2A-D). As a consequence, milk protein ingestion resulted in a quicker rise in circulating insulin concentrations (Figure 3.3A), also a postprandial systemic condition expected to facilitate muscle protein anabolism (Abdulla et al., 2016). In line with our hypothesis, we observed a robust stimulation of mixed muscle protein synthesis rates in response to both protein sources (Figure 3.6A). However, we report a greater stimulation of mixed muscle protein synthesis rates following mycoprotein compared with milk protein ingestion (Figure 3.6A). Moreover, when expressing these data as the change in muscle protein synthesis rates from postabsorptive to postprandial (Figure 3.6B), the response to mycoprotein ingestion was more than double that of milk protein ingestion. Given this response occurred despite

‘inferior’ postprandial plasma amino acid kinetics, it is of interest to consider *why* this was observed.

In studies where isolated protein sources are ingested (i.e. with little or no additional macronutrients), the rate and/or magnitude of leucinemia generally predicts subsequent rates of muscle protein synthesis (Pennings et al., 2011b, Churchward-Venne et al., 2013, Burd et al., 2012, Phillips, 2016). However, in studies where protein was co-ingested with carbohydrate or fat, plasma leucine concentrations were less predictive of the subsequent muscle protein synthetic response. For example, carbohydrate co-ingestion can markedly attenuate the peak (and total) magnitude of postprandial leucinemia, but does not inhibit the muscle protein synthetic response compared with protein ingestion alone (Gorissen et al., 2014, Hamer et al., 2013, Koopman et al., 2007, Churchward-Venne et al., 2015, Staples et al., 2011). This suggests, within the context of a mixed meal, plasma leucine kinetics alone do not dictate the postprandial muscle protein synthetic response. Further, co-ingestion of additional macronutrients *per se* do not confer additional stimulation of muscle protein synthesis rates (Gorissen et al., 2014, Hamer et al., 2013, Koopman et al., 2007, Churchward-Venne et al., 2015, Staples et al., 2011), and therefore neither the additional macronutrients nor energy content of the mycoprotein can (solely) explain its *greater* anabolic response in the present study. In line with our findings, a recent study reported that protein consumed within a ‘whole food’ matrix (i.e. whole eggs) led to a delayed and lower peak magnitude of postprandial leucinemia, but *greater* rates of postprandial muscle protein synthesis compared with the protein ingested within the egg white only (van Vliet et al., 2017). It is therefore possible that the present data, and those from van Vliet and colleagues (van Vliet et al., 2017), are a result of a potentiating effect of consuming protein within a more complete food matrix/whole food meal. Whether this is an effect of the food matrix *per se* (Churchward-Venne et al., 2015), a combined effect of the presence of additional macronutrients (or their specific subclasses (Castellano et al., 2017, Sawan et al., 2018,

Yasuda et al., 2014)), fibre (mycoprotein is high in fibre composed of a 2:1 ratio of β-glucan and chitin), higher energy content, or an effect of other (micronutrient) factors (Salles et al., 2013, Zhao et al., 2016, Marzani et al., 2008) is unclear but clearly warrants future investigation.

To investigate how mycoprotein enabled such a potent muscle protein synthetic response, we used the remaining muscle tissue to probe various myocellular signalling responses (Figure 3.7 and 3.8). Aside from amino acids, it has been suggested that systemic rises in non-protein nutrients (e.g. specific lipids (Castellano et al., 2017, Yasuda et al., 2014)), micronutrients (Salles et al., 2013)) may also exert influence over mTOR, the central molecular signalling pathway controlling muscle protein synthesis (Walker et al., 2011). Despite the divergent amino acid (and presumably other nutrients) profiles across the two nutritional conditions we observed no differences between groups in postprandial mTOR phosphorylation. We cannot discount the possibility that mTOR phosphorylation may have differed during the early postprandial period (Churchward-Venne et al., 2012, Drummond et al., 2012, West et al., 2011), that the translocation and subcellular location of mTOR may have differed across conditions (Sawan et al., 2018), that investigating different phosphorylation sites may have yielded different results (Figueiredo et al., 2019), that downstream targets of mTOR may have differed independently, or that our data are not appropriately powered to detect small, but physiologically relevant differences in phosphorylation status (particularly given we did not have a complete dataset due to limited tissue availability). Indeed, recent data suggest the subcellular locality of mTOR is important in regulating the (postprandial) muscle protein synthetic response (Hodson et al., 2017, Abou Sawan et al., 2018a), and that non-protein dietary components may influence translocation and the regulation of post-exercise mRNA translation (Sawan et al., 2018).

To gain insight into the potential adaptive response of the cell we examined the muscle postprandial and post-exercise transcriptional response. Exercise and protein ingestion

resulted in a coordinated transcriptional response, demonstrated by the mRNA expression of 27 of the 46 genes of interest changing 4 h post protein ingestion with/without exercise. The upregulation in expression of genes involved in amino acid transport (e.g. CAT1, LAT2, SNAT2) and protein synthesis (e.g. TSC1, TSC2, DDIT4) underline the rapid transcriptional responses that occur with nutrition and exercise (Wall et al., 2016b, Graber et al., 2017, Wall et al., 2016c, Drummond et al., 2011, Borack et al., 2016, Mitchell et al., 2017) which, at least in part, restore cellular homeostasis and direct the adaptive response. Additionally, the early inhibition of myostatin expression with exercise supports the concept that low myostatin expression facilitates an anabolic environment (Rodriguez et al., 2014). Only a single gene, the E3 ubiquitin ligase TRIM32, differentially responded to the different protein sources; being expressed to a lesser degree following mycoprotein ingestion. TRIM32 preferentially ubiquitinates actin and desmin filaments, with reduced levels of TRIM32 reducing the loss of these proteins (Cohen et al., 2012, Kudryashova et al., 2005). This may be indicative of a more potent ability of mycoprotein to suppress excessive post-exercise muscle protein breakdown, although given the lack of a coordinated response of other proteolytic genes (e.g. MAFBx, MuRF1 etc.) this remains conjectural. Clearly, the ability of prolonged mycoprotein consumption to support resistance training-induced hypertrophy warrants future investigation. Worthy of note is one caveat of interpreting our cell signalling data in the present work is the increased risk of making a type 1 error due to due to multiple testing outcomes.

Two other factors that could have contributed to our findings are worthy of consideration. First, the mycoprotein drink contained ~20% more protein than the milk protein beverage (31.5 vs 26.2 g). In young men the muscle protein synthetic response to dietary protein ingestion plateaus at ~20 g protein (Moore et al., 2008, Witard et al., 2013) (or 0.3 $g{\cdot}kg^{-1}$; (Stokes et al., 2018)), which appears to be more due to leucine content, rather than total protein *per se* (Devries et al., 2018), at least when ample protein is provided (Fuchs et al.,

2019). We therefore assume, since we fed in excess of 20 g / 0.3 g·kg^{-1} in both conditions, that ample protein was available to negate protein *amount* being a significant contributing factor. In agreement, systemic amino acid concentrations were *lower* following mycoprotein compared with milk protein ingestion, again indicating non-protein/amino acid factors are likely responsible for the greater muscle protein synthetic effect of mycoprotein. Second, whilst it is generally assumed that leucine (and the other BCAAs) is the primary nutritional anabolic trigger (Norton et al., 2009, Stokes et al., 2018, Devries et al., 2018, Crozier et al., 2005), it is clear that other essential amino acids (Churchward-Venne et al., 2012), such as arginine (Saxton et al., 2016, Chantranupong et al., 2016, Saxton and Sabatini, 2017), may also play a role in initiating muscle protein synthesis rates. Therefore, it is also plausible that the amino acid profile of mycoprotein was simply more anabolic than milk protein. This, however, would be contrary to the consensus of *in vivo* human data (van Vliet et al., 2015b), and necessitate that specific amino acids, with a greater preponderance in the mycoprotein drink, possess anabolic signalling roles that are as yet undocumented in humans.

To conclude, the bolus consumption of mycoprotein stimulated resting and post-exercise muscle protein synthesis rates in young men, and to a greater extent than a leucine-matched bolus of milk protein. These novel data show mycoprotein represents a viable, sustainably produced non-animal-derived alternative dietary protein source to support acute tissue remodelling in response to exercise. Our work implies that mycoprotein could be incorporated into the habitual diet of those undertaking prolonged resistance training to facilitate muscle hypertrophic responses.

CHAPTER 4

BRANCHED-CHAIN AMINO ACID FORTIFICATION DOES NOT RESTORE MUSCLE PROTEIN SYNTHESIS RATES FOLLOWING INGESTION OF LOWER-COMPARED WITH HIGHER-DOSE MYCOPROTEIN.

The work contained within this chapter is published:

Monteyne, A.J., Coelho, M.O., Porter, C., Abdelrahman, D.R., Jameson, T.S., Finnigan, T.J., Stephens, F.B., Dirks, M.L. and Wall, B.T., 2020. branched-chain amino acid fortification does not restore muscle protein synthesis rates following ingestion of lower-compared with higher-dose mycoprotein. *The Journal of Nutrition*.

Abstract

Background: We have shown that ingesting a large bolus (70 g) of the fungal-derived, wholefood mycoprotein robustly stimulates muscle protein synthesis rates (MPS).

Objective: The aim of this study was to determine if a lower dose (35 g) of mycoprotein enriched with branched-chain amino acids (BCAA) stimulates MPS to the same extent as 70 g of mycoprotein in resistance-trained young men.

Methods: Nineteen males (age: 22±1 y, BMI: 25±1 kg/m^2) took part in a randomised, double-blind, parallel-group study. Participants received primed, continuous infusions of L-[*ring*-$^2H_5$]phenylalanine and ingested either 70 g mycoprotein (31.5 g protein; MYCO; n=10) or 35 g BCAA enriched mycoprotein (18.7 g protein: matched on BCAA content; ENR; n=9) following a bout of unilateral resistance exercise. Blood and bilateral quadriceps muscle samples were obtained before exercise and protein ingestion, and during a 4 h postprandial period to assess MPS in rested and exercised muscle. Two and three way ANOVAs were used to detect differences in plasma amino acid kinetics and mixed muscle FSRs, respectively.

Results: Postprandial plasma BCAA concentrations increased more rapidly and to a larger degree in ENR compared with MYCO. MPS increased with protein ingestion ($P \leq 0.05$) but to a greater extent following MYCO (from 0.025±0.006 to 0.057±0.004%·h^{-1} in rested, and from 0.024±0.007 to 0.072±0.005%·h^{-1} in exercised muscle; $P<0.0001$) compared with ENR (from 0.031±0.003 to 0.043±0.005%·h^{-1} in rested, and 0.027±0.005 to 0.052±0.005%·h^{-1} in exercised muscle; $P<0.01$) ingestion. Postprandial MPS rates were greater in MYCO compared with ENR ($P<0.01$).

Conclusion: The ingestion of lower-dose BCAA enriched mycoprotein stimulates resting and post-exercise MPS rates, but to a lesser extent compared with the ingestion of a BCAA matched 70 g mycoprotein bolus in healthy young men.

INTRODUCTION

Adequate dietary protein intake is required to maintain skeletal muscle mass and to facilitate the remodelling of muscle tissue in response to exercise stimuli. Mechanistically, this is achieved via the ingestion of protein transiently (2-5 h) increasing muscle protein synthesis (MPS) rates which facilitates postprandial net muscle protein accretion following each meal (Pennings et al., 2011b, Moore et al., 2008, Rennie et al., 2002). A single bout of (resistance-type) exercise independently stimulates MPS rates and also acts concurrently with protein ingestion by sensitising the muscle to the anabolic effects of amino acids immediately, and for at least ~24-48 h (Phillips et al., 1997, Biolo et al., 1995a, Burd et al., 2011a).

Whilst most data concerning dietary protein ingestion and muscle protein turnover have been obtained from experiments using animal-derived protein sources, there is increasing societal and research interest in evaluating the efficacy of non-animal-derived alternative protein sources. Mycoprotein is a sustainable, protein-rich whole food source produced by the continuous cultivation of the filamentous fungus *Fusarium venenatum*. We recently reported that the ingestion of a large (70 g; containing 31.5 g protein) bolus of mycoprotein robustly stimulates MPS rates in rested and exercised muscle of young men (Monteyne et al., 2020b). From a practical and sustainability perspective, it is relevant to investigate and maximise the MPS response to the ingestion of smaller boluses of effective alternative dietary protein sources.

Plasma elevation of amino acids, and specifically of the branched-chain amino acids (BCAA; i.e. leucine, isoleucine and valine), appears to be the primary nutritional signal regulating postprandial MPS rates (Pennings et al., 2011b, Norton et al., 2009, Burd et al., 2012, Koopman et al., 2009a, Tang et al., 2008). Indeed, the ingestion of merely 5.6 g of BCAAs alone stimulates MPS rates in recovery from resistance exercise in young males (Jackman et al., 2017). However, this effect is short-lived (2 h) compared with a BCAA matched bolus

of a complete protein source (5 h) (i.e. containing all other essential [and non-essential] amino acids (Fuchs et al., 2019)). The latter suggests that, while BCAAs alone clearly act as potent signalling molecules, in the absence of a full complement of amino acids, substrate availability will become limiting to postprandial MPS rates. In line with this interpretation, fortification of reduced-quantity suboptimal amounts (1.5-6.25 g) and/or types (i.e. casein) of dietary protein with one or more of the BCAAs has previously been shown to be an effective strategy to augment postprandial MPS rates (Wall et al., 2013b, Churchward-Venne et al., 2013, Churchward-Venne et al., 2012, Bukhari et al., 2015, Wilkinson et al., 2018).

In the present work, we tested the hypothesis that the ingestion of a lower-dose mycoprotein beverage (35 g), fortified with BCAAs (resulting in 18.7 g total protein, 2.5 g leucine, 1.5 g isoleucine and 1.9 g valine) would stimulate resting and post-exercise mixed MPS rates to the same extent as a larger (70 g; 31.5 g protein) BCAA matched bolus of mycoprotein in healthy, resistance-trained young men.

METHODS

Participants

Nineteen young, healthy (age: 22±1 y, body mass: 82±2 kg, BMI: 25±1 $kg \cdot m^{-2}$) men volunteered to take part in the present study. Participants' characteristics are displayed in **Table 4.1**. Participants were recreationally active and experienced with resistance training (at least 3 times per week for at least 3 months prior to participation). Participants were deemed healthy based on their blood pressure (≤140/90mmHg), BMI (18-30 $kg \cdot m^{2}$) and responses to a routine medical screening questionnaire (absence of any diagnosed metabolic impairment, cardiovascular disease, or motor disorders), and were informed of the experimental procedures, potential risks, and the purpose of the study prior to providing full written consent. Participants were all 'tracer naïve' having not undergone any previous stable isotope amino acid infusion protocols. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (REF NO. 161026/B/05) in accordance with standards set out for human research as outlined in the declaration of Helsinki. The study was registered at ClinicalTrials.Gov (ID: 660065600). This was part of a wider trial exploring the muscle protein synthetic responses to mycoprotein ingestion.

Table 4.1. Participant characteristics[1].

	MYCO (n = 10)	ENR (n = 9)
Age (y)	22 ± 1	22 ± 1
Body mass (kg)	81 ± 3	84 ± 3
Height (cm)	182 ± 2	180 ± 2
BMI (kg·m^{-2})	25 ± 1	26 ± 1
Fat (% of body mass)	9 ± 2	13 ± 3
Lean mass (kg)	73 ± 2	72 ± 3
Total work done[2] (kJ)	30.7 ± 2.06	27.7 ± 1.88

[1]Values are mean ± SEM. BMI (body mass index), ENR (lower-dose BCAA enriched beverage ingestion condition), MYCO (mycoprotein beverage ingestion condition). [2]The amount of work done during the unilateral experimental exercise protocol.

Pre-testing

Following screening and acceptance onto the study, all participants underwent a single pre-testing session, which took place at least 5 days prior to the experimental trial. Participants reported to the laboratory for this pre-testing session to be familiarised with the exercise equipment and exercise protocol, and to determine body composition. Body fat and lean mass were determined by Air Displacement Plethysmography (BodPod, Life Measurement, Inc. Concord, CA, USA). Participants were familiarised with the unilateral resistance-type exercise that was employed in the experimental protocol. This consisted of 5 sets of 30 repetitions of maximal concentric isokinetic leg extension and leg flexion contractions on a Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, New York, USA) at a speed of 60° per second over a central 80° range of motion using their dominant leg. Verbal encouragement was provided throughout the familiarisation and experimental testing to encourage maximal effort through every repetition. Work done (J) was recorded for each completed set, and fatigue was calculated as the percentage decrement in work done between the first and last set.

Experimental protocol

Participants were randomly assigned to two parallel-groups, and completed a single experimental trial in a double-blind fashion. An overview of the experimental protocol is shown in **Figure 4.1**. Participants were directed to abstain from vigorous physical activity and alcohol consumption in the 48 h preceding the trial. All participants were provided with a standardised meal to consume ~10.5 h prior to the start of the experimental trial (744 kcal [3.1 MJ], 29% energy (%En) fat, 20% En protein, 51% En carbohydrate). On the day of the trial, participants arrived at the laboratory at 07:00-08:00 after a 10 h overnight fast. A Teflon™ cannula was inserted into an antecubital vein of one arm in preparation for stable isotope infusion, before which a baseline venous blood sample was taken from this site to measure background isotope enrichments. Following baseline blood sampling (t = -210 min) the phenylalanine and tyrosine pools were primed with a single intravenous dose of L-[*ring*-$^{2}H_{5}$]phenylalanine (2.12 $\mu mol \cdot kg^{-1}$) and L-[3,3-$^{2}H_{2}$]tyrosine (0.75 $\mu mol \cdot kg^{-1}$). Thereafter, continuous tracer infusion was initiated and maintained at a rate of 0.035 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for L-[*ring*-$^{2}H_{5}$]phenylalanine and 0.012 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ L-[3,3-$^{2}H_{2}$]tyrosine for the duration of the protocol. Once the infusion was begun, a second Teflon™ catheter was inserted into a dorsal hand vein of the contralateral arm and placed in a warmed air hand unit (55°C) for the collection of repeated arterialised venous blood samples (Abumrad et al., 1981). Arterialised venous blood samples were collected throughout the experimental protocol at the following time points: t = -180, -120, -60, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 min. A baseline muscle biopsy sample was collected after 90 min of the infusion (t = -120 min) from the non-dominant leg (designated as the ‘resting leg’). Muscle biopsies were collected from the mid-region of the *m. vastus lateralis* (approx. 15 cm above the patella) with a modified Bergström suction needle under local anaesthesia (2% lidocaine). All biopsy samples were immediately freed from any visible blood, adipose and connective tissue, frozen in liquid nitrogen (within 30 s), and stored at –80°C until subsequent analysis.

Eighty-five min after the initial biopsy (t = -35 min) participants completed the unilateral resistance-type exercise protocol (described above) which took 30 min. Immediately following exercise bilateral muscle biopsies were collected (i.e. from both the rested and exercised leg). Following this (t = 0 min) participants consumed a beverage containing either a larger (70 g; MYCO) or lower-dose mycoprotein beverage (35 g; ENR), with the ENR condition enriched with BCAAs to match BCAA content in MYCO. Beverages were consumed within an allotted 5 min period and administered in a double-blind manner. Thereafter, participants rested in a semi-supine position for 4 h, after which further bilateral muscle biopsies were collected 1–2 cm proximal to the previous incisions (t = 240 min).

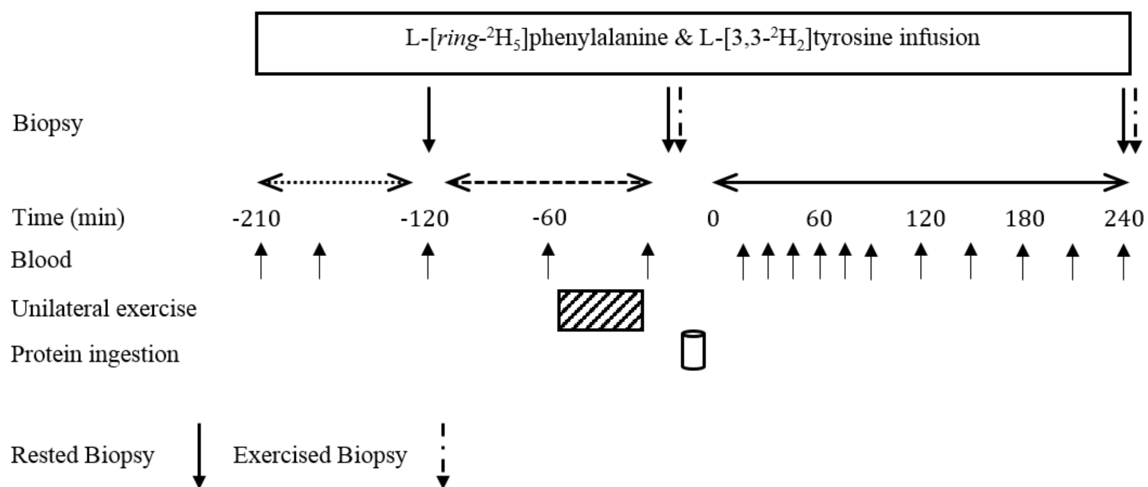

Figure 4.1. Schematic representation of the experimental protocol.

Experimental beverage preparations

Freeze-dried mycoprotein was produced by and obtained from Marlow Foods Ltd, Quorn Foods, Stokesley, UK, and was independently analysed for energy, macronutrient content and amino acid composition (Premier Analytical Services, High Wycombe, UK). The experimental beverages were prepared the evening before the experimental trial. The mycoprotein was assimilated with 400 mL water and 10 g of artificial energy-free flavouring (Myprotein, Manchester, UK), blended for approximately 2 min, topped up with water to make a total final beverage volume of 600 mL and refrigerated overnight. Drinks were enriched (2.5%) with L-[*ring*-$^2H_5$]phenylalanine to account for postprandial tracer dilution by non-labelled phenylalanine and therefore to maintain a systemic isotopic steady-state following protein ingestion. Following drink consumption by the participant, an additional 50 mL of water was then added to 'wash' the bottle and ensure that all protein had been consumed, making a total volume of 650 mL consumed by participants. All drinks were well tolerated, consumed within the allotted time (i.e. 5 min) and resulted in no adverse effects during or after the test day. Double blinding of the drinks was achieved by having a different researcher from the individual running the infusion trial prepare the drinks in an opaque bottle ready for consumption. The larger dose mycoprotein beverage (MYCO) contained 70 g of mycoprotein which contained 31.5 g total protein (providing 2.5 g of leucine, 1.5 g isoleucine and 1.9 g valine). The lower-dose enriched mycoprotein beverage (ENR) contained 35 g of mycoprotein (i.e. half that of MYCO; containing 15.75 g protein) and was enriched with free BCAAs (Iron Science Direct, Cheadle, UK; Bulk Powders, Colchester, UK) to match MYCO resulting in a total protein content of 18.7 g. The detailed nutritional content and amino acid composition of the two beverages are displayed in **Table 4.2**.

Table 4.2. Nutritional content of the experimental drinks.[1]

	MYCO	ENR[2]
Macronutrients		
Protein (g)	31.5	18.7
Fat (g)	9	4.6
Carbohydrate (g)	7	3.5
Fibre (g)	17.5	8.8
Energy (kcal)	238	131
Energy (kJ)	996	547
Amino acid content (g)		
Alanine	2.0	1.0
Arginine	2.2	1.1
Aspartic acid	3.3	1.7
Glutamic acid	3.9	2.0
Glycine	1.5	0.8
Histidine	0.8	0.4
Isoleucine	1.5	1.5
Leucine	2.5	2.5
Lysine	2.6	1.3
Phenylalanine	1.5	0.7
Proline	1.6	0.8
Serine	1.6	0.8
Threonine	1.7	0.9
Tryptophan	1.2	0.6
Tyrosine	1.2	0.6
Valine	1.9	1.9
EAA	13.7	9.8
NEAA	17.4	8.7
BCAA	5.9	5.9

[1]Beverage volume of 600 mL in both conditions. [2]Values for ENR include the energy, macronutrients and amino acids from the added BCAAs. Energy content was calculated using Atwater factors (1944).

Blood sample collection and analyses

Ten mL of arterialised venous blood was collected into a syringe at each sampling point. For each blood sample, six mL was aliquoted into lithium heparin containing tubes (BD vacutainer LH; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged immediately (3000 *g*, 4°C, 10 min). Blood plasma was aliquoted and immediately frozen at –80°C for subsequent analysis. The remaining 4 mL of blood was aliquoted into additional vacutainers (BD vacutainers SST II, Becton, Dickinson and Company) which were left to clot at room temperature for at least 30 min and then centrifuged (3000 *g*, 4°C, 10 min) to obtain blood serum. Serum was aliquoted before freezing at –80°C for subsequent analyses.

Serum insulin concentrations were analysed using a commercially available ELISA kit (DRG Insulin ELISA, EIA-2935, DRG International Inc, Springfield, USA.). Plasma branched-chain amino acid (BCAA; leucine, isoleucine and valine), phenylalanine and tyrosine concentrations, and L-[*ring*-$^{2}H_5$]phenylalanine, L-[3,3-$^{2}H_2$]tyrosine, and L-[*ring*-$^{2}H_4$]tyrosine enrichments were determined by gas chromatography-mass spectrometry (GC-MS) as described previously (Wolfe and Chinkes, 2005, Monteyne et al., 2020b).

Skeletal muscle tissue analyses

Muscle biopsy tissue samples were analysed for protein-bound and free intracellular L-[*ring*-$^{2}H_5$]phenylalanine, determined by GC-MS, as previously described (Wolfe and Chinkes, 2005, Monteyne et al., 2020b). Briefly, 20-30 mg of frozen muscle tissue was homogenised by a mechanical tissue grinder. The supernatant was removed and used for the determination of intracellular labelled phenylalanine enrichment. The remaining pellet was dried, hydrolysed and used to determine the enrichment of bound L-[*ring*-$^{2}H_5$]phenylalanine. The samples were analysed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies) in duplicates using electron impact ionisation and selected ion monitoring for measurement of isotope ratios (Zabielski et al., 2013).

Calculations

The fractional synthetic rates (FSR) of mixed muscle proteins were calculated using the standard precursor-product equation (Wolfe and Chinkes, 2005):

$$FSR\ (\% \cdot h^{-1}) = \left[\frac{\Delta E\text{p}}{E_{\text{precursor}} \times t}\right] \times 100$$

(Where ΔEp is the increment in L-[*ring*-$^2H_5$]phenylalanine enrichment in mixed muscle protein between two biopsies, $E_{precursor}$ is the average L-[*ring*-$^2H_5$]phenylalanine enrichment in the plasma or intracellular precursor pool over time, and t indicates the tracer incorporation time (h) between two muscle biopsies.)

L-[3,3-$^2H_2$]tyrosine and arterialised venous blood sampling were used to assess phenylalanine hydroxylation rates (the initial step in phenylalanine oxidation), which were calculated using modified Steele's equations, as previously described (Gorissen et al., 2017).

Statistical analyses

A two-sided power analysis based on previous research (Tang et al., 2009) showed that n= 9 per condition was sufficient to detect expected differences in postprandial muscle protein synthesis (MPS) rates between dietary protein conditions (MYCO vs ENR) when using a repeated measures ANOVA ($P \leq 0.05$, 90% power, f = 0.67; G*power version 3.1.9.2). Our primary outcome measure was postprandial muscle protein synthesis rates. The delta change in muscle protein synthesis rates from fasted to fed, plasma amino acid concentrations, serum insulin concentrations, phenylalanine hydroxylation, intracellular and mixed muscle protein-bound L-[*ring*-$^2H_5$]phenylalanine represent secondary measures. Differences in participant characteristics and background mixed muscle protein-bound L-[*ring*-$^2H_5$]phenylalanine were analysed using independent t-tests. Plasma amino acid and

serum insulin concentrations, plasma L-[*ring*-$^2H_5$]phenylalanine, L-[3,3-$^2H_2$]tyrosine, and L-[*ring*-$^2H_4$]tyrosine enrichments were tested by two-factor (treatment [mycoprotein vs lower-dose mycoprotein] × time) repeated-measures analysis of variance (ANOVA). Mixed muscle FSRs and mixed muscle protein-bound L-[*ring*-$^2H_5$]phenylalanine enrichments were analysed using three-factor (treatment × time × exercise/rest) ANOVAs. When significant interaction effects were observed Sidak post hoc tests were performed to locate individual differences. Total postprandial amino acid concentrations were calculated as incremental area under curve (iAUC), with baseline set as t = 0, and analysed using independent t-tests. Data were tested for sphericity, and where violations occurred the Greenhouse-Geisser correction was automatically applied. Violations of normality were tested for using the Shapiro-Wilk test, and no considerable violations were found ($P > 0.05$). Where plasma time point data were absent, missing data analyses were performed using regression imputation. Statistical significance was set at $P \leq 0.05$. Calculations were performed using GraphPad 7.1. All data are expressed as means ± SEM.

RESULTS

Participants' characteristics

No differences in age, weight, height, BMI, body composition, or habitual nutritional intake were detected between groups (all P > 0.05; **Table 4.1**). No differences in total work performed during the experimental resistance exercise bout (P > 0.05; Table 4.1) or in fatigue decrement (26±4% in MYCO vs 18±6 in ENR; P > 0.05) were detected between groups.

Plasma amino acid and serum insulin concentrations

Plasma total BCAA, leucine, valine, isoleucine, phenylalanine and tyrosine concentrations during the experimental period are shown in **Figure 4.2**. Plasma total BCAA concentrations (Figure 4.2A) and each of the individual BCAAs (Figure 4.2C, 2E, and 2G) all showed similar kinetic responses. Specifically, from similar postabsorptive values across conditions, plasma BCAA concentrations (and each of the individual BCAAs) increased with protein ingestion (all P<0.0001), but to a greater degree in ENR compared with MYCO (P-interaction all <0.0001), which led to a significant difference between conditions (all P<0.0001). Plasma BCAA, leucine, valine and isoleucine all peaked more rapidly (30 vs 75, 30 vs 75, 30 vs 90 min, and 45 vs 120 min, respectively), to a greater extent (939±37 vs 608±28, 355±15 vs 244±11, 222 ±13 vs 112±7, and 372±11 vs 257±11 $\mu mol \cdot L^{-1}$, respectively) and were higher from 15-75 and at 240 min, 15-75 and at 240 min, 15-75, 180 and at 240 min, and 15-120 and at 240 min, respectively (P ≤ 0.05), following ingestion of ENR compared with MYCO. Total postprandial plasma BCAAs, leucine, isoleucine, and valine availability (iAUC) were all significantly greater in ENR compared with MYCO (P<0.001; Figure 4.2B, 2H, 2D & 2F**)**. Plasma tyrosine and phenylalanine concentrations increased with protein ingestion (both P<0.0001) and to a greater extent in MYCO compared with ENR (P-interaction <0.0001), resulting in a significant difference between conditions (P≤0.05). Plasma tyrosine

concentrations (Figure 4.2I) were greater from 60-150 min ($P \leq 0.05$), and plasma phenylalanine concentrations (Figure 4.2K) from 75-120 min ($P \leq 0.05$) in MYCO compared with ENR. Both plasma phenylalanine (Figure 4.2L) and tyrosine (Figure 4.2J) total postprandial availabilities (iAUC) were higher in MYCO compared with ENR ($P < 0.0001$).

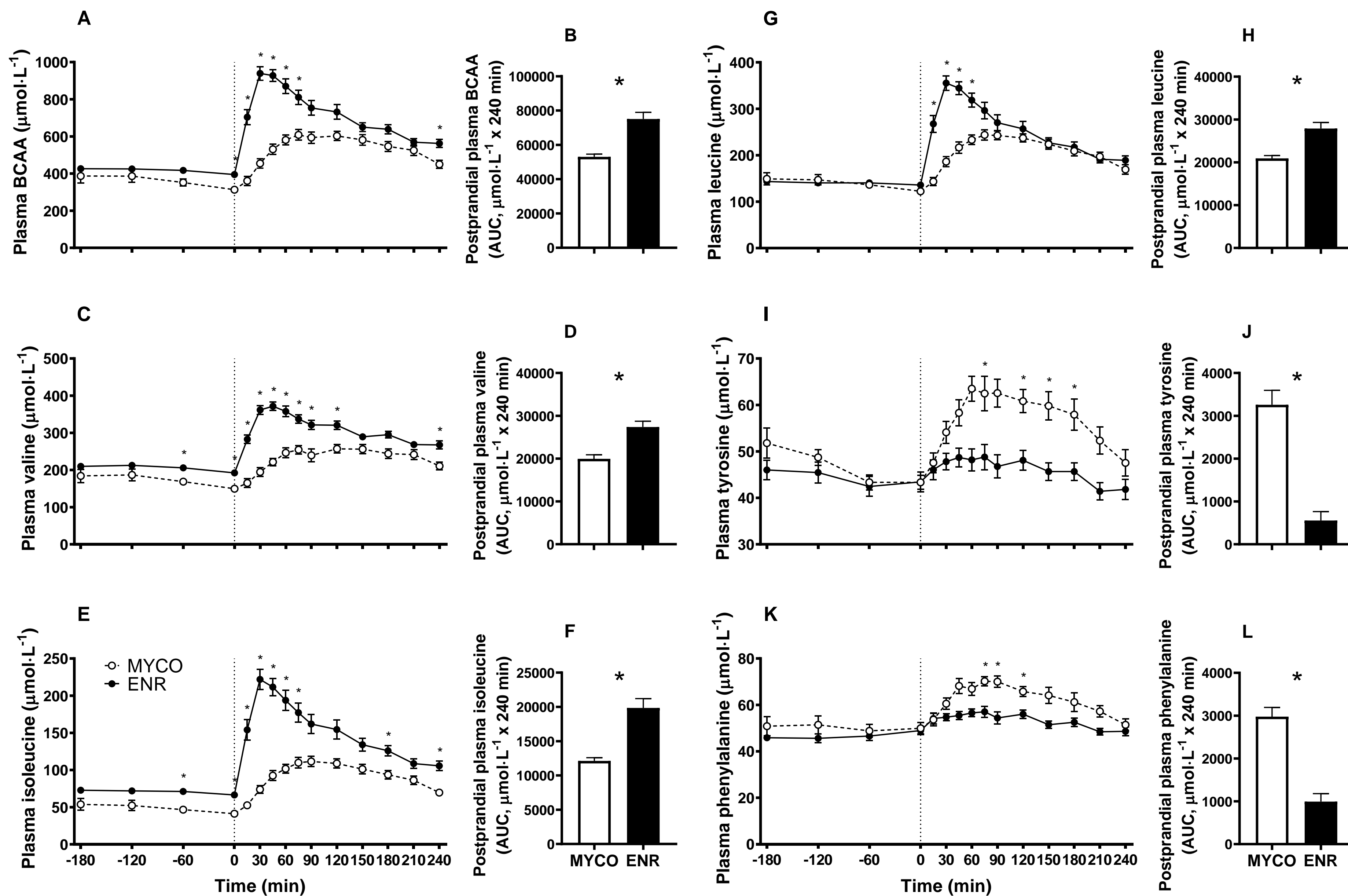

Figure 4.2. The time-course and incremental AUC (iAUC) of plasma total branched-chain amino acid (BCAA; A and B), leucine (C and D), isoleucine (E an

d F), valine (G and H), tyrosine (I and J) and phenylalanine (K and L) concentrations during a 3 h postabsorptive period (time-course graphs only) and a 4 h postprandial period in healthy young men. iAUC graphs represent total 4 h postprandial plasma availability above postabsorptive values. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 31.5 g mycoprotein (MYCO; n=9) or 18.7 g mycoprotein enriched (to match MYCO) with BCAA (ENR; n=9), where a single bout of unilateral leg extension exercise was also performed immediately prior to protein ingestion. Values are mean ± SEM. *Means differ between conditions, ($P \leq 0.05$).

Serum insulin concentrations during the experimental period are displayed in **Figure 4.3**. From similar fasting concentrations (16±2 and 14±2 $mU \cdot L^{-1}$ in MYCO and ENR, respectively) serum insulin increased with protein ingestion ($P<0.0001$) and to a greater degree in MYCO compared with ENR (P-interaction <0.0001) which led to a significant difference between conditions ($P \leq 0.05$). In MYCO serum insulin concentrations peaked at 30 min post-ingestion (36±4 $mU \cdot L^{-1}$) and remained elevated until 60 min ($P<0.0001$) before returning to postabsorptive levels. In ENR serum insulin concentrations also peaked at 30 min (25±5 $mU \cdot L^{-1}$; $P \leq 0.05$) but returned to fasting levels thereafter. As a result, serum insulin concentrations in MYCO were greater than ENR at 30 and 45 min of the postprandial period ($P \leq 0.05$), and MYCO elicited a greater overall postprandial insulin response (iAUC; $P \leq 0.05$; Figure 4.3B).

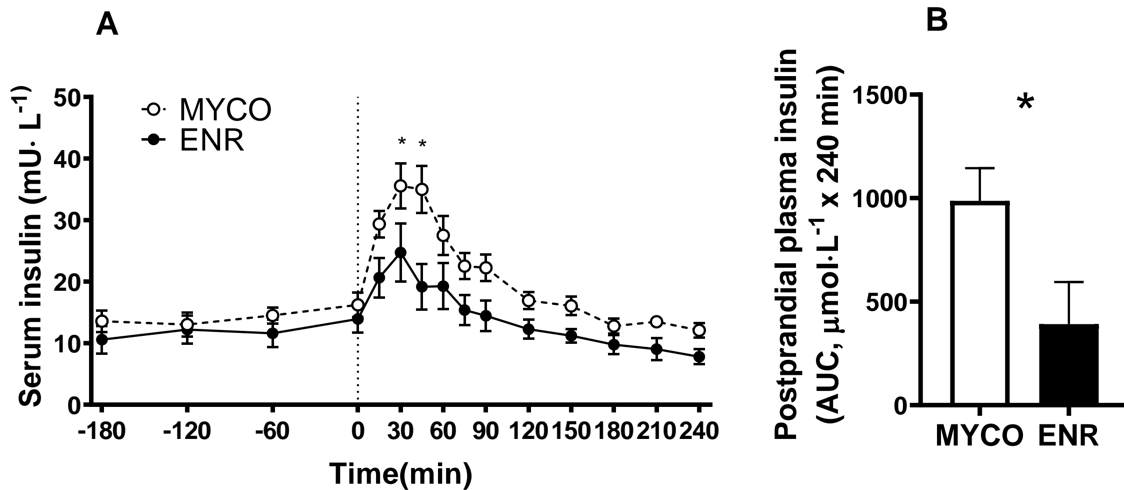

Figure 4.3. The time-course and incremental AUC (iAUC) of serum insulin concentrations during a 3 h postabsorptive period (time-course graph only) and a 4 h postprandial period in healthy young men, with iAUCs representing total 4 h postprandial plasma availability above postabsorptive values. The vertical line indicates the transition from postabsorptive to postprandial conditions via the ingestion of 31.5 g mycoprotein (MYCO; n=9) or 18.7 g mycoprotein enriched (to match MYCO) with BCAA (ENR; n=9), where a single bout of unilateral leg extension exercise was also performed. Values are mean ± SEM. *Means differ between condition, (P≤0.05).

Plasma tracer enrichments

The time-courses of plasma L-[*ring*-$^{2}H_5$]phenylalanine, L-[3,3-$^{2}H_2$]tyrosine, and L-[ring-$^{2}H_4$]tyrosine enrichments are illustrated in **Figure 4.4**. During the postabsorptive period, plasma L-[*ring*-$^{2}H_5$]phenylalanine (Figure 4.4A) remained in a steady state at ~4–5 MPE (mole percent excess) in MYCO and ~5-6 MPE in ENR. Plasma L-[*ring*-$^{2}H_5$] phenylalanine enrichments increased transiently after protein ingestion (P<0.0001) and to a greater extent in ENR compared with MYCO (P-interaction ≤0.05). Specifically, plasma L-[*ring*-$^{2}H_5$]phenylalanine enrichments increased above postabsorptive levels for 30 min post protein ingestion in both groups (P<0.01). Plasma L-[*ring*-$^{2}H_5$]phenylalanine enrichments

were higher in ENR compared with MYCO at 120, 150, 210 and 240 min ($P \leq 0.05$). Plasma L-[3,3-$^2H_2$]tyrosine enrichments (Figure 4.4B**)** changed similarly in both groups over time ($P \leq 0.05$, *P*-interaction >0.05) but were significantly greater in the ENR compared with MYCO condition ($P \leq 0.05$). Plasma L-[*ring*-$^2H_4$]tyrosine enrichments (Figure 4.4C) increased over time ($P < 0.0001$) with no group differences or interaction effects.

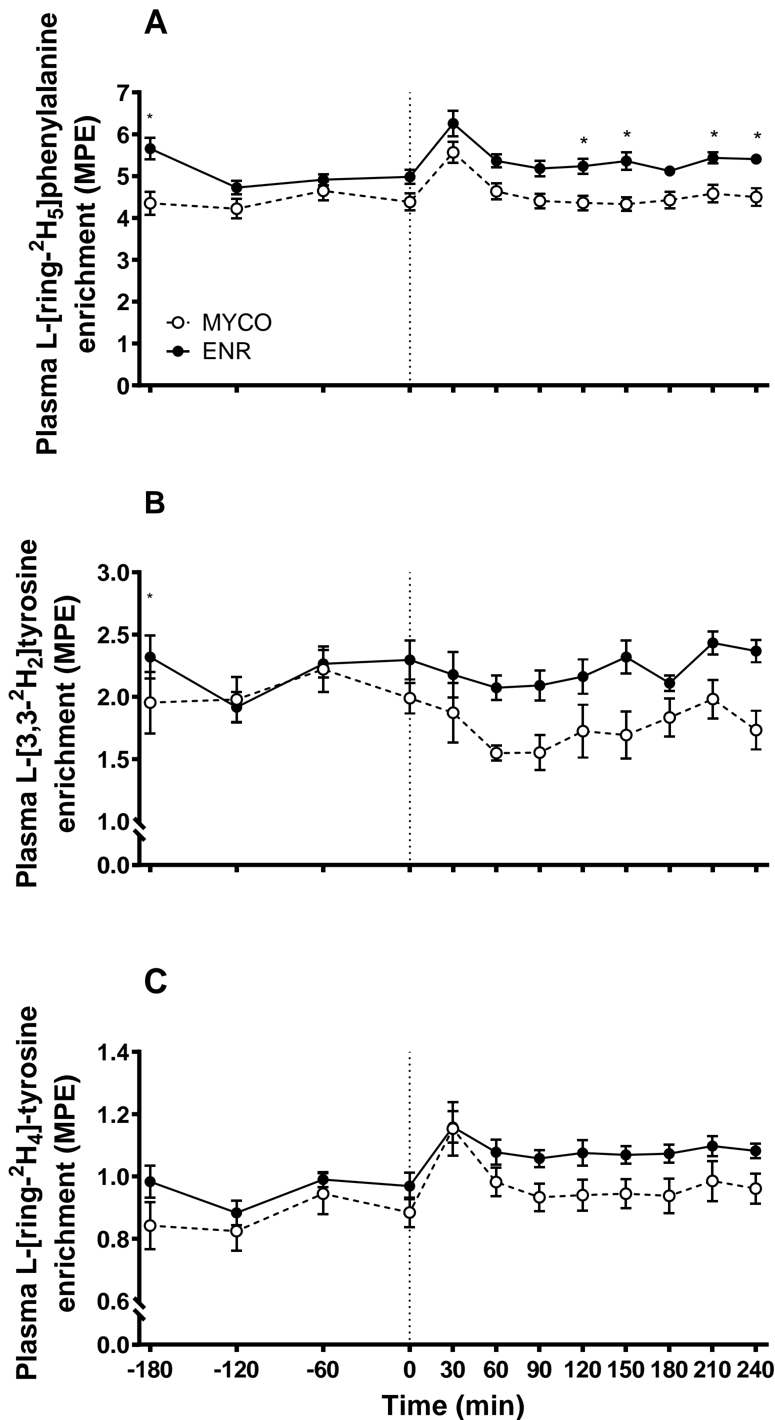

Figure 4.4. L-[ring-$^{2}H_{5}$]phenylalanine (A), L-[3,3-$^{2}H_{2}$]tyrosine (B), and L-[ring-$^{2}H_{4}$]tyrosine (C) enrichments during a stable isotope experimental test day in healthy young men.. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 31.5 g mycoprotein (MYCO; n=9) or 18.7 g mycoprotein enriched (to match MYCO) with BCAA (ENR; n=9), where a single bout of unilateral leg extension exercise was also performed. Values are mean ± SEM. *Means differ between conditions, ($P \le 0.05$). [b]Means differ from fasting (t = 0 min) for MYCO.

Phenylalanine hydroxylation rates **(Figure 4.5)** increased following protein ingestion (*P*<0.01), with rates increasing to a greater extent following the ingestion of MYCO compared with ENR (*P*-interaction <0.01). This resulted in phenylalanine hydroxylation rates being greater in MYCO (*P*≤0.05) specifically at 60, 210 and 240 min (*P*≤0.05).

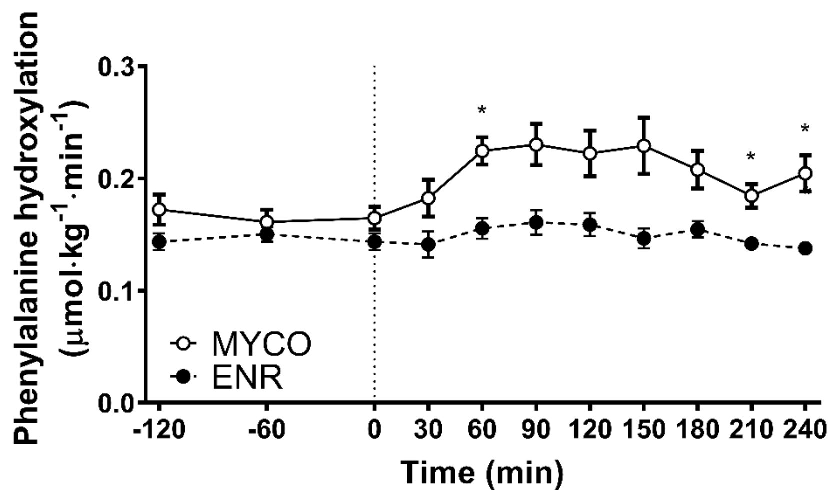

Figure 4.5. Phenylalanine hydroxylation during a 3 h postabsorptive period and a 4 h postprandial period in healthy young men. The vertical line indicates the transition from postabsorptive to postprandial conditions via the ingestion of 31.5 g mycoprotein (MYCO; n=9) or 18.7 g mycoprotein enriched (to match MYCO) with BCAA (ENR; n=9), where a single bout of unilateral leg extension exercise was also performed. Values are mean ± SEM. *Means differ between conditions, (P≤0.05).

Skeletal muscle tracer analyses

One participants' samples were excluded from the MYCO condition due to insufficient tissue resulting in the reporting of muscle data for *n*=9 in both conditions. Intracellular L-[*ring*-$^{2}H_{5}$]phenylalanine enrichments increased over time (*P*<0.01) with no differences between groups (treatment and *P*-interaction >0.05). Baseline mixed muscle protein-bound L-[*ring*-$^{2}H_{5}$]phenylalanine enrichments did not differ between conditions (*P* > 0.05). Mixed muscle

protein L-[*ring*-$^2H_5$]phenylalanine enrichments increased during the postabsorptive period in the rested leg (from 0.0034±0.0006 to 0.0058±0.0006 MPE in MYCO and 0.0032±0.0006 to 0.0065±0.0005 in ENR; P<0.0001) to the same extent in each group (treatment and P-interaction; both >0.05). Mixed muscle protein L-[*ring*-$^2H_5$]phenylalanine enrichments increased with protein ingestion (from 0.0058±0.0006 to 0.0167±0.0012 MPE in rested, and 0.0055±0.0006 to 0.0187±0.0011 MPE in exercised muscle in MYCO, and from 0.0065±0.0005 to 0.0161±0.0014 MPE in rested, and 0.0058±0.0006 to 0.0168±0.0013 MPE in exercised muscle in ENR) with a trend for a greater increase over time in MYCO (P-interaction =0.08).

Mixed muscle FSRs and the change from postabsorptive to postprandial periods, calculated using the mean plasma L-[*ring*-$^2H_5$]phenylalanine enrichment as the precursor pool, are displayed in **Figure 4.6**. Protein ingestion increased mixed muscle FSRs in rested and exercised muscle in both conditions (P<0.0001) with exercising muscle increasing to a greater extent (P-interaction ≤0.05). Mixed muscle FSRs increased from 0.025±0.006 to 0.057±0.004%$\cdot h^{-1}$ and 0.024±0.007 to 0.072±0.005%$\cdot h^{-1}$ in rested and exercised muscle, respectively, in MYCO (P<0.0001), and from 0.031±0.003 to 0.043±0.005%$\cdot h^{-1}$ and 0.027±0.005 to 0.052±0.005%$\cdot h^{-1}$ in rested and exercised muscle, respectively, in ENR (P<0.01). The increase in mixed muscle FSR was greater in the MYCO compared with ENR condition (P-interaction ≤0.05), with the divergence located as a difference in postprandial FSRs between conditions (P≤0.05). This divergence in postprandial FSR resulted in the delta postabsorptive to postprandial rises in mixed muscle FSR also being greater in MYCO compared with ENR (Δ 0.031±0.008 and 0.048±0.008%$\cdot h^{-1}$ in rested and exercised muscle, respectively, in MYCO, and Δ 0.013±0.005 and 0.025±0.009%$\cdot h^{-1}$ in rested and exercised muscle, respectively, in ENR; treatment and exercise effects; both P≤0.05).

The group differences became slightly less pronounced when comparing muscle FSRs derived from the L-[*ring*-$^2H_5$]phenylalanine intracellular precursor pool. Protein ingestion

increased FSR (P<0.0001) and there was a trend for this to be to a greater extent in MYCO compared with ENR (P-interaction =0.06). Exercise interacted with protein ingestion to increase FSR above that of the rested leg (P-interaction ≤0.05). Mixed muscle FSRs increased from to 0.031±0.007 to 0.070±0.006%·h^{-1} in rested, and 0.028±0.008 to 0.082±0.008%·h^{-1} in exercised muscle in MYCO, and from 0.040±0.010 to 0.060±0.007%·h^{-1} in rested, and 0.050±0.012 to 0.058±0.007%·h^{-1} in exercised muscle in ENR.

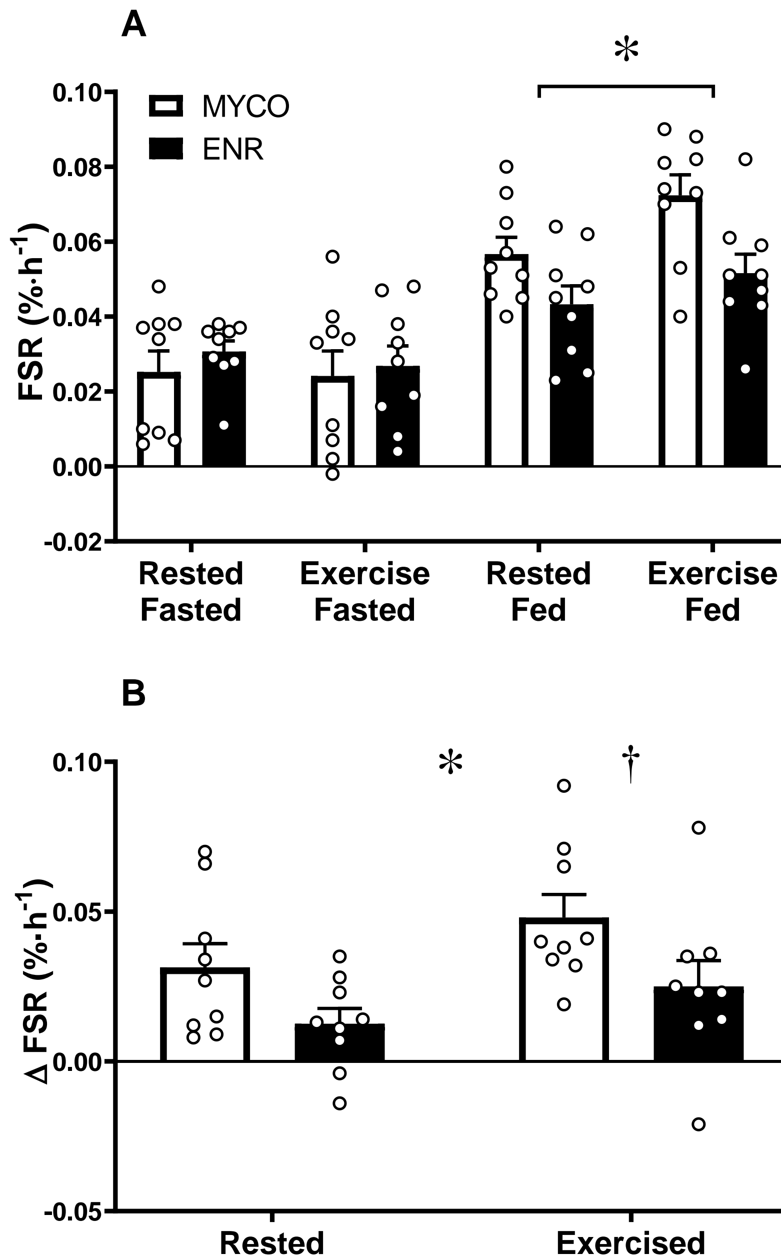

Figure 4.6. Mixed muscle protein fractional synthesis rates (FSRs; A) calculated from the plasma L-[*ring*-$^{2}H_{5}$]phenylalanine precursor pool in the postabsorptive (fasted) and postprandial (fed) state, in rested and exercised (single bout of unilateral concentric leg extensions) muscle in healthy young men. Postprandial state represents a 4 h period following via the ingestion of 31.5g mycoprotein (MYCO; n=9) or 18.7 g mycoprotein enriched (to match MYCO) with BCAA (ENR; n=9), where a single bout of unilateral leg extension exercise was also performed. The delta change in FSR in response to protein ingestion (B), representing the transition from postabsorptive to postprandial conditions in both groups is also presented. Values are mean ± SEM. *Means differ between conditions, (P≤0.05). Means differ between postabsorptive and postprandial rates, in both conditions. †Means differ between exercised and rested legs.

DISCUSSION

We assessed *in vivo* muscle protein synthetic responses to the ingestion of a large bolus of mycoprotein (MYCO; 70 g; containing 31 g protein) compared with a lower-dose branched chain amino acid (BCAA) fortified mycoprotein beverage (ENR; 35 g; containing 18.7 g protein and BCAA enriched to match the larger dose) within resting and exercised skeletal muscle of healthy and trained young men. Whilst the lower-dose enriched mycoprotein beverage robustly stimulated resting and post-exercise muscle protein synthesis (MPS) rates, it did so to a lesser extent compared with the ingestion of the larger bolus. This was observed despite a more rapid and greater increase (both peak magnitude and overall availability) in postprandial plasma concentrations of all three BCAAs following the ingestion of the lower-dose enriched mycoprotein beverage.

We have previously shown mycoprotein to be a sustainable non-animal-derived alternative protein source that, when consumed in a relatively large bolus (i.e. 70 g), is capable of robustly stimulating (post-exercise) MPS rates (Monteyne et al., 2020b). Previous work has suggested that, providing sufficient substrate (i.e. a full complement of necessary amino acids to support sustained postprandial incorporation within a polypeptide chain) is present from the ingestion of a complete protein source, BCAA content (or perhaps leucine alone) is the primary nutritional anabolic stimulus (Tang et al., 2009, Pennings et al., 2011b, Koopman et al., 2009a, Burd et al., 2012). Specifically, it has been proposed that the postprandial speed, magnitude and/or overall availability of BCAA or leucinemia following dietary protein ingestion is the key determinant that modulates the magnitude of the postprandial rise in MPS rates (Pennings et al., 2011b, Norton et al., 2009, Churchward-Venne et al., 2013, Koopman et al., 2009a, Burd et al., 2012, Tang et al., 2009). We therefore hypothesised that the ingestion of a lower-dose mycoprotein beverage fortified with crystalline BCAA to match the total BCAA content of a larger dose would result in comparable postprandial MPS rates. Predictably, due to the lack of requirement for

digestion of ~50% of the ingested BCAAs, we observed considerably more rapid intestinal BCAA absorption following ingestion of the lower-dose enriched beverage, demonstrated by quicker (and greater) elevations of plasma leucine, isoleucine and valine concentrations (Figure 4.3). Despite these divergent postprandial plasma amino acid profiles, the MPS response was greater following the ingestion of the larger-dose mycoprotein beverage (Figure 4.7A). Indeed, when expressing these data as the (delta) increase in MPS from postabsorptive to postprandial conditions, the response to the larger-dose mycoprotein beverage was more than double that of the lower-dose enriched beverage (Figure 4.7B). These data run contrary to our hypothesis that BCAA enriching the lower-dose would 'rescue' the MPS response, due to sufficient total amino acid substrate coupled with equivalent (or greater) BCAA stimulation of translation initiation compared with the larger dose. As a result, our data suggest that factors other than plasma BCAA or leucine responses alone drive postprandial MPS rates, a conclusion that is consistent with an increasing number of recent studies (van Vliet et al., 2017, Chan et al., 2019). Admittedly, the absence of a 35 g *non-enriched* mycoprotein condition precludes us demonstrating the extent to which (if any) the additional BCAAs augmented the MPS response to the lower dose. We speculate, however, that fortification would have augmented the response, given that the BCAA contents of the lower dose (i.e. 1.25, 0.77 and 0.95 g of leucine, isoleucine and valine, respectively) are below those previously reported (particularly leucine) to be necessary for optimal stimulation of MPS (Witard et al., 2013).

The most obvious explanation for our findings is simply that the larger-dose mycoprotein condition contained more total protein compared with the lower-dose enriched condition, and therefore provided more substrate (and/or non-BCAA amino acid signalling stimulus) to initiate or sustain the MPS response. Previous studies have shown a dose-response relationship exists with respect to protein consumed and consequent magnitude of the MPS response (Moore et al., 2008, Witard et al., 2013). Such work has shown that ingestion of

20 g dietary protein (egg or whey) maximally stimulates resting and post- (unilateral leg extension) exercise MPS rates, with amino acids available in excess of that seemingly directed primarily towards oxidation (Moore et al., 2008, Witard et al., 2013). Participants in the present work consumed marginally below (i.e. 18.7 g protein; ENR) and considerably above (i.e. 31 g; MYCO) this 20 g threshold. In line with the previous dose-response data (Witard et al., 2013, Moore et al., 2008), we observed a greater acceleration of whole-body amino acid oxidation in the larger-dose mycoprotein condition (Figure 4.6), though increased amino oxidation also occurred in response to the lower-dose enriched beverage, suggesting ample amino acid substrates for MPS were present under both conditions. However, despite identical BCAA ingestion across conditions, the larger-dose mycoprotein condition ingested 3.9 g more total essential amino acids, and 8.7 g more non-essential amino acids, leaving open the possibility that providing significantly more overall substrate for MPS played a role. This was illustrated by more pronounced postprandial plasma phenylalanine (essential) and tyrosine (non-essential) responses in the larger- compared with lower-dose conditions (see Figure 4.3). Substrate limitation may have been further exacerbated in the lower-dose condition as the co-ingestion of leucine has previously been shown to decrease circulating concentrations of other essential amino acids (Koopman et al., 2008). While non-essential amino acids have been reported not to offer any stimulatory effect on MPS rates (Tipton et al., 1999), we also cannot discount the possibility that signalling roles of other (non-BCAA) essential amino acids may explain the more anabolic effect of the larger-dose mycoprotein beverage. Conversely, despite mycoprotein representing a ‘complete/balanced’ protein source (Table 4.2; (Coelho et al., 2019)), such signalling (or substrate requirement) roles of specific (rather than total) non-BCAAs could also plausibly have limited the MPS response within the lower-dose enriched condition. A caveat to this interpretation, however, is that similar studies to the present work demonstrated parity in MPS responses between the ingestion of lower dose (1.5-6.25 g) BCAA (or leucine only) enriched protein (or EAA)

beverages compared with larger, more optimal doses (20-40 g) of complete proteins (e.g. whey protein) (Churchward-Venne et al., 2013, Churchward-Venne et al., 2012, Bukhari et al., 2015, Wilkinson et al., 2018).

An alternative perspective of the present data concerns the concept of mycoprotein representing a 'whole food' rather than an isolated protein source, and therefore containing a variety of non-protein macro- and micro- nutrients present within a unique food matrix. Additional carbohydrate, fat (or energy) in the larger-dose mycoprotein beverage are candidates for augmenting the MPS response. We observed a more pronounced postprandial circulating insulin response in the larger-dose mycoprotein condition compared with the lower-dose enriched mycoprotein condition (Figure 4.4), presumably due to the greater amount of carbohydrate present. Though insulin can directly activate the Akt/mTORC1/P70S6K signalling axis (Navé et al., 1999, Inoki et al., 2002, Manning et al., 2002, Menon et al., 2014) (the central myocellular anabolic signalling pathway (Drummond et al., 2009, Bodine, 2006)), data demonstrating that even modest (i.e. approx. 5 $mU \cdot L^{-1}$; below that already achieved in the low dose condition) insulin responses are sufficient to support maximal postprandial MPS rates (Greenhaff et al., 2008) makes this unlikely to be fully explanatory of the present findings. Indeed, carbohydrate co-ingestion does not augment the MPS response to protein ingestion (Gorissen et al., 2014, Hamer et al., 2013, Staples et al., 2011). Furthermore, fat co-ingestion also does not potentiate postprandial MPS rates following protein ingestion (Gorissen et al., 2017), collectively making the greater energy content *per se* in the larger-dose mycoprotein condition an unsatisfactory explanation. More intriguing perhaps, we (Monteyne et al., 2020b) and others (van Vliet et al., 2017) have recently proposed that protein ingested as part of a whole food may potentiate the MPS response compared with isolated proteins. Specific mechanisms that may underpin an augmented anabolic effect of whole food ingestion remain to be elucidated, although hypotheses exist around signalling roles for specific lipids (Castellano et al., 2017,

Yasuda et al., 2014, Abou Sawan et al., 2018b, Elliot et al., 2006) and/or micronutrients within the whole food matrix (Salles et al., 2013, Zhao et al., 2016, Marzani et al., 2008), or an interaction of multiple such factors (Abou Sawan et al., 2018b). In the present work we provided *more* of a whole food in the larger-dose condition, and therefore any impact of whole food or food matrices on MPS would presumably have been augmented. Furthermore, such an interpretation would also explain the apparent discrepancy between our results and those studies where MPS responses of lower-dose isolated protein sources were rescued with BCAA or leucine fortification (Churchward-Venne et al., 2013, Churchward-Venne et al., 2012, Bukhari et al., 2015, Wilkinson et al., 2018).

In the current study we measured MPS rates in both rested and exercised muscle, as achieving *optimal* postprandial muscle protein accretion (at least in young adults) is perhaps most relevant to those seeking to maximise muscular adaptations during prolonged resistance exercise training. We observed the typical augmentation of postprandial MPS rates following resistance exercise (Phillips et al., 1997), but the greater MPS response of larger-dose mycoprotein condition compared with the lower-dose enriched mycoprotein condition was present irrespective of prior exercise. Our data therefore imply that chronic mycoprotein consumption represents a viable non-animal-derived protein source to support longer-term muscle tissue remodelling. However, our data also suggest that consuming larger amounts of mycoprotein per meal may be required to facilitate *optimal* adaptive responses (Monteyne et al., 2020b). This may be problematic in individuals with concerns regarding appetite and/or energy intake (e.g. weight loss, periods of muscle disuse or older adults), which may even be exacerbated by the satiating properties of mycoprotein (Burley et al., 1993, Bottin et al., 2016, Williamson et al., 2006, Turnbull et al., 1993). In such instances, consuming a lower dose of mycoprotein enriched with BCAAs may therefore represent a pragmatic approach. Future work should establish the translatability of consuming larger boluses of mycoprotein to longer-term tissue remodelling outcomes and

alternative strategies to augment the MPS response to reduced doses in more compromised individuals.

In conclusion, a lower-dose BCAA enriched mycoprotein beverage stimulates resting and post-exercise MPS rates in healthy young men, but to a lesser extent compared with a larger (BCAA matched) bolus of mycoprotein. Given these greater postprandial MPS responses to the larger mycoprotein bolus occurred despite lower and slower rises in plasma BCAA concentrations, we propose plasma BCAA or leucine concentrations are not solely responsible for regulating postprandial MPS rates. While the BCAA fortification of a lower-dose mycoprotein beverage results in a clear stimulation of MPS upon ingestion, it does not fully compensate for the anabolic capacity of consuming a larger bolus of mycoprotein.

CHAPTER 5

A MYCOPROTEIN BASED HIGH-PROTEIN VEGAN DIET SUPPORTS EQUIVALENT DAILY MYOFIBRILLAR PROTEIN SYNTHESIS RATES COMPARED WITH AN ISONITROGENOUS OMNIVOROUS DIET IN OLDER ADULTS.

The work contained within this chapter is published:

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Abstract

Background: Animal-derived dietary protein ingestion and physical activity stimulate myofibrillar protein synthesis rates in older adults.

Objective: To determine whether a non-animal-derived diet can support daily myofibrillar protein synthesis rates to the same extent as an omnivorous diet.

Methods: Nineteen healthy older adults (age 66±1 y; BMI 24±1 $kg\cdot m^{-2}$; m=12, *f*=7) participated in a randomised, parallel-group, controlled trial during which they consumed a 3-day isocaloric high-protein (1.8 g·kg body $mass^{-1}\cdot d^{-1}$) diet, where the protein was provided from predominantly (71%) animal (OMNI; *n*=9; m=6, *f*=3) or exclusively vegan (VEG; *n*=10; m=6, *f*=4; mycoprotein providing 57% of daily protein intake) sources. During the dietary control period participants conducted a daily bout of unilateral resistance-type leg extension exercise. Prior to the dietary control period participants ingested 400 mL deuterated water, with 50 mL doses consumed daily thereafter. Saliva samples were collected throughout to determine body water deuterium (^2H) enrichments, and muscle samples were collected from rested and exercised muscle to determine daily myofibrillar protein synthesis rates.

Results: Deuterated water dosing resulted in body water ^2H enrichments of ~0.78±0.03%. Daily myofibrillar protein synthesis rates were 13±8 (P=0.169) and 12±4% (P=0.016) greater in the exercised compared with rested leg (1.59±0.12 vs 1.77±0.12%$\cdot d^{-1}$ and 1.76±0.14 vs 1.93±0.12%$\cdot d^{-1}$) in OMNI and VEG groups, respectively. Daily myofibrillar protein synthesis rates did not differ between OMNI and VEG in either rested or exercised muscle (P>0.05).

Conclusion: Over the course of a three-day intervention, omnivorous or vegan derived dietary protein sources can support equivalent rested and exercised daily myofibrillar protein synthesis rates in healthy older adults consuming a high-protein diet.

INTRODUCTION

Ageing is associated with a progressive loss of skeletal muscle mass, termed sarcopenia (Evans, 1995a). The association between muscle loss and increased incidence of falls, fractures, and metabolic disease indicates that the burden of our ageing society on health-care systems will increase dramatically in the coming decades (WHO, 2008, Evans, 1995a, Janssen et al., 2004, Sousa et al., 2016). Importantly, it also underlines the critical role that muscle mass and quality play in healthy ageing.

Muscle mass is regulated by the dynamic balance between daily muscle protein synthesis (MPS) and breakdown (MPB) rates. Ageing muscle displays a blunted responsiveness to the major acute anabolic stimuli; dietary protein ingestion (Wall et al., 2016a, Cuthbertson et al., 2005, Guillet et al., 2004, Katsanos et al., 2005a) and physical activity (particularly resistance exercise) (Kumar et al., 2009, Fry et al., 2011a). This 'anabolic resistance' is now generally accepted as a key physiological mechanism responsible for age-related sarcopenia (Wall et al., 2014a). Consuming dietary protein and performing physical activity in close temporal proximity can synergistically augment muscle protein synthesis rates in older adults (Pennings et al., 2011c, Holwerda et al., 2016). Moreover, by increasing the per meal dose of protein (Pennings et al., 2012a, Moore et al., 2014a, Yang et al., 2012b), consuming high quality or fortified protein sources (Pennings et al., 2011a, Wall et al., 2013a), or strategic timing of protein ingestion (Kouw et al., 2017), the muscle protein synthesis response to each meal can be augmented, though how this translates to daily muscle protein synthesis rates has received less attention.

Our understanding of how dietary protein and physical activity regulate muscle protein turnover in older adults is largely derived from studies using animal-derived protein sources. Indeed, studies evaluating the anabolic potential of animal versus non-animal-derived proteins typically compare meat, milk, casein and/or whey with soy (Tang et al., 2009) or wheat (Gorissen et al., 2016a, Yang et al., 2012b) proteins only. Despite limited non-animal

protein sources having been investigated, there is the widespread assumption that animal-derived proteins are more anabolic compared with plant-based proteins (van Vliet et al., 2015a). Mycoprotein is a sustainably produced protein-rich whole food source, cultivated by the continuous flow fermentation of the filamentous fungus Fusarium venenatum, that is relatively high in protein (45% protein, 20.9% EAA, 24.6% NEAA, 9% BCAA, 3.9% leucine), high in fiber (25%; two-thirds b-glucan and one-third chitin), and with a relatively low-energy-density (Finnigan, 2011). A full description of the nutritional properties of mycoprotein can be found in the review by Coelho and colleagues (2019) (Coelho et al., 2019). We recently reported that an ingested bolus of mycoprotein is effectively digested and its amino acids absorbed (Dunlop et al., 2017), which results in a robust stimulation of muscle protein synthesis rates in rested and exercised muscle of young men (Monteyne et al., 2020b). This suggests that mycoprotein may be a suitable alternative to animal or plant derived proteins to incorporate within the diet of older adults to support daily muscle protein synthesis rates.

In the present work, we applied an oral deuterated water approach (Kilroe et al., 2019) to determine whether a mycoprotein-based high-protein vegan diet could support rested and exercised daily muscle protein synthesis in older adults to the same extent as an isonitrogenous omnivorous protein diet. We hypothesised that in older adults consuming a high-protein diet, exercise would increase daily muscle protein synthesis rates compared with rested muscle, and by a similar extent irrespective of whether dietary protein was primarily obtained from animal or non-animal sources.

METHODS

Participants

Nineteen healthy older adults (age: 66±1 y, BMI: 24±1 kg·m^{-2}, 7 f and 12 m) were included in the study, and their characteristics are presented in **Table 5.1**. Participants (within the age range of 55-75 y) attended the laboratory for a medical screening, where height, body mass and blood pressure were measured, a fasting venous blood sample collected, and a general medical questionnaire was completed, all to assess their eligibility for participation and to ensure no adverse health conditions were present. Exclusion criteria included; a (family) history of deep vein thrombosis/cardiovascular disease, metabolic disorders (e.g. type-2 diabetes), musculoskeletal/orthopedic disorders, a body mass index of above 30 kg·m^{-2} or below 18 kg·m^{-2}, hypertension (defined as >150/90 mmHg), participation in a structured resistance training programme within 6 months prior to the study, any musculoskeletal injury of the legs within 12 months prior to the study, habitual use of anticoagulants, and consumption of any nutritional supplement shortly prior to the study. Blood markers were screened to exclude any participants who displayed evidence of impaired renal function, blood/clotting, autoimmune disorders or high glycated haemoglobin. Overall 34 participants were screened of which 1 was excluded based on the above criteria, and 14 either did not take part or did not complete the study. Participants completed the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003a), and were provided with a diet diary to record habitual nutritional intake for 3 days (two weekdays and one weekend day). Detailed instructions from a member of the research team were provided to assist participants in collecting these data. Dietary analyses for the calculation of energy and macronutrient intakes were completed using specialised nutrition software (Nutritics Professional Nutritional Analysis Software; Swords, Co. Dublin). All subjects were informed of the nature and possible risks of the experimental procedures before providing written informed consent. This study was registered as a clinical trial with clinicaltrials.gov

(NCT04325178). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Sport and Health Sciences Ethics committee of the University of Exeter (180314/B/01).

Pre-testing

Following screening and admittance, all participants underwent a single pre-testing session, which took place ≥5 days before the start of the experimental period. Participants reported to the laboratory for familiarisation with the exercise equipment and unilateral resistance-type exercise protocol to be used, and to determine body composition (using Air Displacement Plethysmography; BodPod, Life Measurement, Inc. Concord, CA, USA). The exercise protocol consisted of 5 sets of 30 repetitions of maximal concentric isokinetic leg extension contractions, with 90 seconds rest between each set, on a Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, New York, USA) at a speed of 60° per second over a central 80° range of motion using their dominant leg. The dominant leg was selected as we deemed it likely that they would be better able to execute contractions in a leg that they were more accustomed to loading, and therefore create more mechanical tension (Tang et al., 2008). The exercise protocol was selected as we have previously shown that it stimulates muscle protein synthesis rates (Monteyne et al., 2020a), allowing us to model the effect on rested tissue alongside exercised tissue. As the exercise is maximally concentric in nature, we would expect it to largely obviate any muscle damage, and therefore represent a protein accretional stimulus as opposed to one directed at correcting muscle damage (Damas et al., 2016). In addition, the lack of muscle damage permitted maximal exercise on three consecutive mornings, which was practical for the population in question, and allowed us to stimulate cumulative myofibrillar protein synthesis rates over the three days. Verbal encouragement was provided throughout the familiarisation and experimental testing to encourage maximal effort. Work done (J) was

recorded for each completed set, and fatigue was calculated as the percentage decrement in work done between the first and last set.

Experimental design

Participants were randomly assigned to two parallel-groups, A or B, by the lead investigator and completed a single condition, unless their habitual dietary choices (i.e. vegan) precluded their inclusion in the group that would receive the omnivorous diet The study was an open-label design, with it not practically possible to blind the participants or the investigators. We took a theoretically optimal approach to stimulating daily muscle protein synthesis rates, providing a high-protein diet distributed throughout the day, and with protein consumed post resistance exercise (Holwerda et al., 2018a, Murphy et al., 2015, Mamerow et al., 2014b). This necessitated that we clamped both protein and energy intake across groups. Participants received one of two dietary interventions which differed with respect to the primary sources of dietary protein consumed: predominantly animal-derived protein sources including milk protein supplementation (OMNI; n=9), or exclusively vegan-derived protein sources including mycoprotein supplementation (VEG; n=10). Three participants were vegetarian, and were therefore allocated to the VEG group. A graphical representation of the experimental study design can be seen in **Figure 5.1**. All participants were asked to refrain from alcohol and caffeine consumption, and from strenuous exercise (except for the exercise prescribed within the protocol) for 2 days prior and throughout the experimental protocol, though to keep all other daily habitual activities as normal.

Participants attended the laboratory each day for a 5-day (Mon-Fri) experimental period with days 2-4 (i.e. 3 days, Tue-Thu, inclusive) involving the dietary control. Each day of the 3-day dietary control period participants attended the laboratory to conduct a single bout of unilateral leg extension exercise as described above. Immediately afterwards volunteers received a protein-rich breakfast (~20 g protein), and were then provided with their food for

the remainder of the day. To measure daily myofibrillar protein synthesis rates participants underwent a deuterium oxide dosing protocol (described below), in line with our previous work (Kilroe et al., 2019), and muscle biopsies were collected before commencing the controlled diet (i.e. Tue ~0800 h; single muscle biopsy from the [to-be] rested leg) and following (i.e. Fri ~0800 h; bilateral biopsies from the rested and exercised legs). We chose a duration of three days to reduce the burden on participants, in terms of sampling and dietary imposition, whilst also remaining confident that this allowed sufficient time to robustly measure daily muscle protein synthesis rates (Kilroe et al., 2020c) and detect differences between groups.

Muscle biopsies were obtained under local anaesthesia, using a percutaneous Bergstrom biopsy needle technique (6), from the *m. vastus lateralis* ~15 cm above the patella and ~3 cm below the fascia. Muscle tissue was quickly assessed and any blood or non-muscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

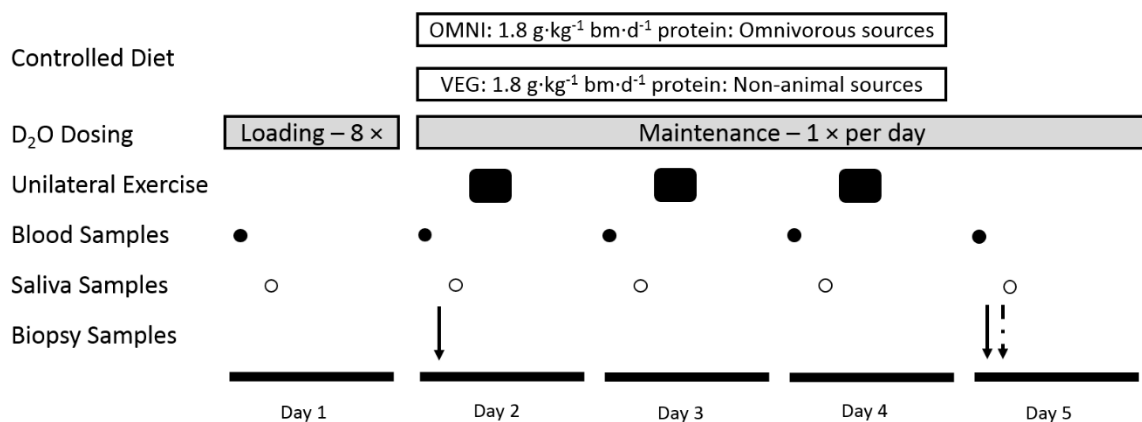

Figure 5.1. Schematic representation of the experimental protocol. Nineteen healthy older adults (age, 66±1 y) consumed a 3-day fully controlled, eucaloric and high-protein (1.8 g·kg body mass^{-1}·d^{-1}) diet, where the protein was provided predominantly from animal (OMNI; *n*=9) or exclusively non-animal (VEG; *n*=10) sources. During the dietary control period (Days 2-4) participants conducted a single bout of unilateral isokinetic knee extension exercise (5 x 30 contractions) each morning. On Day 1 participants loaded 400 mL deuterated water,

with 50 mL maintenance doses consumed daily thereafter. Saliva (open circles) and blood samples (closed circles) were collected daily, and muscle biopsies were collected from both the rested (straight arrow) and exercised (dashed arrow) legs to determine daily myofibrillar protein synthesis rates.

Dietary intervention

Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (Henry, 2005a). The IPAQ was used to calculate a physical activity level (PAL) factor (Westerterp, 1999). Individual energy requirements were then calculated by multiplying the participant's BMR by their PAL factor. Thereafter, an individual 3-day meal plan was designed for each participant with all food prepared, weighed, and packaged in-house in the Nutritional Physiology Unit's research kitchen facility. Nutritional information for the two diets is provided in **Table 5.2 and 5.3**. Subjects consumed a diet containing 1.8 g of protein per kg of body mass (bm) per day ($g \cdot kg\ bm^{-1} \cdot d^{-1}$, with 23-26% of energy being provided by fat and 49-54% from carbohydrates in OMNI, and with 23-28% and 42-52% of energy being provided by fat and carbohydrates, respectively, in VEG (variation due to different energy requirements and the matching of protein intake). The meals were identical between the two groups, aside from meat or dairy providing the primary protein source in lunches and dinners for the OMNI group and this being replaced by Quorn Foods™ mycoprotein containing products or supplementary mycoprotein in the VEG group (provided by Marlow Foods Ltd, Stokesly, North Yorkshire, UK). The OMNI group received 39 g supplemental milk protein daily (31 g protein, 2 g carbohydrate, <1 g fat, 548 kJ (131 kcal)) to drink prior to sleep, which was replaced with 70 g of supplementary mycoprotein (31 g protein, 7 g carbohydrate, 9 g fat, 971 kJ (232 kcal)) to drink in the VEG group. A small amount of mycoprotein was also added to the breakfast in the VEG group to more closely equate the protein in the breakfast meal across groups. Breakfast was consumed within 1 hour of completing the unilateral resistance-type exercise, and provided 19±1 and 21±1 g

protein per day in OMNI and VEG, respectively. The OMNI group consumed meals based on chicken, pork, and dairy. In the VEG group, this was substituted for Quorn deli ham, Quorn pieces, Quorn burgers, Quorn BBQ strips, Quorn sausages, and Quorn nuggets from their vegan range (which does not contain any egg products). A document and diary detailing the plan were provided to the subjects to log mealtimes and provide recipe information/instructions. Compliance to the intervention was ascertained verbally on each morning of the intervention during a detailed discussion with the researcher. There were no major deviations from the diet and no major incidents of GI distress reported by participants who completed the study. Three out of the four participants who withdrew from the intervention (all in VEG) did so because they disliked the diet with one citing bloating as the primary reason. Participants body mass was measured wearing light clothing at the start and end of the three-day control diet period (seca 703 column scale, seca GmbH & Co. KG, Hamburg, Germany). Each morning the researchers discussed with the participants any questions or issues that may have arisen, before the next day of food was provided.

Deuterated water dosing protocol

The deuterated water dosing protocol was based on our previous work (Kilroe et al., 2019). Day 1 (Mon) of the experimental protocol acted as a D_2O loading day where participants consumed 400 mL of 70% D_2O separated over the day as 8 x 50 mL boluses (CK Isotopes Ltd, Leicestershire, UK). Upon arrival at the laboratory (0730 h) background saliva samples were collected before the first bolus of D_2O was ingested. The first dose of D_2O was consumed at ~0800 h with the remaining loading day doses being consumed every 1 hour (doses 2 and 3) and then every 1.5 h thereafter. Participants stayed at the laboratory until 4 out of the 8 loading day D_2O doses had been consumed, with the remaining D_2O doses being consumed at home under instruction of timings (i.e. leaving 1.5 h between each). Every day following the loading day participants consumed a maintenance dose of D_2O (50 mL) upon waking (~0800 h). A small proportion of participants reported mild feelings of

dizziness during the latter part of the loading day, which subsided by the following morning (4/19). At least 90 minutes (~09:30 h) after the daily D_2O maintenance dose a daily saliva sample was collected using a cotton mouth swab (Celluron, Hartmann, Germany) which the participant lightly chewed for ~1 min until saturated with saliva. The saturated sponge was placed into an empty syringe where the swab was squeezed to release the saliva into a collection tube, and stored at -80°C until further analysis. The saliva samples were used to assess the body water ^2H enrichment. To ensure uniformity and compliance with the D_2O protocol, participants were provided with bottles of D_2O labelled with the specific time and date to be taken, and were required to return bottles each day.

Body water deuterium enrichment

Body water deuterium enrichment was measured using the saliva samples collected daily throughout the study, at the University of Texas Medical Branch. A ThermoFisher Delta V Advantage Isotope Ratio mass spectrometer (IRMS) (Bremen, Germany), equipped with a Finnigan GasBench II (Thermo Fisher Scientific, Waltham, MA, USA), was used for stable hydrogen isotope ratio measurements. After uncapping a 12-mL Exetainer (Labco Limited, Lampeter, UK), 5 mg of activated charcoal (Thermo Fisher Scientific) and 200 mg of copper powder (Thermo Fisher Scientific) were introduced into the Exetainer followed with a platinum catalytic rod (Thermo Fisher Scientific). The activated charcoal and copper powder were added to remove any potential contaminants in the samples that might poison the platinum catalyst. After putting 200uL of sample into the Exetainer, the Exetainer was recapped and placed into the GasBench II and flushed with 2% H2 in helium for 7 min. The sample was allowed to equilibrate with the flushed gas at room temperature (~4-6 hours). At the end of the equilibration, an aliquot of the headspace in the Exetainer was injected into the Thermofisher Delta V Advantage mass spectrometer system for stable hydrogen isotope ratio measurement against the reference gas H2. A standard calibration curve was prepared using 99.9% deuterium-enriched water (Sigma-Aldrich, St. Louis, MO, USA), and the

deuterium (^2H) enrichment in duplicate saliva samples was determined (Wong and Clarke, 2012).

Myofibrillar bound ^2H alanine enrichments

Myofibrillar protein-enriched fraction was extracted from ~50 mg of wet weight muscle tissue by hand-homogenisation on ice using a pestle in a standard homogenisation buffer (TRIS-HCL 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerophosphate 10 mM, NaF 50 mM, activated sodium orthovanadate 0.5mM, cOmplete protease inhibitor cocktail tablet (Roche Holding AG, Basel, Switzerland)) (7.5 µL/mg). The samples were centrifuged at 2,200 *g* for 10 min at 4°C, the pellet was then washed with 500 µl of homogenisation buffer, and centrifuged at 700 *g* for 10 min at 4°C. The myofibrillar protein was solubilised by adding 750 µl of 0.3 M NaOH and heating for 30 min at 50°C with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 10,000 *g* and 4°C, the supernatant containing the myofibrillar protein was kept and the collagen protein pellet was discarded. The myofibrillar proteins were precipitated by the addition of 500 µL of 1 M PCA and centrifuged at 700 *g* and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolysed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids from the hydrolysed myofibrillar protein pellet were dried under vacuum with a Speed-Vac rotary dryer (Savant Instruments, Farmingdale, NY, USA) for 3 h at 80°C radiant cover. The free amino acids were subsequently dissolved in 1.5 mL 25% acetic acid solution and passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA) and eluted with 6 M NH_4OH. Following this, the purified amino acids were dried and derivatised to tert-butyldimethylsilyl derivatives via the addition of 50 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 50 µl acetonitrile, which was then vortex mixed and heated at 95°C for 40 min. The samples were transferred to a GC vial. The level of enrichment of D4-alanine was analysed using a ThermoFisher Delta V Advantage Isotope Ratio mass spectrometer (IRMS) fitted with a Trace 1310 GC with an

on-line high-temperature thermal conversion oven (HTC) at 1420°C. The sample (1µl) was injected in splitless mode at an injection port temperature of 250°C. The peaks were resolved on a 30m × 0.25mm ID × 0.25µm film Agilent Technologies DB-5 capillary column (temperature programme: 110°C for 1 min; 10°C·min^{-1} ramp to 180°C; 5°C·min^{-1} ramp to 220°C; 20°C·min^{-1} ramp to 300°C; hold for 2 min) prior to pyrolysis. Helium was used as the carrier gas with a constant flow of 1ml/min. Any amino acid eluting from the gas chromatograph was converted to H2 before entry into the IRMS. The enrichment of the alanine tracer was measured by monitoring the Ion masses 2 and 3 to determine the 2H/1H ratios in the samples and referenced to the calibration curve. The calibration curve consisted of a series of known concentrations of d4-alanine and was applied to assess both the linearity of the mass spectrometer and to control for the loss of tracer. The isotopic abundances were expressed as the delta notation, δ^2H per mil (‰) deviation from VSMOW (Vienna Standard Mean Ocean Water) standard (Wong and Clarke, 2012). Values of delta per mil given by the IRMS were transformed into MPE.

Calculations

Myofibrillar fractional synthesis rates (FSR) were calculated based on the incorporation of the mean body water deuterium enrichment over the 3-day intervention as a precursor pool into myofibrillar bound proteins. Ourselves and others have previously shown the body water deuterium pool is a valid precursor pool for the calculation of myofibrillar protein synthesis rates (corrected by a factor of 3.7 based on deuterium labelling of alanine during *de novo* synthesis) which shows excellent agreement with either plasma or muscle free [$^2H_4$] alanine as alternative precursor pool selections (Dufner et al., 2005, Kilroe et al., 2019, Holwerda et al., 2018a). FSR was calculated using the standard precursor-product method and expressed as daily rates as follows:

$$FSR\ (\% \cdot \text{day}^{-1}) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor}\ x\ t}\right) x\ 100$$

where E_{m1} and E_{m2} are the myofibrillar muscle protein-bound enrichments pre- (one leg only) and post- (either the rested or exercised leg) the dietary intervention. $E_{precursor}$ represents mean body water deuterium enrichment corrected by a factor of 3.7. t represents the time between biopsies (i.e. 3 days).

Statistics

A two-sided power analysis based on previous research (Holwerda et al., 2018a) showed that n=8 per groups was sufficient to detect expected differences in myofibrillar protein synthesis rates between rested and exercised legs when using a two-factor ANOVA ($P < 0.05$, 95% power, f=1.68; G*power version 3.1.9.2). Our primary measure was myofibrillar protein synthesis rates, with all other measures representing secondary measures. All data are presented as mean ± SEM and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). An independent samples *t*-test was used to compare each of the participants' characteristics across groups. A two-way (OMNI vs VEG and time [pre-post]) ANOVA was used to compare body mass during the nutritional intervention. A two-way (OMNI vs VEG and time [days 1-4]) ANOVA was used to compare changes in body water deuterium enrichments. A three-way (OMNI vs VEG, pre vs post and rested vs exercised leg) mixed-effects model was used to compare myofibrillar protein-bound [^{2}H] alanine enrichments. A two-way (OMNI vs VEG and rested vs exercised leg) ANOVA, independent samples *t*-tests, and paired *t*-tests were used to compare myofibrillar protein synthesis rates. When a significant interaction was found, Sidak or Tukey *post hoc* tests were applied to locate individual differences. Statistical significance was set at $P<0.05$.

RESULTS

Participants' characteristics and daily exercise protocol

No differences in age, weight, height, BMI, or body composition were detected between groups (all *P*>0.05) and groups were also well balanced for sex (Table 5.1). Data relating to work done during the exercise protocol are displayed in Table 5.1. No differences in total work performed during the experimental resistance exercise bouts (32,547±3,450 J in OMNI vs 27,851±2,809 J in VEG; *P*=0.302), or in fatigue during each trial (all *P*>0.05) or over the week (*P*=0.392) were detected between groups. Total work done was lower on day 2 than day 3 (*P*=0.026), with no differences detected between days 1 and 2 (*P*=0.503), or 1 and 3 (*P*=0.934). Body mass did not change in response to the nutritional intervention in either group (*P*=0.703 and *P*=*0*.175 in OMNI and VEG, respectively).

Table 5.1. Participant characteristics and work done during three consecutive days of unilateral resistance-type exercise (15 sets of 30 maximal isokinetic extension contractions).

	OMNI (*n* = 9) *3♀ / 6♂*	**VEG** (*n* = 10) *4♀ / 6♂*	***P* Value**
Age (y)	64±2	68±2	*P=0.21*
Body mass (kg)	70±3	69±3	*P=0.86*
Height (cm)	172±3	166±3	*P=0.17*
BMI (kg·m^{-2})	23.6±0.6	25.1±0.7	*P=0.16*
Fat (% body mass)	20±3	25±3	*P=0.27*
Lean mass (kg)	56±4	52±4	*P=0.45*
Total work done (J)	32,457±3,450	26,657±2,809	*P=0.30*

Values represent mean ± SEM. OMNI, omnivorous diet; VEG, non-animal-derived diet.

Nutritional intervention

Habitual diet did not differ between groups for energy intake, protein intake or carbohydrate intake (all *P*>0.05), although fat intake was higher in the OMNI group than VEG (*P*=0.039; Table 5.2). Energy intake did not change between participants' habitual diets and the diet they received during the intervention (*P*=0.142). Daily protein intake was higher (by design) during the intervention diet than in participants' habitual diets (1.8±0.0 vs 1.2±0.1 g.day^{-1}, respectively; *P*<0.0001). Daily carbohydrate intake was also higher during the intervention diet than in participants' habitual diets (*P*=0.037). Time and interaction effects were detected (both *P*<0.05) such that fat intake decreased from habitual levels during the intervention diet in the OMNI group only (*P*<0.0001).

During the intervention diet participants consumed 10±0.6 and 9.6±0.6 MJ (0.14±0.00 and 0.14±0.00 MJ/kg; 2382±139 and 2296±137 kcal) per day in OMNI and VEG, respectively, with no differences between groups (*P*=0.667). By design, daily protein intake (i.e. 127±5 and 125±5 g per day, respectively) was identical between groups during the intervention. Participants consumed 305±20 and 274±20 g carbohydrate, and 65±4 and 67±4 g fat per day in OMNI and VEG, respectively, with no differences between groups (both *P*>0.05). Fibre intake was higher in the VEG intervention diet than the OMNI intervention diet (68±3 g vs 32±2 g; *P*<0.0001) as a result of the high natural fibre content of mycoprotein (Table 5.3).

In VEG, of the 125±6 g protein consumed per day, 71±2 g was derived from mycoprotein (30±2 g from mycoprotein within 329±23 g of Quorn products, and 41±1 g protein from supplementary isolated mycoprotein) corresponding to 57±1% total protein intake. Remaining protein was provided by wheat and potato protein in Quorn products, and from the protein present in the other elements of the diet. Overall, Quorn products provided 50±3 g daily protein and accounted for 39±2% total daily protein intake.

In OMNI, of the 127±5 g protein consumed per day, 90±3 g was provided by animal-derived sources and 38±3 g from non-animal sources, corresponding to 71±1% and 29±1% from animal and non-animal-derived sources, respectively. Meat products provided 40±0 g and dairy products (including the milk protein supplement) provided 50±3 g protein per day, corresponding to 32±1 and 39±1% of total protein, respectively. The milk protein supplement alone provided 31±0 g protein per day, 25±1% of total protein.

Table 5.2. The nutritional content of the participants' habitual diets and of the intervention diets. In OMNI, participants consumed an omnivorous diet with the majority of their protein coming from animal-derived sources. In VEG, participants consumed a diet derived from non-animal sources with the majority of their protein coming from Quorn products and mycoprotein. Both groups received 1.8 g·kg bm^{-1}·day^{-1} protein.

	OMNI	**VEG**	***P* Value**
	(*n* = 9) *3♀ / 6♂*	(*n* = 10) *4♀ / 6♂*	
Habitual Diet			
Energy (MJ·day^{-1} (kcal·day^{-1}))	9.7±0.7 (2,314±164)	8.2±0.6 (1,949±145)	*0.12*
Protein (g·day^{-1})	81±6	82±6	*0.90*
Protein (g·kg bm^{-1}·day^{-1})	1.17±0.08	1.27±0.10	*0.42*
Carbohydrate (g·day^{-1})	264±32	222±20	*0.31*
Fat (g·day^{-1})	94±6	72±8	*0.04**
Fibre (g·day^{-1})	27±2	31±2	*0.22*
Intervention Diet			
Energy (MJ·day^{-1} (kcal·day^{-1}))	10±0.6 (2,382±139)	9.6±0.6 (2,296±136)	*0.67*
Protein (g·day^{-1})	127±5	125±6	*0.79*
Protein (g·kg bm^{-1}·day^{-1})	1.8-±0.00	1.8-±0.00	*N/A*
Carbohydrate (g·day^{-1})	305±20	274±20	*0.28*
Fat (g·day^{-1})	65±4	67±4	*0.60*
Fibre (g·day^{-1})	32±2	68±3	*<0.0001**

Values represent mean ± SEM. OMNI, omnivorous diet; VEG, non-animal-derived diet. * indicates a difference between groups *(P*<0.05*).*

Table 5.3. Dietary intake, meal-by-meal, during the intervention. In OMNI, participants consumed an omnivorous diet with the majority of their protein coming from animal-derived sources. In VEG, participants consumed a diet derived from non-animal sources with the majority of their protein coming from Quorn products and mycoprotein. Both groups received 1.8 g·kg bm^{-1}·day^{-1} protein.

	Energy		Protein		CHO		Fat		Fibre
	kcal	**kcal/kg**	**g**	**g/kg**	**g**	**g/kg**	**g**	**g/kg**	**g**
OMNI									
Breakfast	500 ± 27	7.1 ± 0.3	19 ± 1	0.3 ± 0.0	75 ± 4	1.1 ± 0.0	11 ± 1	0.2 ± 0.0	11 ± 1
Lunch	493 ± 35	6.9 ± 0.4	33 ± 2	0.5 ± 0.0	67 ± 6	0.9 ± 0.1	9 ± 1	0.1 ± 0.0	4 ± 0
Dinner	917 ± 55	13.0 ± 0.5	40 ± 3	0.6 ± 0.0	91 ± 6	1.3 ± 0.1	41 ± 3	0.6 ± 0.0	11 ± 1
Snacks	473 ± 56	6.6 ± 0.5	35 ± 1	0.5 ± 0.0	73 ± 10	1.0 ± 0.1	3 ± 1	0.0 ± 0.0	6 ± 1
Total	2382 ± 139	33.6 ± 0.7	127 ± 5	1.8 ± 0.0	305 ± 20	4.3 ± 0.1	65 ± 4	0.9 ± 0.0	32 ± 2
VEG									
Breakfast	448 ± 26	6.5 ± 0.3	21 ± 1	0.3 ± 0.0	61 ± 4	0.9 ± 0.1	11 ± 1	0.2 ± 0.0	15 ± 1
Lunch	590 ± 35	8.5 ± 0.3	33 ± 2	0.5 ± 0.0	74 ± 5	1.1 ± 0.0	15 ± 1	0.2 ± 0.0	13 ± 1
Dinner	842 ± 58	12.1 ± 0.5	39 ± 3	0.6 ± 0.0	95 ± 7	1.4 ± 0.1	30 ± 2	0.4 ± 0.0	19 ± 1
Snacks	416 ± 46	5.9 ± 0.5	33 ± 1	0.5 ± 0.0	45 ± 8	0.6 ± 0.1	11 ± 1	0.2 ± 0.0	21 ± 1
Total	2296 ± 136	33.0 ± 0.9	125 ± 5	1.8 ± 0.0	274 ± 20	3.9 ± 0.2	67 ± 4	1.0 ± 0.0	68 ± 3

Values represent mean ± SEM. OMNI, omnivorous diet; VEG, non-animal-derived diet

Body water deuterium enrichments

Saliva deuterium enrichments throughout the experimental protocol are depicted in **Figure 5.2**. From baseline (i.e. background) enrichments of 0.00044±0.00017 and 0.00030±0.00010% in OMNI and VEG, respectively, body water deuterium enrichments increased (*P*<0.0001; effect of time) and reached 0.77±0.03, 0.79±0.03, 0.79±0.04 and 0.85±0.03% on days 2-5 in OMNI, and 0.70±0.06, 0.75±0.05, 0.74±0.05 and 0.78±0.05% on days 2-5 in VEG, with no differences between groups (treatment and treatment × time interaction both *P*>0.05).

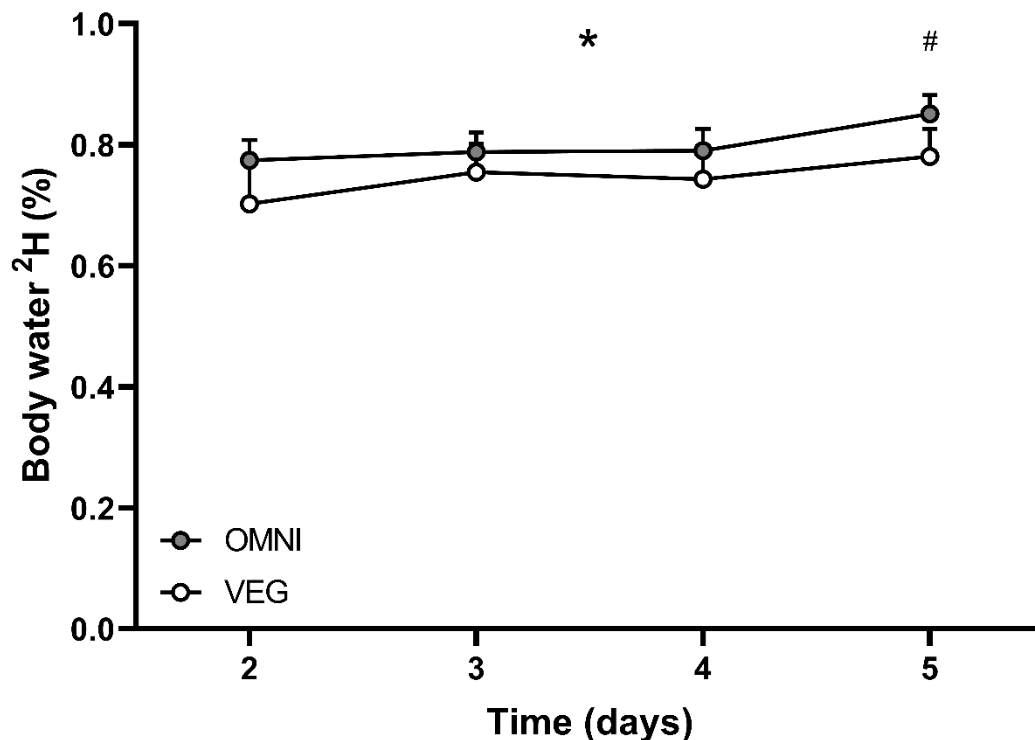

Figure 5.2. Saliva deuterium enrichments over time during oral deuterated water dosing in nineteen healthy older adults (age, 66±1 y) consuming a 3-day fully controlled eucaloric high-protein (1.8 g·kg $bm^{-1}\cdot d^{-1}$) diet, where the protein was provided predominantly from animal (OMNI; *n*=9) or exclusively non-animal (VEG; *n*=10) sources. During the dietary control period (Day 2-4) participants conducted a single bout of maximal unilateral concentric isokinetic knee extension exercise (5 x 30 contractions) each morning. On Day 1 participants were loaded with a total of 400 mL deuterated water, with 50 mL maintenance doses consumed daily thereafter.

Data were analysed with mixed-effects ANOVA. Values are means, with their standard errors represented by vertical bars. * indicates a main effect of time ($P<0.05$). # indicates a significant difference to preceding time points ($P<0.05$). Treatment × time interaction effect; $P=0.5565$, Treatment; $P=0.3847$, Time; $P=0.0026$.

Daily myofibrillar protein synthesis rates

Myofibrillar protein-bound [^{2}H] alanine enrichments increased over time ($P<0.0001$) and to a greater extent in the exercised compared with control leg (time x leg interaction; $P=0.015$). Myofibrillar protein-bound [^{2}H] alanine enrichments increased in the OMNI group by 358±76% (from 0.058±0.014 to 0.206±0.027 MPE) in rested and 394±74% (from 0.058 ±0.014 to 0.216±0.023 MPE) in exercised muscle, and in the VEG group by 263±42% (from 0.070±0.014 to 0.229±0.0.031 MPE) in rested and 299±44% (from 0.070±0.014 to 0.235±0.028 MPE) in exercised muscle, with no differences between groups (all OMNI vs VEG interactions; $P>0.05$)

Saliva deuterium enrichments were used as a precursor pool and myofibrillar protein-bound [^{2}H] alanine enrichments as the product to calculate daily myofibrillar fractional synthetic rates (FSRs) (**Figure 5.3**). Daily myofibrillar FSRs were 13±8 ($P=0.169$) and 12±4% ($P=0.016$) greater in the exercised compared with rested leg (1.59±0.12 vs 1.77±0.12%·d^{-1} and 1.75±0.14 vs 1.93±0.12%·d^{-1}) in OMNI and VEG groups, respectively. Daily myofibrillar protein synthesis rates did not differ between groups in either rested ($P=0.38$) or exercised ($P=0.33$) muscle.

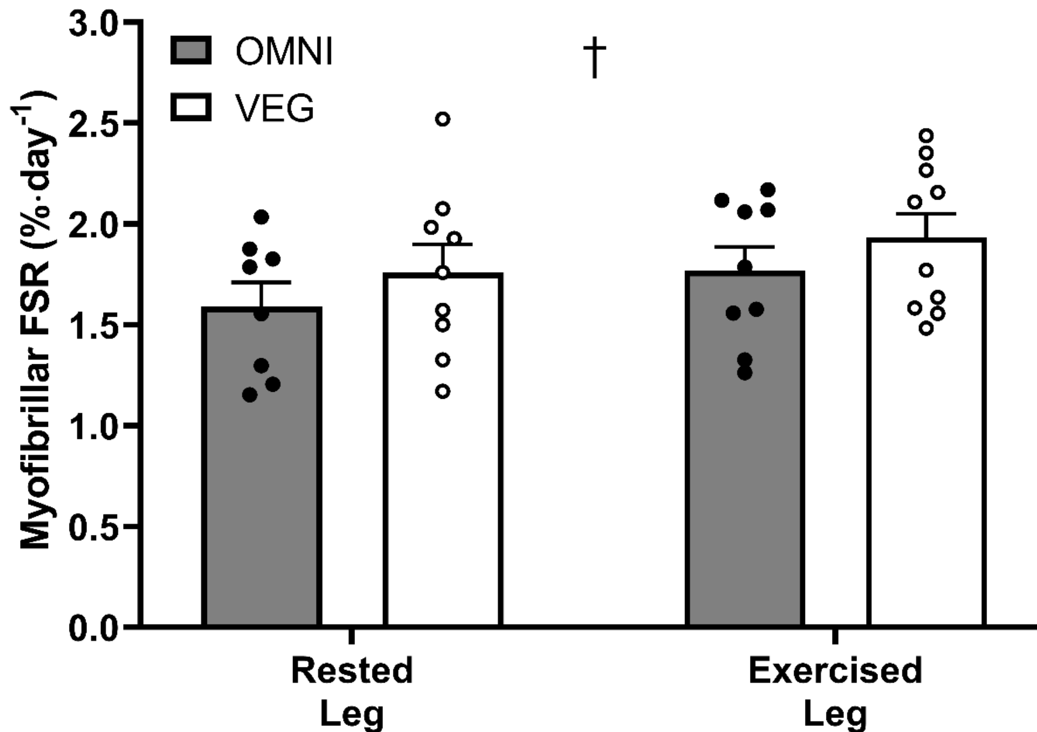

Figure 5.3. Daily free-living myofibrillar protein fractional synthesis rates (FSRs) calculated from the body water deuterium precursor pool in nineteen healthy older adults (age, 66±1 y) consuming a 3-day fully controlled eucaloric high-protein (1.8 g·kg $bm^{-1}\cdot d^{-1}$) diet, where the protein was provided predominantly from animal (OMNI; n=9) or exclusively non-animal (VEG; n=10) sources, in rested and exercised (single bout of 5 x 30 maximal unilateral isokinetic knee extension contractions on three consecutive days) muscle. Data were analysed with two-way ANOVA, with paired *t*-tests used to detect differences between rested and exercised legs in each respective groups. Values are means, with their standard errors represented by vertical bars. † indicates an effect of exercise in the VEG groups ($P<0.05$). Treatment × exercise interaction effect; $P=0.9917$, treatment; $P=0.1874$, Exercise; $P=0.1632$. OMNI paired *t*-test, rested leg vs exercised leg, $P=0.1694$. VEG paired *t*-test, rested leg vs exercised leg, $P=0.0162$†.

DISCUSSION

The present study demonstrates that performing a single bout of unilateral knee extensor resistance exercise daily for a 3-day period modestly stimulates daily myofibrillar protein synthesis rates compared with the rested control leg in older adults consuming a high-protein (1.8 g·kg $bm^{-1}·d^{-1}$) diet. Importantly, we report the novel finding that daily myofibrillar protein synthesis rates in both rested and exercised muscle of older adults during the dietary intervention period were equivalent regardless of whether dietary protein was obtained primarily from animal or exclusively from non-animal sources.

The continuous infusion of stable isotopically labelled amino acids into human volunteers to measure hour-to-hour muscle protein synthesis rates has revealed important insights concerning the physiological aetiology of age-related sarcopenia. Specifically, a failure of senescent muscle tissue to respond appropriately to the anabolic properties of bolus dietary protein ingestion ('anabolic resistance') is now widely accepted as a key physiological mechanism responsible for sarcopenia (Wall et al., 2014a, Cuthbertson et al., 2005, Burd et al., 2013). Such experimental approaches have also demonstrated that anabolic resistance can effectively be overcome (or compensated for) on a meal-by-meal basis by consuming higher amounts of protein (Pennings et al., 2012a, Yang et al., 2012a) and/or consuming protein in close temporal proximity to resistance-type exercise (Pennings et al., 2011c, Yang et al., 2012a). However, such studies have relied on single time point measurements made over only a few hours and confined to laboratory conditions. Fewer data are available exploring whether increased dietary protein intake with concurrent physical activity in older adults translates to improvements in muscle protein synthesis rates throughout multiple days (including basal, postprandial and overnight periods), and thus encompassing all free-living influences (e.g. multiple meals, habitual physical activity, diurnal metabolic fluctuations, sleep etc.). In the present work, we applied a deuterated water stable isotope approach in older adults consuming a fully controlled high-protein (1.8 g·kg $bm^{-1}·d^{-1}$) diet and daily

unilateral resistance exercise (with the non-exercised leg serving as a rested, within-subject control) to determine daily and free-living myofibrillar protein synthesis rates over a 3-day experimental period. In agreement with our previous work in young adults (Kilroe et al., 2019), we observed the deuterated water dosing regimen rapidly increased body water deuterium enrichments to near steady-state levels of ~0.78%, with a slight increase over time. The steady-state enrichment of body water served as a precursor for the calculation of daily myofibrillar protein synthesis rates from muscle biopsy samples (Figure 5.2). We report a single bout of unilateral resistance exercise performed daily each morning for 3 days increased daily myofibrillar protein synthesis rates by ~13% in healthy older men and women (Figure 5.3), though this effect only reached statistical significance in the vegan group (though numerically similar across groups).

The absence of myofibrillar protein synthesis data collected under habitual (i.e. lower; 1.2 g·kg $bm^{-1}·d^{-1}$ for the present volunteers) protein intake conditions precludes us from confirming that the high-protein dietary intervention *per se* (i.e. irrespective of protein source) had a stimulatory effect. However, recent work has reported that higher protein diets in the absence of exercise (1.8 vs 1.0 g·kg $bm^{-1}·d^{-1}$; achieved via protein supplementation, and in line with the intervention vs habitual intakes of the present volunteers) elevates daily, free-living myofibrillar protein synthesis rates in older adults (Oikawa et al., 2020a). Further, such studies have also demonstrated that resistance exercise can elevate daily muscle protein synthesis rates across a range of low-moderate or protein supplemented protein intakes (1.0-1.8 g·kg $bm^{-1}·d^{-1}$) in older adults of varying health statuses (Murphy et al., 2016a, Murphy et al., 2018, Oikawa et al., 2020a, McKendry et al., 2019b). Our data, therefore, support and extend on these observations by demonstrating that daily resistance exercise retains the capacity to augment daily myofibrillar protein synthesis rates (though perhaps more modestly) in healthy older adults consuming a strategically timed (i.e. protein consumed directly after exercise, distributed throughout the day and before sleep (Mamerow

et al., 2014a, Areta et al., 2013a, Kouw et al., 2017)) high-protein diet designed to nutritionally maximise daily myofibrillar protein synthesis rates. Collectively, therefore, the data indicate that chronic maintenance of higher daily muscle protein synthesis rates mechanistically underpin the cross-sectional and longitudinal observational studies that reliably show more active (Strasser et al., 2018, McKendry et al., 2019a, Mckendry et al., 2018) and/or higher protein consuming (at least above the currently accepted RDA/RDIs) (Isanejad et al., 2015, Beasley et al., 2013) older adults experience slower rates of annual muscle loss.

Aside from total daily (or per meal) dietary protein intake, an important contemporary research focus is from *where* dietary protein should (or could) be obtained. Government and societal priorities are increasing the demand to reduce animal-derived protein consumption in favour of sustainable alternatives (Allès et al., 2017). This is particularly pertinent for older adults where the scientific consensus is advocating for a near 50% increase in the UK/US RDI for dietary protein (Phillips, 2017a, Wall et al., 2014a, Paddon-Jones et al., 2015a). Limited comparisons to date have suggested that, on a gram-for-gram basis, plant-based protein sources are inferior to animal-derived protein sources with respect to their capacity to stimulate muscle protein synthesis rates upon ingestion, attributed to their typically lower leucine contents (Gorissen et al., 2016a, Yang et al., 2012b, Tang et al., 2009). This implies that more total protein would be required within a vegan diet to support equivalent daily muscle protein synthesis rates, a circumstance itself that has implications for environmental sustainability and dietary feasibility. Indeed, by supplementing with more acutely anabolic proteins (whey vs collagen) it has been shown that daily muscle protein synthesis rates can be augmented to a greater degree, even under isonitrogenous conditions (Oikawa et al., 2020a). We have recently shown that the fungal-derived protein source, mycoprotein, robustly stimulates muscle protein synthesis rates in young men. In the present work, we therefore hypothesised that the incorporation of mycoprotein within a vegan, high-protein

diet, would support daily myofibrillar protein synthesis rates to the same extent as a protein-matched diet based (more typically) upon animal-derived protein consumption in older men and women. In support of this hypothesis, comparable daily free-living rates of myofibrillar protein synthesis between omnivorous and vegan diets were observed in both rested (1.59 vs 1.76%, respectively) and exercised (1.77 vs 1.93%, respectively) muscle (Figure 5.3). Our data therefore provide the proof-of-concept that higher protein vegan diets can be adopted in healthy older adults without compromising rested myofibrillar protein synthesis rates, implying vegan diets can be equivalently capable of supporting the maintenance of muscle mass during ageing. Further, our data also indicate that for older adults living more active lifestyles and/or participating in structured (resistance) exercise training, implementing a higher protein vegan diet would not compromise prolonged muscle tissue adaptive responses. We therefore show that non-animal-derived dietary proteins (the vast majority of which have not been investigated in relation to their impact on muscle protein synthesis) are not *necessarily* inferior in their capacity to stimulate daily myofibrillar protein synthesis rates when incorporated into the daily diet, even in older adults. Our work also captures the total muscle anabolism achieved with two divergent diets in a free-living scenario over multiple days, thereby incorporating the diurnal variation and multiple feeding-fasting cycles that are potentially missed by intravenous isotope infusion studies. The necessarily short-term nature of such metabolic studies as presently reported (i.e. 3 days) means we cannot rule out the possibility that a longer duration of study (or increased statistical power) may have yielded significant differences across groups. However, it is also true that numerically our data indicated greater rates of myofibrillar protein synthesis in the vegan group. Irrespective, it is of clear importance that future work is performed to confirm our mechanistic findings as to the longer-term impact of such diets on muscle mass and strength in older adults.

Some important design aspects and limitations of this study require further consideration and context. We chose to provide a dietary protein intake (amount, type, timing and distribution) that we considered (close to) 'optimal' for maximising daily myofibrillar protein synthesis rates. While this amount (i.e. 1.8 g·kg bm^{-1}·d^{-1}) is well above the RDA, it is in line with the currently asserted optimal dietary protein intakes to support active and healthy muscle ageing (Lancha Jr et al., 2017, Traylor et al., 2018), and is only ~50% above habitual intakes typically reported by healthy and active UK adults (Smeuninx et al., 2020), including those within this study (see Table 5.2). Though the feasibility of applying such a diet in community-dwelling older adults can be debated, the approach allowed us to perform a proof-of-concept experiment investigating whether manipulating the *type* of protein only would impair daily myofibrillar rates under these "optimal" conditions. As a result, we can conclude that protein type may become less relevant when consuming a high-protein diet, where the amount and timing of protein is at an "optimal" level. Further research is necessary to establish whether such findings hold true under lower and/or less optimal protein intake conditions. Indeed, given the typically lower leucine (and total EAA/BCAA) contents of non-animal protein sources (van Vliet et al., 2015b), it may be expected that differences across sources would become most apparent when protein intake is suboptimal. However, due to the relatively high leucine content of mycoprotein and the proportion of protein across groups that was obtained from identical sources (i.e. the control group was omnivorous), we estimated that daily leucine intakes were sufficient (and perhaps surplus) and comparable across groups (~10 g leucine a day, an average of ~2.5 g split between four meals, in both groups). The latter may, at least in part, explain the lack of differences between diets in daily myofibrillar protein synthesis rates. Lastly, as we did not measure habitual physical activity during the three days of controlled diet, we cannot discount that inter-individual differences in physical activity might have affected daily muscle protein synthesis rates.

The inclusion of mycoprotein as the basis for the vegan dietary intervention is also of relevance. To date, mycoprotein is the only vegan protein source that's bolus ingestion has been shown to acutely stimulate postprandial muscle protein synthesis rates to a comparable extent as an animal-derived control (Monteyne et al., 2020b). Accordingly, it cannot be assumed that our current data are generalisable to vegan diets predicated on other protein sources, particularly those lower in leucine and/or other essential amino acids. Future work is required to investigate the impact of a range of vegan protein sources both on acute postprandial and daily muscle protein synthesis rates. Finally, we employed unilateral isodynamic maximal concentric contractions as a model of exercise to ensure a daily stimulation of muscle protein synthesis rates in line with our previous work (Monteyne et al., 2020a). We reasoned that this would increase the cumulative stimulatory effect across the 3-day intervention without inducing excessive muscle damage. While effective for the experimental purposes herein, there will be value in translating our data to multiple modalities of exercise to further the ability to make applied recommendations to support active and healthy ageing.

To conclude, the present work reports that a single bout of resistance exercise performed daily each morning increases daily myofibrillar protein synthesis rates in older men and women consuming a high-protein diet. Obtaining the majority of dietary protein from animal-derived sources compared with exclusively vegan-based sources (primarily mycoprotein) did not modulate daily myofibrillar protein synthesis rates in rested or exercised muscle. Our data indicate that obtaining dietary protein from animal-derived sources is not an essential prerequisite to support daily myofibrillar protein synthesis rates in older adults.

CHAPTER 6

A HIGH-PROTEIN NON-ANIMAL DIET SUPPORTS EQUIVALENT DAILY MYOFIBRILLAR PROTEIN SYNTHESIS RATES, RESISTANCE TRAINING INDUCED HYPERTROPHY, AND STRENGTH GAINS AS A HIGH-PROTEIN OMNIVOROUS DIET IN YOUNG ADULTS.

Abstract

Introduction: It remains unclear whether non-animal-derived dietary protein sources, and non-animal-derived diets, can support resistance training-induced skeletal muscle remodelling to the same extent as animal-derived protein sources.

Methods: In Phase 1, twenty-one healthy young adults (*m=11*, *f=10*) (age: 23±1 y, body mass: 69±2 kg, BMI: 23±1 kg/m^2) completed a 3-day dietary intervention, alongside unilateral resistance exercise, where they consumed a high-protein diet (1.8 g·kg bm^{-1}·day^{-1}) derived from omnivorous (OMNI) or exclusively non-animal (VEG) sources. Resting and exercised daily muscle protein synthesis rates were assessed using an oral deuterium oxide tracer approach. In Phase 2, twenty-two healthy young adults (*m=11*, *f=11*) (age: 24±1 y, body mass: 70±2 kg, BMI: 23±0 kg/m^2) completed a progressive resistance exercise programme 5 d/week for 10 weeks, whilst consuming an omnivorous or majority non-animal-derived high-protein diet (~2 g·kg bm^{-1}·day^{-1}). Muscle fibre CSA, DXA whole-body lean mass, MRI thigh muscle volume, muscle strength, and muscle function were determined pre and post-intervention.

Results: Daily myofibrillar protein synthesis rates were 27±7 (P=0.098) and 11±7% (P=0.107) higher in the exercised compared with rested leg (2.19±0.14 vs 2.46±0.11%·d^{-1} and 2.01±0.17 vs 2.39±0.09%·d^{-1}) in OMNI and VEG groups, respectively. Daily myofibrillar protein synthesis rates did not differ between OMNI and VEG in either rested or exercised muscle (P>0.05). Resistance training increased lean mass in both groups by a similar magnitude (OMNI 2.6±0.3 kg, VEG 3.1±0.8 kg; P>0.05). Likewise, training equivalently increased thigh muscle volume (OMNI 8±1%, VEG 8.2±1.4%; P>0.05), and muscle fibre CSA (OMNI 33±10%, VEG 32±17%; P>0.05). Both groups increased deadlift (OMNI 17±3%, VEG 25±3%; P>0.05), back squat (OMNI 19±3%, VEG 34±7%; P>0.05) and incline bench press (OMNI 18±2%, VEG 28±4%; P=0.04) 1RM strength.

Conclusion: Over the course of a three-day intervention, omnivorous or non-animal-derived dietary protein sources can support equivalent rested and exercised daily myofibrillar protein synthesis rates in healthy young adults consuming a high-protein diet. Moreover, during a 10-week progressive resistance exercise training programme, omnivorous or non-animal-derived diets support equivalent increases in muscle mass and strength in healthy young adults consuming a high-protein diet.

INTRODUCTION

In healthy young individuals, muscle mass is regulated through the interaction of contractile stimuli and protein ingestion. It is well established that protein ingestion, primarily through an increase in plasma essential amino acid concentrations, increases muscle protein synthesis rates (Witard et al., 2013, Moore et al., 2008, Tipton et al., 1999, Gorissen et al., 2020). Resistance exercise, through an increase in mechanical tension, also upregulates muscle protein synthesis and sensitises the muscle to plasma essential amino acids (Phillips et al., 1997, Burd et al., 2011b, Pennings et al., 2012b). If this combination of mechanical and nutritional stimuli is regularly repeated, effecting elevations in hourly and, therefore, daily muscle protein synthesis rates, there are prolonged periods of positive net muscle protein balance, leading to an appreciable accrual of contractile protein and an increase in muscle fibre size (Damas et al., 2016). In order to facilitate this anabolic environment those adhering to a structured resistance exercise training programme are advised to consume 1.6 $g{\cdot}kg\ bm^{-1}{\cdot}day^{-1}$ of protein or above, an amount double that of the RDA (Morton et al., 2018). The question arises as to where this protein should come from? There is a drive, both at a societal and governmental level, to reduce meat consumption, in response to the associated environmental cost of industrial-scale farming. Concomitantly, almost the entirety of the research available to us on nutritional strategies to optimise muscle hypertrophy is derived from studies utilising animal-derived protein sources. This is problematic, as we have little to establish whether non-animal-derived proteins are able to comparably stimulate daily muscle protein synthesis rates, and subsequently facilitate tissue accrual. Indeed, it may be that those following a non-animal-derived diet require specific guidelines, for which an evidence base is yet to be adequately established.

It has been suggested that non-animal-derived dietary protein sources are inferior in their capacity to support skeletal muscle adaptive responses to prolonged resistance training (van Vliet et al., 2015b), though minimal data is available. Soy protein supplementation has been

shown to be inferior with respect to the hypertrophic gains that it elicits in comparison to dairy protein supplementation (Hartman et al., 2007, Volek et al., 2013). For example, Hartman and colleagues demonstrated a 4.4% compared to a 6.2% increase in fat-free mass following soy and milk protein supplementation, respectively. This is supported by data demonstrating concurrently lower postprandial muscle protein synthesis rates (Wilkinson et al., 2007, Tang et al., 2009). Contrastingly, there is evidence demonstrating that pea protein supplementation promotes a hypertrophic response similar to that of whey protein supplementation (Babault et al., 2015). This suggests that not all plant-based sources are homogenously-inferior with respect to their ability to elicit hypertrophy, and although we lack a supporting quantification of muscle protein synthesis for the ingestion of pea protein, we know that it is rich in essential amino acids and therefore may support skeletal muscle anabolism (Babault et al., 2015). We have recently demonstrated that a single bolus of mycoprotein robustly stimulates post-exercise muscle protein synthesis, so there is reason to believe that mycoprotein would support daily muscle protein synthesis rates and resistance exercise adaptation (Monteyne et al., 2020b).

The aforementioned studies are of limited scope as they do not consider the diet as a *whole*, delineating condition on protein supplementation alone. Indeed, emerging data suggests that protein might act differently when consumed as part of a whole food (van Vliet et al., 2017, Burd et al., 2019), an interaction that is likely to become more involuted when considering the complexities of a diet. In turn, such studies only partially inform on the ability of non-animal proteins to support hypertrophic adaptation, and the adaptive responses to more stringent dietary patterns (i.e. vegans). Given the increased prevalence of non-animal-derived diets (Allès et al., 2017), it is important that we develop an evidence base for non-animal-based *diets,* both with respect to their ability to support muscle protein synthetic responses over several days, and to support resistance training adaptation.

The aim of this study was twofold. Firstly, in Phase 1, to investigate the cumulative muscle protein synthetic response to an entirely non-animal-derived mycoprotein-rich high-protein diet in free-living conditions alongside resistance exercise. Secondly, in Phase 2, to comprehensively investigate the adaptive response to a majority non-animal-derived diet alongside 10 weeks of structured progressive resistance exercise. We hypothesise that a mycoprotein-rich non-animal diet will support daily muscle protein rates in exercised muscle, and this will translate into increases in muscle size and strength.

METHODS

Participants

Participants were recreationally active and had resistance exercise experience, having formally completed structured resistance exercise training regimens for extended periods of time. Participants attended the laboratory for a medical screening, where height, body mass and blood pressure were measured, and a general medical questionnaire was completed, to assess their eligibility for participation and to ensure no adverse health conditions were present. Participants were deemed healthy based on their blood pressure (≤140/90 mmHg), BMI (18–30), and the absence of any diagnosed metabolic impairment, cardiovascular disease, or motor disorders. Participants completed the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003a). Participants recorded habitual nutritional intake for 3 days, either prior to starting the study or shortly after completing it. All subjects were informed of the nature and possible risks of the experimental procedures before providing written informed consent. This study was registered as a clinical trial with clinicaltrials.gov (NCT04325178). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the NHS Health Research Authority Research Ethics Committee (18/LO/0374).

Pre-testing

Following screening and admittance, all participants underwent a period of pre-testing, which took place ≥5 and ≤10 days prior to the start of the experimental period. Participants reported to the laboratory for strength testing, muscle function testing, and for familiarisation with the exercise equipment and unilateral resistance-type exercise protocol to be used.

Participants completed 1 repetition maximum (1RM) tests for the barbell back squat, conventional deadlift, and for the incline (30°) barbell bench press. The participant

performed a warm-up with a self-selected load that allowed them to complete 6-10 submaximal repetitions. Participants then rested for 2 minutes before selecting a weight, based on the previous effort, which allowed them to perform 3 submaximal repetitions. After a further 2 minutes rest participants were instructed to increase the load and perform a single submaximal repetition. A series of single maximum attempts were then completed until a 1RM was achieved, with load increments increasing ~5-20% for each attempt, 3 minutes rest between attempts, and with 1RM achieved within 3-7 attempts. The researcher overseeing the testing ensured that proper lifting technique was demonstrated and practised throughout, noting range of motion and individual variation in technique. For the barbell back squat participants were instructed to squat to parallel (midline of the thigh parallel with the floor), if this range of motion was not possible they were asked to move through the maximum achievable range of motion. Range of motion in maximum attempts was matched to that performed during the submaximal warm-up sets, such that if participants produced a range of motion significantly shorter than demonstrated previously it was deemed unacceptable. The conventional deadlift was deemed acceptable contingent on continued upward momentum until upright. The lift was deemed unacceptable if there was downward movement of the bar before completion of the lift, failure to stand erect with shoulders back, failure to lock the knees straight, and if the bar was supported on the thighs without upward momentum. For the incline bench press participants were instructed to lower the bar as far as was comfortable, with range of motion in maximum efforts matched to submaximal efforts, and then pressing upwards until the elbows were locked out. The lift was deemed unacceptable if there was any downward movement of the bar in the course of being pressed out.

Immediately following 1 repetition maximum testing participants completed a number of unilateral exercise tests on an isokinetic dynamometer (Biodex Medical Systems, Shirley, New York, USA). Firstly, after undertaking an incremental warm-up protocol, participants

completed three 3 second maximum isometric voluntary contractions (MVC) at a 70° joint angle, with peak isometric torque recorded. Participants then completed three maximal concentric isokinetic leg extension contractions at a speed of 60° per second over a central 80° range of motion using their dominant leg, with peak isokinetic torque recorded. This was followed by 5 sets of 30 repetitions of maximal concentric isokinetic leg extension contractions at a speed of 60° per second over a central 80° range of motion using their dominant leg. Work done (J) was recorded for each completed set, and fatigue was calculated as the percentage decrement in work done between the first and last set. Verbal encouragement was provided throughout testing to encourage maximal effort.

Experimental design

Experimental testing was divided into two phases. Phase 1, 3 days of dietary control with the application of deuterium oxide to measure muscle protein synthesis. Phase 2, 10 weeks if dietary manipulation alongside structured resistance exercise. A graphical representation of the experimental study design can be seen in **Figure 6.1**. Participants were randomly assigned to two parallel-groups, A or B, by the lead investigator, unless their habitual dietary choices (i.e. vegan) precluded their inclusion in the group that would receive the omnivorous diet. A number of participants who volunteered already adhered to a non-animal-derived diet, and were assigned to the non-animal-derived dietary group (3/12 in Phase 1, 2/10 in Phase 2). Participants who were habitual vegans prior to the study responded similarly to non-vegans in all measures during the study.

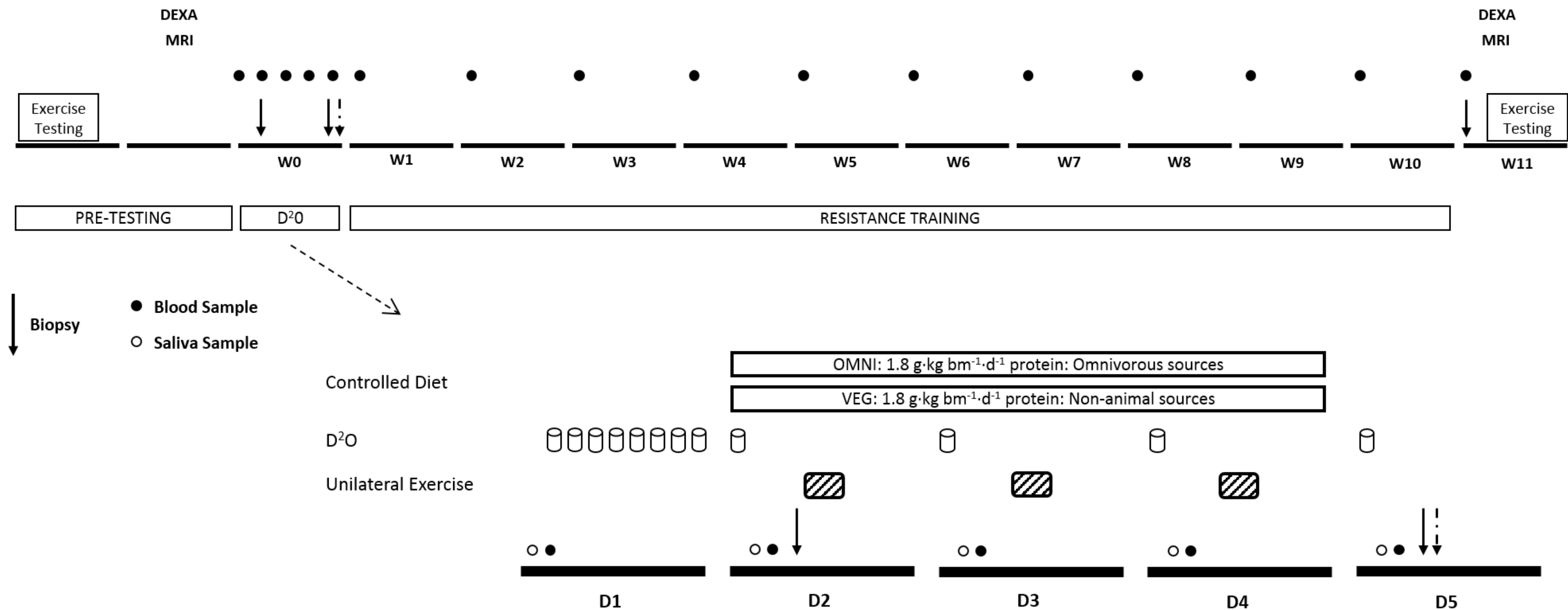

Figure 6.1. Schematic representation of the experimental protocol. Phase 1, Twenty-one healthy young adults consumed a 3-day fully controlled, eucaloric and high-protein (1.8 g·kg $bm^{-1}·d^{-1}$) diet, where the protein was provided predominantly from animal (OMNI; *n*=9) or exclusively non-animal (VEG; *n*=10) sources. During the dietary control period (Days 2-4) participants conducted a single bout of unilateral isokinetic knee extension exercise (5 x 30 contractions) each morning. On Day 1 participants loaded 400 mL deuterated water, with 50 mL maintenance doses consumed daily thereafter. Saliva (open circles) and blood samples (closed circles) were collected daily, and muscle biopsies were collected from both the rested (straight arrow) and exercised (dashed arrow) legs to determine daily myofibrillar protein synthesis rates. Phase 2, twenty-two healthy young adults completed a 10-week high-intensity resistance exercise training programme, whilst consuming a high-protein omnivorous diet (OMNI; *n*=12) or a majority non-animal-derived diet (VEG; *n*=10). Participants underwent DXA and MRI scans, muscle biopsies, and strength testing, before and after completing the resistance exercise training programme to characterise resistance exercise adaptation.

PHASE 1

Participants received one of two dietary interventions which differed with respect to the primary sources of dietary protein consumed. Predominantly animal-derived protein sources including milk protein supplementation (OMNI; n=9), or exclusively non-animal-derived protein sources including mycoprotein supplementation (VEG; n=10). All participants were asked to refrain from alcohol and caffeine consumption, and from strenuous exercise for 2 days prior and throughout the experimental protocol, though to keep all other daily habitual activities as normal.

Participants attended the laboratory each day for a 5-day (Mon-Fri) experimental period with days 2-4 (i.e. 3 days, Tue-Thu, inclusive) involving the dietary control. Each day of the 3-day dietary control period, participants attended the laboratory to conduct a single bout of unilateral leg extension exercise, the previously described 5 sets of 30 repetitions of maximal concentric isokinetic leg extension contractions, and were then provided with their food for the day. To measure daily myofibrillar protein synthesis rates participants underwent a deuterium oxide dosing protocol (described below), in line with our previous work (Kilroe et al., 2020a, Kilroe et al., 2020c), and muscle biopsies were collected before commencing the controlled diet (i.e. Tue ~0800 h; single muscle biopsy from the [to-be] rested leg) and following (i.e. Fri ~0800 h; bilateral biopsies from the rested and exercised leg. Muscle biopsies were obtained under local anaesthesia, using a percutaneous Bergstrom biopsy needle technique (6), from the *m. vastus lateralis* ~15 cm above the patella and ~3 cm below the fascia. Muscle tissue was quickly assessed and any blood or non-muscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Dietary intervention

Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (Henry, 2005b). The IPAQ was used to calculate a physical activity level (PAL) factor (CRAIG et al., 2003b). Individual energy requirements were then calculated by multiplying the participant's BMR by their PAL factor. Thereafter, an individual 3-day meal plan was designed for each participant with all food prepared, weighed, and packaged in-house in the Nutritional Physiology Unit's research kitchen facility. Nutritional information for the two diets is provided in **Table 6.2.** Subjects consumed a diet containing 1.8 g of protein per kg of body mass (bm) per day ($g{\cdot}kg\ bm^{-1}{\cdot}d^{-1}$, with 24-27% of energy being provided by fat and 50-55% from carbohydrates in OMNI, and with 22-27% and 48-58% of energy being provided by fat and carbohydrates, respectively, in VEG (variation due to different energy requirements and the matching of protein intake). The meals were identical between the two groups, aside from meat or dairy providing the primary protein source in lunches and dinners for the OMNI group and this being replaced by Quorn Foods™ mycoprotein containing products or supplementary mycoprotein in the VEG group. The OMNI group received 39 g supplemental milk protein daily (31 g protein, 2 g carbohydrate, <1 g fat, 131 kcal) to drink prior to sleep, which was replaced with 70 g mycoprotein (31 g protein, 7 g carbohydrate, 9 g fat, 232 kcal) to drink in the VEG group. A small amount of mycoprotein was also added to the breakfast in the VEG group to more closely equate the protein in the breakfast meal across conditions. Breakfast was consumed within 1 hour of completing the unilateral resistance-type exercise, and provided 18±1 and 19±1 g protein per day in OMNI and VEG, respectively. The OMNI group consumed meals based on chicken, pork, and dairy. In the VEG group, this was substituted for Quorn deli ham, Quorn pieces, Quorn burgers, Quorn BBQ strips, Quorn sausages, and Quorn nuggets from their vegan range (which does not contain any egg products). A document and diary detailing the plan were provided to the subjects to log mealtimes and provide recipe information/instructions. Participants body

mass was measured wearing light clothing at the start and end of the three-day control diet period (seca 703 column scale, seca GmbH & Co. KG, Hamburg, Germany). Each morning the researchers discussed with the participants any questions or issues that may have arisen, before the next day of food was provided.

Deuterated water dosing protocol

The deuterated water dosing protocol was based on our previous work (Kilroe et al., 2020a). Day 1 (Mon) of the experimental protocol acted as a D_2O loading day where participants consumed 400 mL of 70% D_2O separated over the day as 8 x 50 mL boluses (CK Isotopes Ltd, Leicestershire, UK). Upon arrival at the laboratory (0730 h) background saliva samples were collected before the first bolus of D_2O was ingested. The first dose of D_2O was consumed at ~0800 h with the remaining loading day doses being consumed every 1 hour (doses 2 and 3) and then every 1.5 h thereafter. Participants stayed at the laboratory until 4 out of the 8 loading day D_2O doses had been consumed, with the remaining D_2O doses being consumed at home under instruction of timings (i.e. leaving 1.5 h between each). Every day following the loading day participants consumed a maintenance dose of D_2O (50 mL) upon waking (~0800 h). At least 90 minutes (~09:30 h) after the daily D_2O maintenance dose a daily saliva sample was collected using a cotton mouth swab (Celluron, Hartmann, Germany) which the participant lightly chewed for ~1 min until saturated with saliva. The saturated sponge was placed into an empty syringe where the swab was squeezed to release the saliva into a collection tube, and stored at -80°C until further analysis. The saliva samples were used to assess the body water ^2H enrichment. To ensure uniformity and compliance with the D_2O protocol, participants were provided with bottles of D_2O labelled with the specific time and date to be taken, and were required to return bottles each day.

Body water deuterium enrichment

Body water deuterium enrichment was measured using the saliva samples collected daily throughout the study, at the University of Texas Medical Branch. A ThermoFisher Delta V Advantage Isotope Ratio mass spectrometer (IRMS) (Bremen, Germany), equipped with a Finnigan GasBench II (Thermo Fisher Scientific, Waltham, MA, USA), was used for stable hydrogen isotope ratio measurements. After uncapping a 12-mL Exetainer (Labco Limited, Lampeter, UK), 5 mg of activated charcoal (Thermo Fisher Scientific) and 200 mg of copper powder (Thermo Fisher Scientific) were introduced into the Exetainer followed with a platinum catalytic rod (Thermo Fisher Scientific). The activated charcoal and copper powder were added to remove any potential contaminants in the samples that might poison the platinum catalyst. After putting 200uL of sample into the Exetainer, the Exetainer was recapped and placed into the GasBench II and flushed with 2% H2 in helium for 7 min. The sample was allowed to equilibrate with the flushed gas at room temperature (~4-6 hours). At the end of the equilibration, an aliquot of the headspace in the Exetainer was injected into the Thermo Delta V Advantage mass spectrometer system for stable hydrogen isotope ratio measurement against the reference gas H2. A standard calibration curve was prepared using 99.9% deuterium-enriched water (Sigma-Aldrich, St. Louis, MO, USA), and the deuterium (^{2}H) enrichment in duplicate saliva samples was determined (Wong and Clarke, 2012).

Myofibrillar bound ^{2}H alanine enrichments

Myofibrillar protein-enriched fraction was extracted from ~50 mg of wet weight muscle tissue by hand-homogenisation on ice using a pestle in a standard homogenisation buffer (TRIS-HCL 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerophosphate 10 mM, NaF 50 mM, activated sodium orthovanadate 0.5mM, cOmplete protease inhibitor cocktail tablet (Roche Holding AG, Basel, Switzerland)) (7.5 µL/mg). The samples were centrifuged at 2,200 g for 10 min

at 4°C, the pellet was then washed with 500 µl of homogenisation buffer, and centrifuged at 700 g for 10 min at 4°C. The myofibrillar protein was solubilised by adding 750 µl of 0.3 M NaOH and heating for 30 min at 50°C with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 10,000 g and 4°C, the supernatant containing the myofibrillar protein was kept and the collagen protein pellet was discarded. The myofibrillar proteins were precipitated by the addition of 500 µL of 1 M PCA and centrifuged at 700 g and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolysed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids from the hydrolysed myofibrillar protein pellet were dried under vacuum with a Speed-Vac rotary dryer (Savant Instruments, Farmingdale, NY, USA) for 3 h at 80°C radiant cover. The free amino acids were subsequently dissolved in 1.5 mL 25% acetic acid solution and passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA) and eluted with 6 M NH4OH. Following this, the purified amino acids were dried and derivatised to tert-butyldimethylsilyl derivatives via the addition of 50 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 50 µl acetonitrile, which was then vortex mixed and heated at 95°C for 40 min. The samples were transferred to a GC vial. The level of enrichment of D4-alanine was analysed using a ThermoFisher Delta V Advantage Isotope Ratio mass spectrometer (IRMS) fitted with a Trace 1310 GC with an on-line high-temperature thermal conversion oven (HTC) at 1420°C. The sample (1µl) was injected in splitless mode at an injection port temperature of 250°C. The peaks were resolved on a 30m × 0.25mm ID × 0.25µm film Agilent Technologies DB-5 capillary column (temperature programme: 110°C for 1 min; 10°C·min^{-1} ramp to 180°C; 5°C·min^{-1} ramp to 220°C; 20°C·min^{-1} ramp to 300°C; hold for 2 min) prior to pyrolysis. Helium was used as the carrier gas with a constant flow of 1ml/min. Any amino acid eluting from the gas chromatograph was converted to H2 before entry into the IRMS. The enrichment of the alanine tracer was measured by monitoring the Ion masses 2 and 3 to determine the $^{2}H/^{1}H$

ratios in the samples and referenced to the calibration curve. The calibration curve consisted of a series of known concentrations of d4-alanine and was applied to assess both the linearity of the mass spectrometer and to control for the loss of tracer. The isotopic abundances were expressed as the delta notation, δ^2H per mil (‰) deviation from VSMOW (Vienna Standard Mean Ocean Water) standard (Wong and Clarke, 2012). Values of delta per mil given by the IRMS were transformed into MPE.

Calculations

Myofibrillar fractional synthesis rates (FSR) were calculated based on the incorporation of the mean body water deuterium enrichment over the 3-day intervention as a precursor pool into myofibrillar bound proteins. We and others have previously shown the body water deuterium pool is a valid precursor pool for the calculation of myofibrillar protein synthesis rates (corrected by a factor of 3.7 based on deuterium labelling of alanine during *de novo* synthesis) which shows excellent agreement with either plasma or muscle free [$^2H_4$] alanine as alternative precursor pool selections (Kilroe et al., 2020a, Holwerda et al., 2018b, Dufner and Previs, 2003). FSR was calculated using the standard precursor-product method and expressed as daily rates as follows:

$$FSR\ (\%\ \cdot\ \mathrm{day}^{-1}) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor}\ x\ t}\right) x\ 100$$

where E_{m1} and E_{m2} are the myofibrillar muscle protein-bound enrichments pre- (one leg only) and post- (either the rested or exercised leg) the dietary intervention. $E_{precursor}$ represents mean body water deuterium enrichment corrected by a factor of 3.7. t represents the time between biopsies (i.e. 3 days).

PHASE 2

Resistance Exercise Programme

Participants completed 5 exercise sessions a week, in a pull-push-lower pattern, training each muscle group twice per week. The barbell back squat, conventional deadlift, and incline bench press were treated distinctly from the rest of the programme, and programming for these three exercises load and repetition ranges were designated in an undulating-periodisation pattern, based upon 1RM loads, to augment muscular strength. Repetitions were designated within a range of 1-5 repetitions, along with a top set where participants were required to perform as many repetitions as possible, with a specified load, whilst maintaining technical quality. Load increased and repetitions decreased across a three-week cycle, before load was subsequently increased in the next cycle. Participants completed three such cycles, and the extent to which the load was increased was adjusted based on progress in the previous cycle. In the final week of the training programme participants completed RPE based single repetitions for these three exercises, in order to inform upon ensuing 1RM testing and to taper training volume prior to post-testing protocols. For the majority of the other exercises in the programme participants were requested to select a load that would cause them to reach muscular failure, the point at which they were not able to complete another repetition, between 8-12 repetitions. For certain exercises, where appropriate, a greater number of repetitions was advised, still with the aim of reaching muscle failure, and for self-bodyweight exercises participants were advised to complete as many repetitions as possible, again with the aim of reaching muscle failure. Intensity techniques (drop sets and rest-pause sets) were also employed on specific exercises, on the final set of the exercise, to accumulate greater volume in a time-efficient manner, to increase the number of stimulating repetitions, and to stimulate a high degree of glycolytic flux and metabolite accumulation.

The first five exercise sessions were completely supervised, allowing proper technique to be taught and discourse on the training programme to take place. Thereafter, participants were allowed to complete exercise sessions at a gym of their choosing, either at their regular fitness establishment or at our own laboratory fitness facility. Participants were required to visit the laboratory fitness facility, or were visited in their own fitness facility, at regular intervals so that a researcher was able to monitor their progress and technique. Given the volume and intensity of the training protocol, it was considered prudent to allow participants to use their regular fitness facility, if they wished, in order to reduce the burden placed upon them and to facilitate compliance. Participants completed a training diary where they were asked to record load, repetitions, and any other pertinent information. Participants trained five times a week for a period of 10 weeks, plus or minus one week. If participants missed training sessions in a week, due to illness, injury or unforeseen circumstances, they were required to recuperate those sessions at a later point. If participants were to miss more than 50% of sessions over a two week period, they would be excluded from the study. On average participants completed 47/50 sessions, over a period of between 9 and 11 weeks.

Dietary Manipulation

Whilst undertaking the resistance exercise training programme participants consumed either an omnivorous diet, with the majority of protein derived from animal-based sources, or a majority non-animal-derived diet. Participants were provided with a caloric target ((BMR × PAL) × 1.1) to place them in a 10% energy surplus, and a protein target to provide 2 $g \cdot kg\ bm^{-1} \cdot d^{-1}$, and asked to matched their food consumption to these targets. Two $g \cdot kg\ bm^{-1} \cdot d^{-1}$ of protein was deemed more than sufficient to support hypertrophic adaptation (Morton et al., 2018), whilst providing some leeway such that even if participants fell below this level they should exceed the ~1.6 $g \cdot kg\ bm^{-1} \cdot d^{-1}$ suggested to be required to optimise resistance training adaptation. This target accounted for dietary fluctuations, and obviated any deleterious effects of days where protein intake was slightly lower. The implementation of a

dietary strategy to create an energy surplus is a commonly advocated practice when attempting to optimise skeletal muscle hypertrophy (Manore et al., 2009, Burke et al., 2000). Whilst experimental evidence is lacking to support this practice, it is logical from the perspective of providing an increased amount of energy to synthesise new tissue, and overcome the metabolic cost of possessing more metabolically active tissue (Slater et al., 2019). Moreover, it is well established that an energy deficit impairs muscle protein synthesis rates (Hector et al., 2018, Pasiakos et al., 2010, Murphy et al., 2015), and we sought to negate this eventuality. Participants in OMNI were instructed to consume an omnivorous diet, focussing their intake on high-quality animal-derived proteins (i.e. beef, pork, lamb, poultry, milk, yogurt, cheese). Participants in VEG were instructed to avoid animal products for 6 days a week, focussing their intake on non-animal-derived protein sources (Quorn, lentils, grains, tofu, meat replacement products). Participants in this group were permitted to eat animal-derived protein sources on one day of the week, however, the majority chose to abstain completely for the duration of the 10 week period. Participants in VEG were provided with a weekly supply of Quorn vegan products, receiving ~one product per day, participants in OMNI were provided with an equivalent weekly supply of chicken or beef. Participants were required to record their dietary intake for a minimum of three days each week, but were requested to record as many days as was practical, so that the researcher had more information available to them to provide feedback. All dietary recording was done via the MyfFtnessPal app (MyFitnessPal; San Francisco, CA, USA), to allow for ease of recording and real-time feedback of caloric and macronutrient intake so that participants were able to orientate themselves to the dietary targets set by the researcher. Participants emailed a copy of their dietary recordings to the lead researcher at the end of each week, such that the researcher could evaluate progress and provide feedback. When participants deviated from their target caloric and protein targets (falling significantly below them) the researcher discussed this with the participants, and proffered advice to amend the issue. If

participants were consistently unable to adhere to the protein target, falling below 1.6 g·kg $bm^{-1}·d^{-1}$, they would be excluded from the study. The OMNI group received 59 g supplemental milk protein daily (47 g protein, 2 g carbohydrate, <1 g fat, 198 kcal) to drink post-training (19.5 g) and prior to sleep (39 g). The VEG group received 105 g mycoprotein (46 g protein, 10 g carbohydrate, 13 g fat, 348 kcal), 35 g post-training and 70 g prior to bed. On non-training days the post-training dose was consumed between meals. The protein supplements were provided to make the protein target more manageable, and ensure that a proportion of the protein participants received in each group was consistent. A moderate serving of protein was provided to stimulate post-exercise muscle protein synthesis rates (Pennings et al., 2010), whilst a larger serving was provided prior to sleep to profit from a pre-sleep protein strategy (Snijders et al., 2019) and optimise protein distribution (Areta et al., 2013b). The two daily servings differed in size as it was deemed, based on pilot testing, that it would be too challenging for participants to consume two 70 g boluses of mycoprotein a day for such a prolonged period of time. Participants also supplemented with creatine throughout the study, consuming five doses of 5 g creatine monohydrate (myprotein) for five days during Week 1, before then consuming a single 5 g dose each day thereafter.

Blood Sampling

Participants reported to the laboratory on a designated each week for a blood sample. Blood samples were obtained via venepuncture, with 8 mL of venous blood collected into liquid heparin-containing tubes (BD vacutainer LH; Becton, Dickinson and Company) and centrifuged immediately (3000 × g, 4◦C, 10 min). Blood plasma was aliquoted and frozen at –80◦C for subsequent analysis. A further 6 mL of blood was collected into SST vacutainers (BD vacutainers SST II, Becton, Dickinson and Company) which were left to clot at room temperature for ≥30 min and then centrifuged (3000 × g, 4◦C, 10 min) to obtain blood serum. Serum was aliquoted before freezing at –80◦C for subsequent analyses. This visit also allowed discourse to take place between the lead researcher and participant, such that they

were able to discuss training, the diet diary they'd provided, and general progress. It also provided an opportunity to provide participants with items that they required for the study.

Magnetic Resonance Imaging

A 1.5 tesla (T) MRI scanner (Intera, Phillips, The Netherlands) was used to obtain images of the right thigh in the axial plane over the full length of the femur. A T1-weighted 3D turbo spin echo sequence was used (field of view, 500 500 mm; reconstructed matrix, 512 512 mm; echo time, 15 ms; repetition time, 645 ms; slice thickness, 5 mm; slice gap, 5 mm) with the subject lying still in the supine position. A four-element sense body radiofrequency (RF) coil was wrapped around both thighs. During the pre-testing scan a specified distance from a bony landmark (the lateral and medial femoral condyles) in the frontal plane was used to centre the axial plane images (Glover et al., 2008). This same distance was used on all subsequent MRI scans to ensure the axial images were in the same location along the length of the thigh on all scans. 3D Slicer MRI software (Slicer 4.10.2; www.slicer.org/software) was used to analyse the images obtained in the axial plane. On average ~45 images were acquired along the length of the femur, with the bottom 25% (from the lateral femoral condyle working proximally) and top 25% (from the greater trochanter working distally) excluded, in line with previous work from our lab (Kilroe et al., 2020d, Kilroe et al., 2020c). The remaining images, in the central 50% portion of the thigh, were processed using a combination of automated thresholding, manual thresholding, and manual segmentation. Briefly, individual slices of the whole thigh were automatically thresholded to provide an approximate outline of the thigh musculature, before being further processed to produce a precise final image. The same experimenter performed all manual segmentation of the images to reduce variability in processing, and therefore ensure consistency across the data set. Thigh muscle volume was automatically calculated using 3D Slicer's segment statistics function. Subsequently, quadriceps, hamstrings and adductor muscles were delineated and manually segmented, before volume was calculated in the same manner as above.

DXA

Participants underwent a whole-body dual-energy X-ray absorptiometry (DXA) scan (Lunar Prodigy) prior to testing and upon completion of the intervention to establish changes in body composition (lean mass, fat mass, and bone mineral density). Participants were scanned at the same time of day, following a 10–12 h overnight fast with no fluid intake that morning. Body position was recorded with the use of bony landmarks and scan table references to ensure that body position was maintained as constant as possible between scans. All participants were scanned with their hands by their sides in a supinated position with their feet 10 cm apart.

Immunohistochemistry

Muscle cross-sections obtained from biopsy samples were cut at -20°C (8 μm) from OCT-embedded samples, and mounted on glass slides. Samples were air-dried for 30 min, fixed with 4% paraformaldehyde (15 min), washed (3 × PBS), blocked (60 min), washed (3 × PBS), and stained with antibodies against Pax7 (PAX7 MIgG1 kappa light chain, Developmental Studies Hybridoma Bank, Iowa City, IA) overnight. After washing (3 × PBS), samples were then incubated (2 h) with primary antibodies against laminin (2E8 MIgG2a kappa light chain, Developmental Studies Hybridoma Bank) and myosin heavy chain type 1 (slow isoform) (A4.84 MIgM0, Developmental Studies Hybridoma Bank). After washing (3 × PBS), samples were incubated with appropriate secondary antibodies (1 h): MHC secondary (Goat anti-mouse IgM mu-chain, Alexa 647, abcam, Cambridge, UK), Laminin secondary (Rabbit anti-mouse IgG2a gamma-chain, Alexa 568, Thermo Fisher Scientific, Waltham, MA, USA), Pax 7 secondary (Goat anti-mouse IgG1 gamma-chain, Alexa 488, Thermo Fisher Scientific) along with DAPI staining. Samples were then washed (3 x PBS), dried, and mounted with cover glasses using Mowiol. All images were captured digitally (LAS X software; Leica Microsystems GmbH, Wetzlar, Germany) using a Leica DMi8 S widefield

fluorescence microscope (Leica Microsystems GmbH) coupled to a Hamamatsu C11440-22C camera (Hamamatsu Photonics, Shizuoka, Japan) at x20 magnification. [Epifluorescence signal was recorded by using excitation filters for DAPI (400 nm), laminin (Texas Red, 540–580 nm), PAX7 (FITC, 465–495 nm), and MHC-I (Y5, 620-650 nm)].

Statistics

In Phase 1 of the study our primary measure was daily muscle protein synthesis rates, with all other measures representing secondary measures. All data are presented as mean ± SEM and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Independent samples t-tests were used to compare each of the participants' characteristics across groups, and dietary data throughout. For Phase 1, a two-way (OMNI vs VEG and time [pre-post]) ANOVA was used to compare body mass during the nutritional intervention. A two-way (OMNI vs VEG and time [days 1-4]) ANOVA was used to compare changes in body water deuterium enrichments. A three-way (OMNI vs VEG, pre vs post and rested vs exercised leg) mixed-effects model was used to compare myofibrillar protein-bound [^{2}H] alanine enrichments. A two-way (OMNI vs VEG and rested vs exercised leg) ANOVA, independent samples t-tests, and paired t-tests were used to compare myofibrillar protein synthesis rates. For Phase 2, independent samples t-tests were used to compare baseline measures of lean mass, muscle volume, muscle fibre CSA, and muscle strength. Two-way ANOVA (OMNI vs VEG and pre vs post) were used to compare pre-post lean mass, muscle volume, muscle fibre CSA, and muscle strength. Independent samples t-tests were used to compare percentage change in lean mass, muscle volume, muscle fibre CSA, and muscle strength. When a significant interaction was found, Sidak or Tukey post hoc tests were applied to locate individual differences. Statistical significance was set at P<0.05.

RESULTS

Participant characteristics for both phases of the study are provided in Table 6.1. One participant was excluded from Phase 2 analysis due to ill health, one participant withdrew from Phase 2 due to personal reasons. In total, 21 participants completed Phase 1 of the study, and 22 completed Phase II of the study. Age, body mass, and BMI did not differ at baseline in either Phase 1 (age: 23±1 y, body mass: 69±2 kg, BMI: 23±1 kg/m^2; all P>0.05) nor Phase 2 (age: 24±1 y, body mass: 70±2 kg, BMI: 23±0 kg/m^2; all P>0.05) of the study, and groups were also well balanced for sex. No differences in total work performed during the experimental resistance exercise bouts in Phase 1 (37754±3094 J in OMNI vs 34787±2579 J in VEG; P=0.47), or in fatigue during each trial (all P>0.05) or over the week (P=0.35) were detected between groups.

Table 6.1. Participant characteristics.

	Phase 1		**Phase 2**	
	OMNI (*n* = 9) *4♀ /5♂*	**VEG** (*n* = 12) *6♀ / 6♂*	**OMNI** (*n* = 12) *6♀ / 6♂*	**VEG** (*n* = 10) *5♀ / 5♂*
Age (y)	23±2	23±2	24±2	24±2
Body mass (kg)	72±3	67±2	71±3	68±3
Height (cm)	174±4	173±3	173±3	172±3
BMI ($kg \cdot m^{-2}$)	24±1	23±0	24±1	23±1
Fat (% body mass)	25±4	23±3	25±3	23±3
Lean mass (kg)	51±5	49±3	50±4	51±4

Values represent mean ± SEM. OMNI, omnivorous diet; VEG, non-animal-derived diet.

Habitual Diet

For both Phase 1 and Phase 2, habitual diet did not differ between groups for energy intake, protein intake or carbohydrate intake (all $P>0.05$), although fat intake was higher in OMNI than VEG in Phase 1 ($P=0.062$), but not Phase 2 **(**Phase 1 habitual diet shown in **Table 6.2**). In Phase 1, energy intake increased slightly between participants' habitual diets and the diet they received during the intervention ($P=0.007$). Daily protein intake was higher (by design) during the intervention diet than in participants' habitual diets (1.8±0.0 vs 1.5±0.1 $g.day^{-1}$, respectively; $P=0.006$). Daily carbohydrate intake was also higher during the intervention diet than in participants' habitual diets ($P<0.0001$). An interaction effect was detected ($P=0.036$) such that fat intake decreased from habitual levels during the intervention diet in the OMNI group only ($P=0.019$).

Table 6.2. The nutritional content of the participants' habitual diets and of the Phase 1 intervention diets. In OMNI, participants consumed an omnivorous diet with the majority of their protein coming from animal-derived sources. In VEG, participants consumed a diet derived from non-animal sources with the majority of their protein coming from Quorn products and mycoprotein. Both groups received 1.8 $g{\cdot}kg\,bm^{-1}{\cdot}day^{-1}$ protein.

	OMNI (*n* = 9) *3♀ / 6♂*	**VEG** (*n* = 10) *4♀ / 6♂*	***P* Value**
Habitual Diet			
Energy ($MJ{\cdot}day^{-1}$ ($kcal{\cdot}day^{-1}$))	10.7±0.8 (2,559±199)	9.0±0.9 (2,156±214)	*0.20*
Protein ($g{\cdot}day^{-1}$)	121±16	95±12	*0.20*
Protein ($g{\cdot}kg\,bm^{-1}{\cdot}day^{-1}$)	1.57±0.14	1.43±0.15	*0.52*
Carbohydrate ($g{\cdot}day^{-1}$)	267±19	276±27	*0.81*
Fat ($g{\cdot}day^{-1}$)	102±11	71±8	*0.04**
Fibre ($g{\cdot}day^{-1}$)	28±6	41±4	*0.07*
Intervention Diet			
Energy ($MJ{\cdot}day^{-1}$ ($kcal{\cdot}day^{-1}$))	11.2±0.5 (2,666±130)	10.8±0.5 (2,582±125)	*0.65*
Protein ($g{\cdot}day^{-1}$)	129±7	122±4	*0.38*
Protein ($g{\cdot}kg\,bm^{-1}{\cdot}day^{-1}$)	1.8±0.00	1.8±0.00	*N/A*
Carbohydrate ($g{\cdot}day^{-1}$)	350±17	335±21	*0.59*
Fat ($g{\cdot}day^{-1}$)	75±4	73±3	*0.69*
Fibre ($g{\cdot}day^{-1}$)	35±2	70±2	*<0.0001**

Values represent mean ± SEM. OMNI, omnivorous diet; VEG, non-animal-derived diet. * indicates a difference between groups ($P<0.05$).

Phase 1 Dietary Intervention

Dietary intake during the intervention period is displayed in Table 6.2. Body mass did not change over the intervention period, in either group (both P>0.05). During the intervention period participants consumed 11.2±0.5 and 10.8±0.5 MJ (0.16±0.00 and 0.16±0.00 MJ·kg bm^{-1}·d^{-1}; 2,666±130 and 2,582±125 kcal) per day in OMNI and VEG, respectively, with no differences between groups (P=0.689). By design, daily protein intake (i.e. 129±7 and 122±4 g per day, respectively) was identical between groups during the intervention. Participants consumed 350±17 and 335±21 g carbohydrate, and 75±4 and 70±2 g fat per day in OMNI and VEG, respectively, with no differences between groups (both P>0.05). Fibre intake was higher in VEG than OMNI (70±2 g vs 35±2 g; P<0.0001) as a result of the high fibre content of mycoprotein.

In VEG, of the 129.7±7 g protein consumed per day, 67±2 g was derived from mycoprotein (27±2 g from mycoprotein within Quorn products, and 40±1 g protein from supplementary isolated mycoprotein) corresponding to 55±1% total protein intake. Remaining protein was provided by wheat and potato protein in Quorn products, and from the protein present in the other elements of the diet. Overall, Quorn/mycoprotein products provided 44±3 g daily protein and accounted for 36±1% total daily protein intake.

In OMNI, of the 122±4 g protein consumed per day, 88±4 g was provided by animal-derived sources and 41±3 g from non-animal sources, corresponding to 69±1% and 31±1% from animal and non-animal-derived sources, respectively. Meat products provided 40±0 g and dairy products (including the milk protein supplement) provided 48±4 g protein per day, corresponding to 32±2 and 37±1% of total protein, respectively. The milk protein supplement alone provided 31±0 g protein per day, 25±1% of total protein.

Body water deuterium enrichments

Saliva deuterium enrichments throughout the experimental protocol are depicted in **Figure 6.2**. From baseline (i.e. background) enrichments of 0.0009±0.0002 and 0.0013±0.0002% in OMNI and VEG, respectively, body water deuterium enrichments increased (time effect; $P<0.0001$) and reached 0.70±0.05, 0.70±0.05, 0.68±0.05 and 0.74±0.05% on days 2-5 in OMNI, and 0.72±0.04, 0.71±0.03, 0.72±0.03 and 0.73±0.03% on days 2-5 in VEG, with no differences between groups (treatment and treatment × time interaction both $P>0.05$).

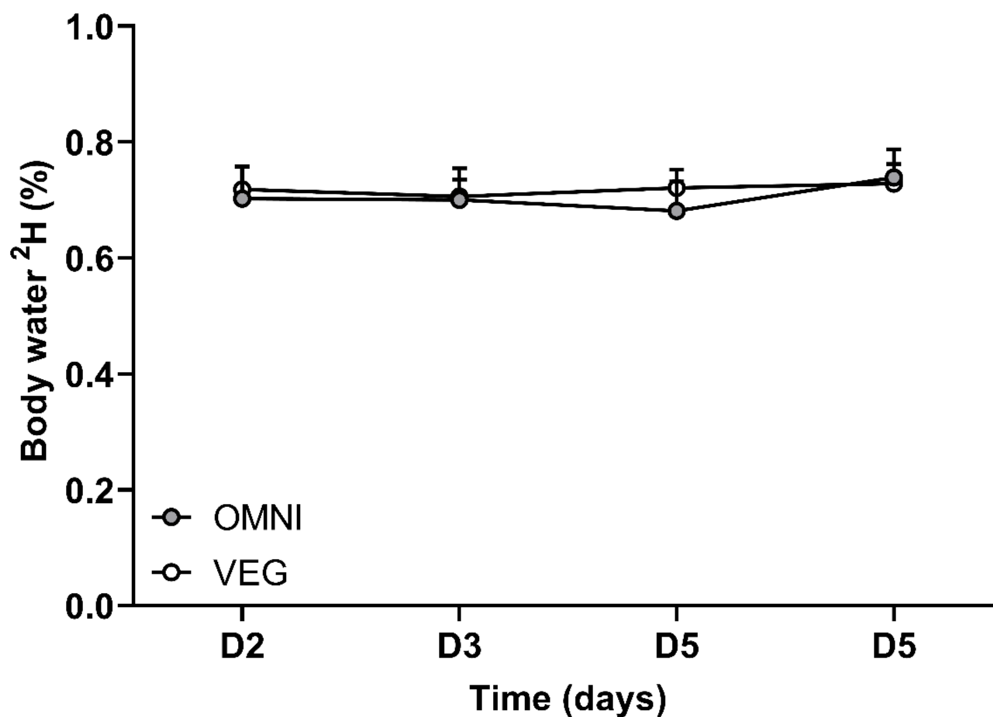

Figure 6.2. Saliva deuterium enrichments over time during oral deuterated water dosing in twenty-one healthy older adults (age, 23±1 y) consuming a 3-day fully controlled eucaloric high-protein (1.8 g·kg bm^{-1}·d^{-1}) diet, where the protein was provided predominantly from animal (OMNI; n=9) or exclusively non-animal (VEG; n=12) sources. During the dietary control period (Day 2-4) participants conducted a single bout of maximal unilateral concentric isokinetic knee extension exercise (5 x 30 contractions) each morning. On Day 1 participants were loaded with a total of 400 mL deuterated water, with 50 mL maintenance doses consumed daily thereafter. Data were analysed with mixed-effects ANOVA. Values are means, with their standard errors represented by vertical bars.

Daily myofibrillar protein synthesis rates

Myofibirillar enrichments and daily myofibrillar protein synthesis rates are displayed for an *n* of 12 (*n*=6 in each group), as the analysis is ongoing at the time of writing. Myofibrillar protein-bound [^{2}H] alanine enrichments increased over time (P<0.0001) and to a greater extent in the exercised compared with control leg (time x leg interaction; P=0.049). Myofibrillar protein-bound [^{2}H] alanine enrichments increased in the OMNI group in rested (from 0.08±0.01 to 0.26±0.02 MPE) and in exercised muscle (from 0.08 ±0.01 to 0.27±0.02 MPE), and in the VEG group (from 0.07±0.01 to 0.23±0.04 MPE) in rested and (from 0.07±0.01 to 0.29±0.02 MPE) in exercised muscle, with no differences between groups (all OMNI vs VEG interactions; P>0.05). Saliva deuterium enrichments were used as a precursor pool and myofibrillar protein-bound [^{2}H] alanine enrichments as the product to calculate daily myofibrillar fractional synthetic rates (FSRs) (**Figure 6.3**). Daily myofibrillar FSRs were 27±7 and 11±7% greater in the exercised compared with rested leg (2.2.±0.14 vs 2.46±0.11%·d^{-1} and 2.01±0.18 vs 2.39±0.09%·d^{-1}) in OMNI and VEG groups, respectively (P=0.035). Daily myofibrillar protein synthesis rates did not differ between groups in either rested (P=0.43) or exercised (P=0.63) muscle.

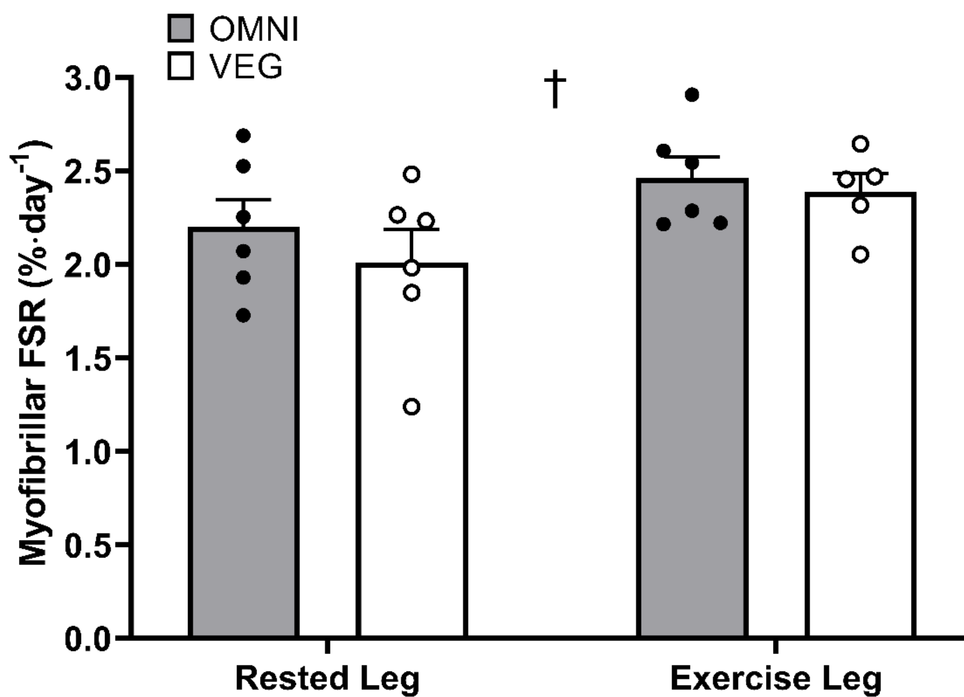

Figure 6.3. Daily free-living myofibrillar protein fractional synthesis rates (FSRs) calculated from the body water deuterium precursor pool in twelve healthy adults consuming a 3-day fully controlled eucaloric high-protein (1.8 g·kg $bm^{-1}·d^{-1}$) diet, where the protein was provided predominantly from animal (OMNI; n=6) or exclusively non-animal (VEG; n=6) sources, in rested and exercised (single bout of 5 x 30 maximal unilateral isokinetic knee extension contractions on three consecutive days) muscle. Data were analysed with two-way ANOVA, with paired *t*-tests used to detect differences between rested and exercised legs in each respective groups. Values are means, with their standard errors represented by vertical bars. † indicates a main effect of exercise ($P<0.05$).

Phase 2 dietary Intervention

Dietary intake during the intervention period is displayed in **Figure 6.4**. The diet was well adhered to, with participants providing dietary records in 99.1% of cases. Energy intake did not differ between groups when expressed in absolute terms, 2882±140.3 in OMNI and 2839±202.9 kcal in VEG (P=0.8605), or relative to body mass, 40.4±1.7 in OMNI and 40.4±1.8 kcal·kg $bm^{-1}·d^{-1}$ in VEG (P=0.9920). Likewise, neither fat intake nor carbohydrate intake differed between the two groups, either in absolute terms or when expressed relative

to body mass (P>0.05). Absolute protein intake did not differ between groups, 163.3±9.9 in OMNI and 145.6±9.6 g in VEG (P=0.2211), or relative to body mass, 2.3±0.08 in OMNI and 2.1± 0.06 in VEG g·kg $bm^{-1}·d^{-1}$ (P=0.0898).

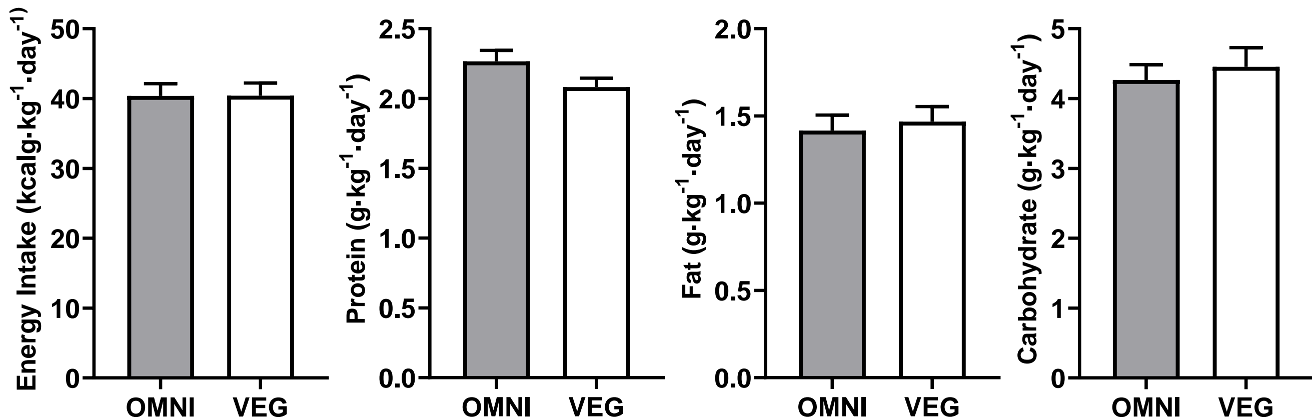

Figure 6.4. Energy intake (kcal·$bm^{-1}·d^{-1}$), protein intake (g·kg $bm^{-1}·d^{-1}$), fat intake (g·kg $bm^{-1}·d^{-1}$) and carbohydrate intake (g·kg $bm^{-1}·d^{-1)}$ during 10 weeks of resistance exercise training in healthy young men and women consuming either a high-protein omnivorous diet (OMNI; n=12) or a majority non-animal-derived diet (VEG; n=9), expressed as both absolute values and percent change. Values are means ± SEMs. † indicates a significant effect of training (P<0.05). * indicates a significant difference between groups (P<0.05).

Body Composition

Whole-body lean mass and fat mass is displayed in **Figure 6.5**. At baseline, neither lean mass nor fat mass were different between groups (P>0.05). Lean mass increased with training (OMNI 50.5±3.6 to 53.1±3.7 kg, VEG 50.6±3.7 to 53.7±4.4 kg) (time effect; P<0.0001), with no differences between groups (treatment and treatment × time effects; P>0.05). Fat mass remained unchanged in response to training and the dietary interventions (time, treatment, and treatment × time effects; P>0.05). Total lean mass increased 5.4±0.8% in OMNI, and 5.6±1.2% in VEG, with no differences between groups (P=0.9298). The

increase in lean mass was apparent in the legs (OMNI 4.7±1.4%, VEG 5.0±1.3%), arms (OMNI 10.6±1.4%, VEG 10.2±1.8%), and trunk (OMNI 5.2±1.3%, VEG 5.3±1.4%), with the percentage change greater in the arms than legs (*P*=0.0008), and trunk (*P*=0.0021), but with no differences between groups (treatment and treatment × region effects; *P*>0.05).

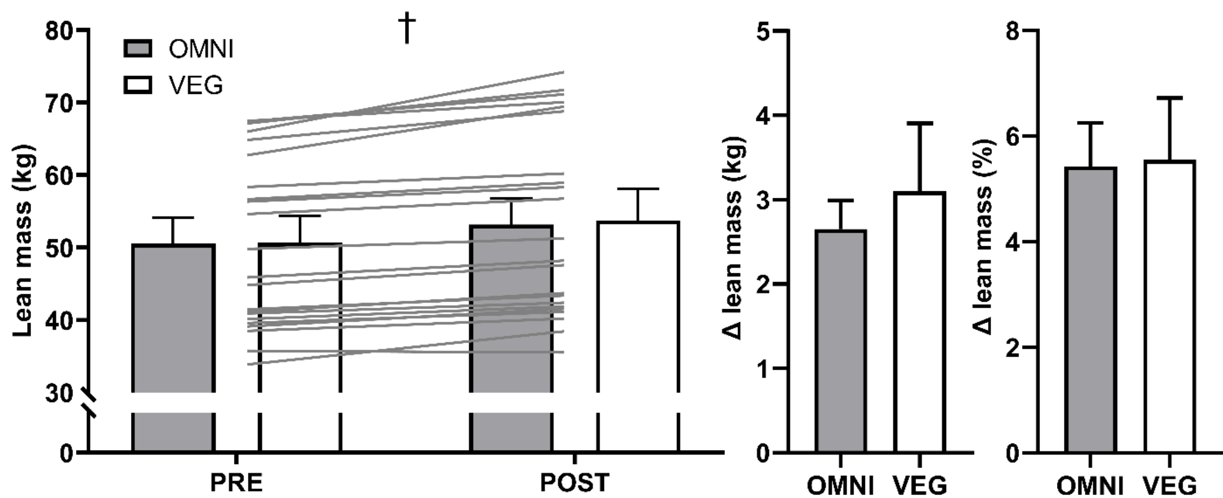

Figure 6.5. Whole-body lean mass before and after 10 weeks of resistance exercise training in healthy young men and women consuming either a high-protein omnivorous diet (OMNI; *n*=12) or a majority non-animal-derived diet (VEG; *n*=10), expressed as both absolute values and percent change. Values are means ± SEMs. † indicates a significant effect of training. No differences were observed between groups.

Skeletal Muscle Size

Whole thigh, quadriceps, hamstring and adductor volumes determined via MRI are displayed in **Figure 6.6**. At baseline, no significant differences in whole thigh, quadriceps, hamstrings, and adductor volume were observed (all *P*>0.05). Whole thigh (OMNI Δ246±43, VEG Δ269±65 cm^3, quadriceps (OMNI Δ 125±23, VEG Δ 127±28 cm^3), hamstring (OMNI Δ 55±11, VEG Δ 64±18 cm^3), and adductor (OMNI Δ 73±12, VEG Δ 77±22 cm^3) volume increased with training in both groups (time effect; *P*<0.0001), with no differences between

groups (treatment and treatment × time effects; $P>0.05$). Likewise, no significant differences in whole thigh (OMNI 8±1%, VEG 8.2±1.4%), quadriceps (OMNI 8.1±1.2%, VEG 7.7±1.1%), hamstring (OMNI 8.4±1.3%, VEG 9.7±2.3%) or adductor (OMNI 8.7±0.9%, VEG 8.5±1.9%) volume percentage change were observed between groups ($P>0.05$).

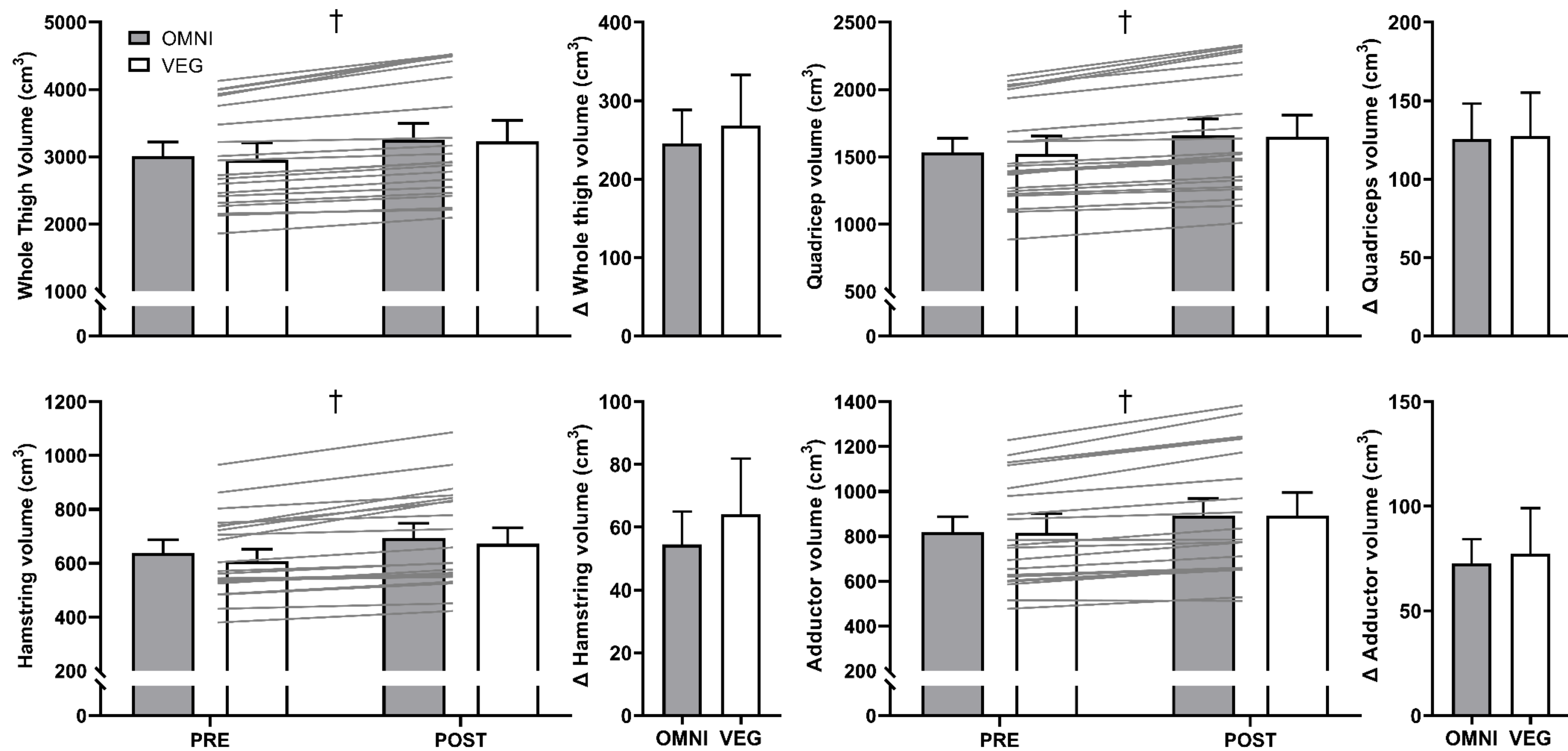

Figure 6.6. Whole thigh, quadriceps, hamstring and adductor muscle volume before and after 10 weeks of resistance exercise training in healthy young men and women consuming either a high-protein omnivorous diet (OMNI; *n*=12) or a majority non-animal-derived diet (VEG; *n*=9). Values are means ± SEMs. † indicates a significant effect of training. No differences were observed between groups.

Skeletal Muscle Fibre Size

Data are displayed for an *n* of 14 (OMNI *n*=6, VEG *n*=8), as data analysis is ongoing at the time of writing. Average, Type I, and Type II muscle fibre cross-sectional areas are displayed in **Figure 6.7**. At baseline, no significant differences in total, type I and type II muscle fibre CSA were observed between groups ($P>0.05$). Total (OMNI Δ 1724±530, VEG Δ 1522±958 μm^2) and Type II (OMNI Δ 1907±556, VEG Δ 1476±932 μm^2) fibre CSA increased with training in both groups (time effect; $P=0.0196$ and $P=0.0160$, respectively), whereas Type I remained unchanged (time effect; $P=0.1003$). No differences were observed between groups for total, type I or type II CSA (treatment and treatment × time effects; $P>0.05$). Likewise, no significant differences in total (OMNI 33±10%, VEG 32±17%), type I (OMNI 29±10%, VEG 24±18%), or type II (OMNI 33±8%, VEG 32±18%) CSA percentage change were observed between groups ($P>0.05$).

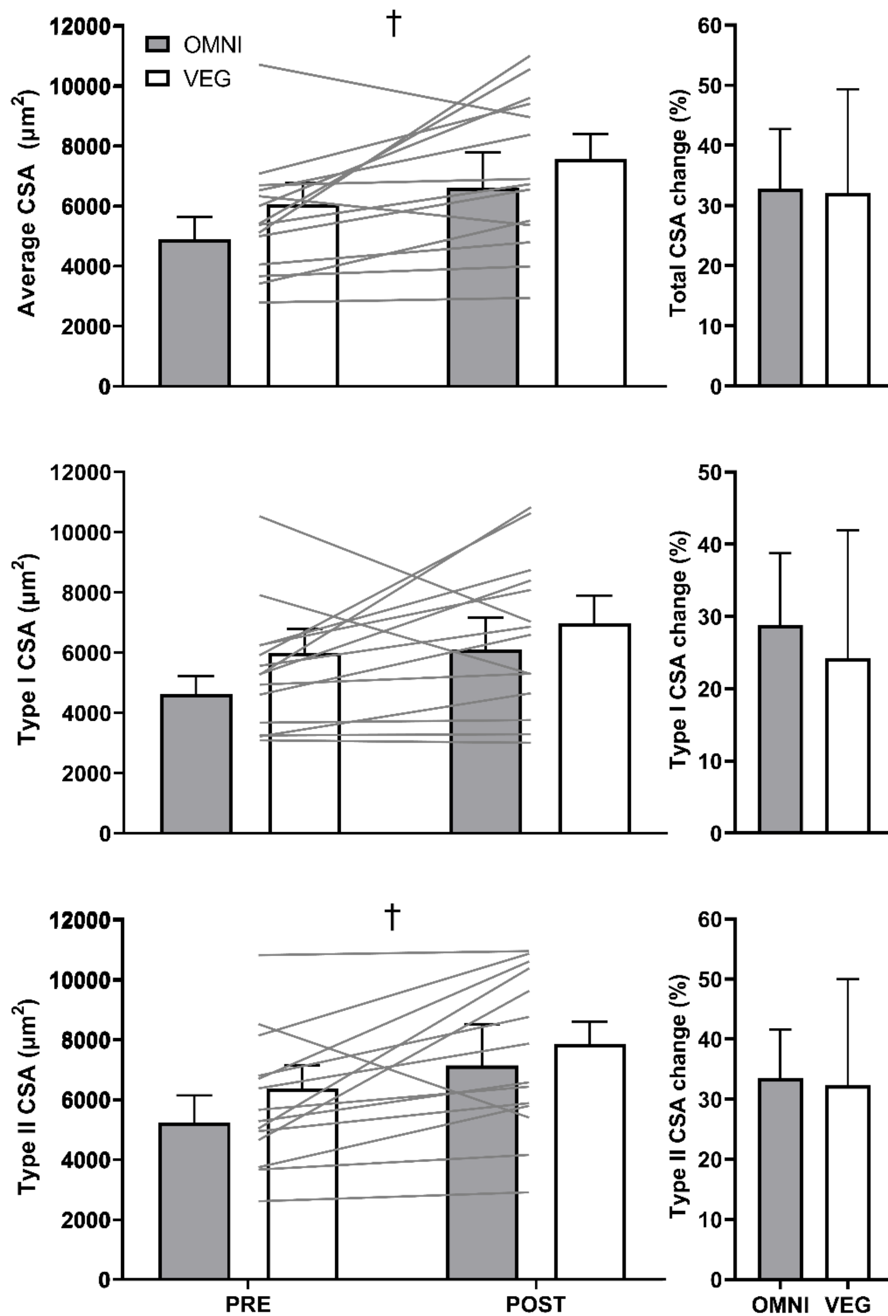

Figure 6.7. Average, Type 1, and Type II muscle fibre cross-sectional area before and after 10 weeks of resistance exercise training in healthy young men and women consuming either a high-protein omnivorous diet (OMNI; *n*=6) or a majority non-animal-derived diet (VEG; *n*=8), expressed as both absolute values and percent change. Values are means ± SEMs. † indicates a significant effect of training. No differences were observed between groups.

Muscle Strength

Strength data are displayed in **Figure 6.8**. At baseline, there were no differences in strength for the deadlift (P=0.4945), squat (P=0.5438), or incline bench press (P=0.6091). Strength increased with training in all three of these measures (time effects; all P<0.0001), with no differences between groups (all P>0.05) or interaction effects (all P>0.05). The percentage change in strength did not differ between groups for the deadlift (P=0.0526) or squat (P=0.1775), but was significantly greater in VEG than OMNI for the incline bench press (P=0.0400). MVC strength increased with training, from 225±17 to 239±18 N·m in OMNI, and 215±22 to 228±24 N·m in VEG (time effect; all P<0.0001), with no differences between groups (all P>0.05) or interaction effects (all P>0.05).

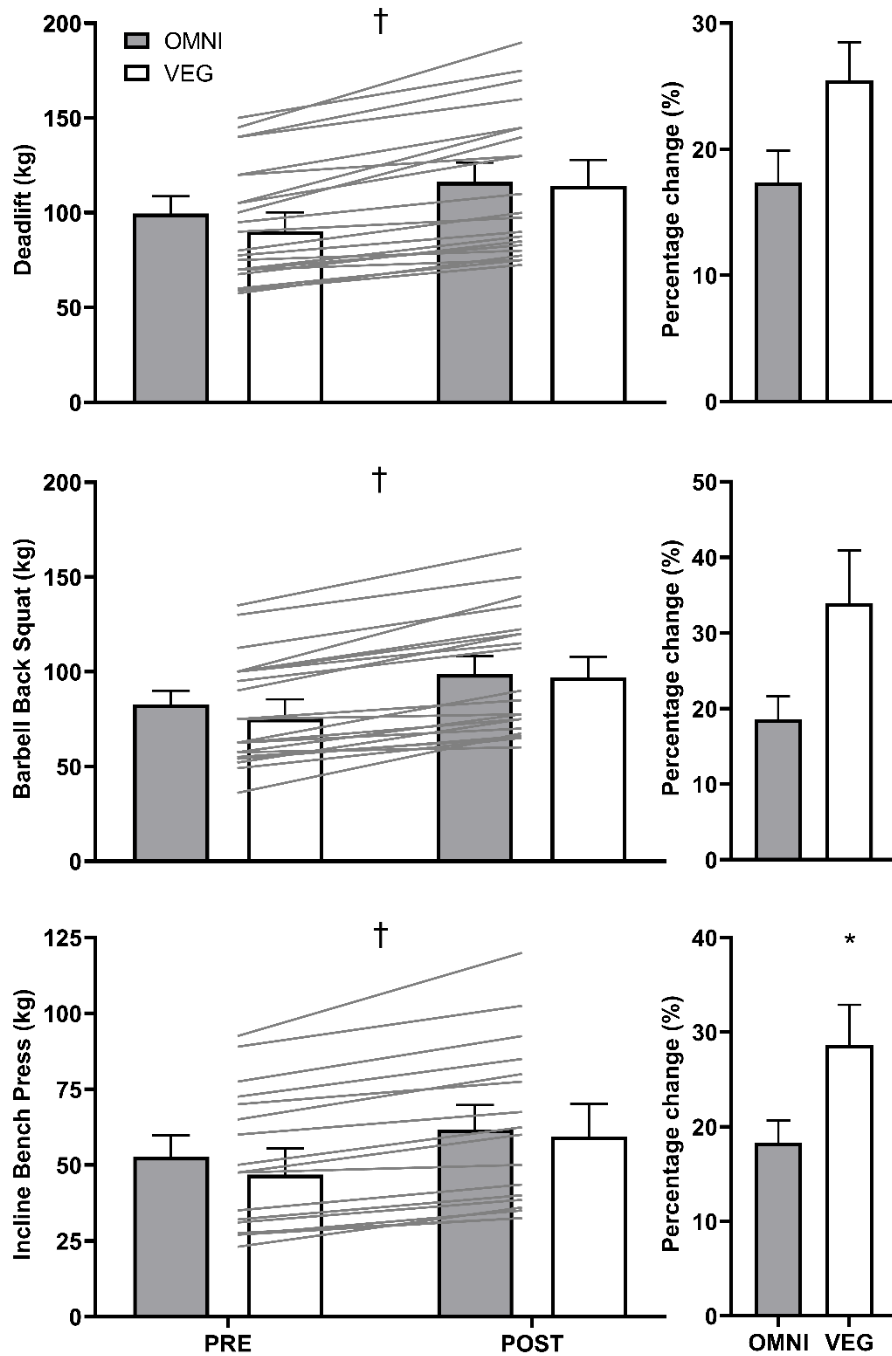

Figure 6.8. One-repetition maximum strength is the deadlift, barbell back squat and incline bench press before and after 10 weeks of resistance exercise training in healthy young men and women consuming either a high-protein omnivorous diet (OMNI; *n*=11) or a majority non-animal-derived diet (VEG; *n*=9), expressed as both absolute values and percent change. Values are means ± SEMs. † indicates a significant effect of training (*P*<0.05). * indicates a significant difference between groups (*P*<0.05).

DISCUSSION

In the current study we demonstrate that a high-protein (1.8 g·kg $bm^{-1} \cdot d^{-1}$), mycoprotein-rich, non-animal-derived diet is capable of facilitating equivalent daily muscle protein synthesis rates as a high-protein omnivorous diet, in both rested and exercised muscle. Moreover, a non-animal-derived, high-protein, mycoprotein-rich diet is able to support equivalent resistance training-induced hypertrophic and strength adaptations to that of a high-protein omnivorous diet. We, therefore, present proof-of-concept that non-animal-derived proteins can support muscle hypertrophy, under dietary and exercise conditions expected to elicit a near-optimal magnitude of hypertrophy. Animal-derived proteins are not an essential prerequisite for optimising resistance training adaptation when consuming a high-protein diet. Our results suggest that if one is to take a careful and considered approach to utilising a vegan diet, that significant increases in lean mass and strength are achievable, in young men and women with prior resistance training experience.

Government, consumer, and commercial priorities are shifting, increasing the impetus for sustainable alternatives to intensively-farmed protein products that incur a significant environmental cost. Research has likewise begun to focus more keenly on *where* we should obtain our dietary protein from, as opposed to simply *when* and *how much* protein. The provenance of protein is particularly pertinent for those involved in structured resistance exercise, where there is a scientific consensus that to (optimally) support skeletal muscle training adaptations individuals should consume protein in an amount above the RDA (Morton et al., 2018). The limited number of studies we have at our disposal suggest, that when dose-matched, plant-based protein sources are inferior to animal-derived protein sources with respect to their ability to stimulate muscle protein synthesis rates (Tang et al., 2009, Gorissen et al., 2016b, Yang et al., 2012b). This would imply that, compared to an omnivorous diet, those adhering to a vegan diet would need to consume more protein to stimulate equivalent daily muscle protein synthesis rates. Indeed, we know that protein

quality can modulate daily muscle protein synthesis rates even under isonitrogenous conditions (Oikawa et al., 2020b). Accordingly, there are implications for those seeking to drive hypertrophic adaptation, for the practicalities of adhering to a non-animal diet if markedly more protein need be consumed to overcome a lower anabolic value, and for the environmental sustainability of such a dietary strategy.

Conversely, we have recently shown that mycoprotein stimulates postprandial muscle protein synthesis rates comparable to the ingestion of milk protein (Monteyne et al., 2020b), and supports equivalent daily myofibrillar protein synthesis rates compared with an isonitrogenous omnivorous diet in older adults (Monteyne et al., 2020c). Due to this anabolic equivalence, we theorised that the incorporation of mycoprotein into a high-protein vegan diet would stimulate equivalent daily myofibrillar protein synthesis rates to an omnivorous diet, in young adults. In line with our hypothesis, we observed comparable daily free-living rates of myofibrillar protein synthesis between animal and vegan diets in both rested (2.20 vs 2.01%, respectively) and exercised (2.46 vs 2.39%, respectively) muscle (Figure 6.3). We, therefore, provide proof-of-concept that a high-protein non-animal-derived diet can be adopted without compromising muscle protein synthesis rates, in young healthy individuals, akin to what we have shown in older adults. Further, our data indicate that a non-animal diet is not inherently inferior in its capacity to support daily muscle protein synthesis rates following resistance exercise. Importantly, the data are collected in free-living conditions over multiple days, thereby encapsulating total muscle protein synthesis in response to the two divergent diets, and in turn incorporating habitual activity, diurnal variation and multiple feeding-fasting cycles. Accordingly, and in extension of our previous data (Monteyne et al., 2020b), the equivalence we observe in daily muscle protein synthesis represents a net equivalency in the muscle protein synthetic response to each and every meal throughout the dietary intervention. Whilst we saw a greater response of mycoprotein compared with milk protein in our previous work, this did not translate into greater daily rates of muscle

protein synthesis in the current investigation. Whilst we can only speculate as to the reasons why this might be, the added complexity of consuming a diet with protein coming from many different foods surely contributed. Mycoprotein *might* have been anabolic than milk protein, however, other elements of the diet may have proved less anabolic in VEG than their equivalents in OMNI; dairy replacement products compared to dairy products, for example.

There is limited direct evidence to suggest that non-animal-derived dietary protein sources might not optimally support optimal skeletal muscle adaptive responses to prolonged resistance training. Soy protein supplementation has been shown to elicit a lower magnitude of hypertrophy than dairy protein supplementation (Hartman et al., 2007, Volek et al., 2013), forming the crux of the narrative for plant-based inferiority. However, this should not be surprising as we know that soy protein is not a particularly potent stimulator of muscle protein synthesis (Tang et al., 2009, Yang et al., 2012b). Moreover, the longitudinal studies on which the narrative of non-animal based protein inferiority is formed consider *supplementation*, not diet. To properly form an evidence base for non-animal proteins, and non-animal protein diets, it is necessary to consider the diet as a whole. Our data demonstrate that a comparable adaptive response can be achieved when consuming a non-animal-derived diet, to consuming an omnivorous diet, provided that enough protein is provided and a proportion of that protein is of sufficiently high quality to robustly stimulate postprandial muscle protein synthesis. Whilst it has been suggested that non-animal proteins may support muscle anabolism if they were consumed in a large enough quantity (van Vliet et al., 2015b, Norton et al., 2009), we are the first to provide empirical evidence confirming this supposition. Significantly, and prototypically, we do so within the context of a practical, self-selected, non-animal-derived diet, as opposed to a conclusion drawn upon supplementation alone.

From the outset of designing the study we endeavoured to take an optimal approach to inducing muscle hypertrophy, both from the perspective of nutrition and training. We

observed an increase in lean mass of 2.8 kg (5.5%), an increase of 8.1% in muscle volume of the thigh (7.9% quadriceps, 8.9% hamstrings, 8.6% adductors), and an increase of 32.4% in muscle fibre CSA. The response that we induced somewhat surpassed our expectations, based on similar studies within the literature. For instance, a systematic review by Benito et al. (2020) suggests resistance exercise improves lean mass gains by 1.6 kg in healthy adult males, whilst the systematic review from Morton and colleagues (2018) suggests an increase in FFM of 1.1 kg with resistance exercise. Although valuable, such analysis compare relatively heterogeneous sets of studies, so it is worth comparing to similar studies within the field. Whilst an exhaustive comparison is not feasible here, Snijders et al. (2015) showed a gain of 1.9 kg in lean mass after 12 weeks of whole-body resistance training (protein intake; 1.9 $g \cdot kg\ bm^{-1} \cdot d^{-1}$), whilst Hartman et al. (2007) observed an increase of 3.9 kg FBFM over the same period of time (protein intake; 1.8 $g \cdot kg\ bm^{-1} \cdot d^{-1}$). Both of these studies were in young men, where greater absolute increases in lean mass are probable due to greater relative lean body mass, whereas our own work included both males and female participants. To provide a point of comparison, Josse et al., (2010) observed a 1.9 kg increase in lean mass following 12 weeks of whole-body resistance exercise training in young women (estimated protein intake; 1.5 $g \cdot kg\ bm^{-1} \cdot d^{-1}$). When delineated by biological sex, we observe increases in lean mass of 3.6±0.7 and 2.1±0.4 kg, with a range of 6.8 and 4.8 kg, in males and females, respectively. Clearly, our results are at the upper end of what might be expected in terms of hypertrophy, and demonstrate that a non-animal-derived diet is able to facilitate the upper demands of potential hypertrophy. Though the individuals in the current study did have experience of resistance training, to differing degrees, they were not highly resistance-trained. This might explain, in part, the extent of the response, as from the outset of training there was ample room for an adaptive response, alongside the necessary training acumen to create a large hypertrophic stimulus.

Significant muscle hypertrophy was seen alongside considerable increases in strength (23% on average), although these measures were far more variable in nature. The reasons for the variability in strength measures, and the divergence in extent compared to measures of muscle hypertrophy, are not entirely clear, but are likely to relate to the multifaceted nature of adaptive changes in muscle strength. Indeed, a change in functional strength in a multi-joint exercise is not just contingent upon a change in muscle size, but, rather, a complex array or neuromuscular adaptations, alongside improvements in technical proficiency (Folland and Williams, 2007, Balshaw et al., 2017). The adaptive increase in strength appears to be slightly greater in VEG than OMNI, although it was also far more variable in this group, particularly in the squat and incline bench press, muddying the waters for interpretation. Moreover, one individual exhibited exceptionally large relative improvements in squat and incline bench press strength (>50%), representing an outlier. Although augmenting strength was of secondary importance to that of hypertrophy in programme design, the observed increase in strength does present a cogent example of the translation of our findings on mycoprotein from a molecular level (Monteyne et al., 2020b, Monteyne et al., 2020a), all the way to the level of functional movement.

One consideration when interpreting our results is that all participants in the study supplemented with creatine, and we cannot discount that there may have been more of a discrepancy between conditions had this not been the case. This was a component of the "optimal" approach that we took (Rawson and Volek, 2003, Cooper et al., 2012), and we reasoned that there is no reason why those adhering to a non-animal-derived diet could not supplement with creatine. Nevertheless, we acknowledge that this may have reduced any potential difference between the two diets *per se*, as those consuming a diet richer in meat typically have a higher concentration of intramuscular phosphocreatine (Burke et al., 2003, Delanghe et al., 1989). Indeed, it might be necessary that those adhering to a non-animal-derived diet supplement with various other nutrients, vitamin B12 for example (Gilsing et al.,

2010). Whilst any deficiencies in the non-animal-derived diet did not inhibit adaptation over the period of investigation, it cannot be discounted that this would not be the case over a longer period of time. One anecdotal finding that we observed in the study was that whilst consuming a high-protein vegan diet is feasible, participants in the non-animal group appeared to find it far more difficult to reach their protein target. This appears to be supported by the literature, where those following vegan and vegetarians have slightly lower habitual protein intakes (Allès et al., 2017, Schmidt et al., 2016, Clarys et al., 2014). Whilst the causation for this is no doubt multifaceted, the relatively low percentage protein content of many plant-based foods surely contributes. Lastly, as we did not measure habitual physical activity during the three days of controlled diet, we cannot discount that differences in physical activity might have affected daily muscle protein synthesis rates.

To conclude, we present proof-of-concept that a non-animal-derived diet can support both equivalent daily muscle protein synthesis rates, and equivalent resistance training-induced skeletal muscle remodelling, as a high-protein omnivorous diet. This demonstrates that under near-optimal nutritional and exercise-training conditions, non-animal-derived diets have the capacity to support muscle tissue anabolism, and facilitate hypertrophic and strength adaptations in healthy young men and women.

CHAPTER 7

GENERAL DISCUSSION

"All generalizations are false, including this one."
Mark Twain

A SUMMARY OF THE THESIS AIMS & PRIMARY FINDINGS

In this thesis I have assessed the capacity of mycoprotein to acutely, intermediately, and chronically support muscle anabolism. Prior to this, there was little appreciation, nor direct measurement, of the capacity of mycoprotein, a novel protein source, to support acute or longitudinal muscle tissue remodelling. I present here that the ingestion of a single dose of mycoprotein stimulates muscle protein synthesis, that enriching a smaller bolus of mycoprotein with BCAAs does not rescue the muscle protein synthetic response, that a vegan-mycoprotein diet supports equivalent cumulative muscle protein synthesis to an omnivorous diet (in young and old individuals), and that a vegan-mycoprotein high-protein diet is able to support equivalent adaptive responses to that of an omnivorous diet. As such, I have comprehensively assessed the role of mycoprotein to support hypertrophic remodelling. Furthermore, the work shown here adds significantly to the paucity of data considering the capacity of non-animal-derived protein sources to support muscle tissue remodelling. Indeed, the data presented herein are the first to show parity in muscle protein synthesis rates between the ingestion of an animal and non-animal-derived protein source. Furthermore, this work is the first to examine the effect of a vegan diet on muscle protein synthesis rates, and subsequently, the first to show parity in cumulative muscle protein synthesis rates between two diets derived from different sources. I thereby translate the muscle protein synthetic response from a single meal basis, in hours, to a whole diet, in days. Additionally, as far as the author is aware we are the first to investigate the hypertrophic remodelling effect to *diets* differing in protein provenance; as opposed to investigation along the lines of amount or supplementation. Herein, there is further evidence to support the emerging idea of a “whole food” effect; a purported non-amino acid derived potentiation of the muscle protein synthetic response. Lastly, the work challenges the ostensible essentiality of a rapid and high magnitude of leucinemia to optimise muscle protein synthesis. In encapsulation, this thesis tracks the impact of mycoprotein ingestion

on skeletal muscle anabolism within hours of ingestion, through to months of consumption within a diet, while simultaneously shedding light on mechanisms of muscle anabolism concerning the role of leucine and the whole food matrix. In essence, from molecule to movement.

Mycoprotein ingestion stimulates protein synthesis rates to a greater extent than milk protein.

In the first study I aimed to quantify the mixed skeletal muscle protein synthetic response to the ingestion of a single bolus of mycoprotein compared with a leucine-matched bolus of milk protein (2.5 g leucine), in rested and exercised muscle of resistance-trained young men. Further, to gain a broader insight into the anabolic response of the muscle cell I examined the postprandial and postexercise transcriptional response to mycoprotein and milk protein ingestion, and resistance exercise, by utilising a 48 gene Taqman low-density microarray genecard. I hypothesised that mycoprotein would robustly stimulate mixed muscle protein synthesis, albeit to a less extent than a leucine matched bolus of milk protein, which previous research had shown to provoke more rapid and higher magnitude hyperaminoacidemia.

Contrary to our hypothesis, mixed muscle protein synthesis rates increased to a greater extent following the ingestion of mycoprotein than milk protein, and the postprandial increase in muscle protein synthesis was greater following the ingestion of mycoprotein than the ingestion of milk (Δ 0.022%·h^{-1}). This occurred despite mycoprotein ingestion resulting in slower and lower rises in plasma amino acid (and leucine) concentrations compared with the ingestion of milk protein. It can therefore be concluded that mycoprotein ingestion stimulates resting and postexercise muscle protein synthesis rates, and it appears to do so to a high magnitude. I would postulate that these results evidence the potential for a "whole food" potentiating effect of mycoprotein, and add credence to suggestions that postprandial

plasma leucinemia is only partially predictive of the subsequent muscle protein synthetic response. Additionally, exercise and protein ingestion resulted in a coordinated transcriptional response, demonstrated by a change in the mRNA expression of 27 of the 46 genes of interest. The upregulation in the expression of genes involved in the regulation of amino acid transporters, protein synthesis, and protein breakdown underline the rapid transcriptional responses that occur with nutrition and exercise. Despite the divergent digestion and absorption kinetics of mycoprotein and milk protein, the two did not lead to any meaningful differences in the transcriptional response of the genes selected. This study identified mycoprotein as a non-animal-derived protein source that supports acute tissue remodelling, and one that might subsequently support tissue remodelling over a longer period of time as a component of the diet. In turn, this work provided the framework, and rationale, for considering longer-term measures of muscle anabolism and phenotypic change.

Branched chain amino acid fortification does not restore muscle protein synthesis rates following ingestion of lower compared with higher dose mycoprotein.

In the second study I aimed to determine if a lower dose (35 g) of mycoprotein enriched with branched-chain amino acids (BCAAs) stimulated mixed skeletal muscle protein synthesis to the same extent as 70 g of mycoprotein in resistance-trained young men. In essence, to investigate whether we could get 'more out of less' anabolically, utilising less product from a sustainability and appetite regulation standpoint. I also sought to further probe the effect of leucinemia on the muscle protein synthetic response, using the two different mycoprotein drinks as a model to produce divergent plasma leucine responses. I hypothesised that the ingestion of the lower-dose mycoprotein beverage (35 g) fortified with BCAAs (resulting in 18.7 g total protein, 2.5 g leucine) would stimulate resting and postexercise mixed muscle

protein synthesis rates to a similar extent as the larger (70 g; 31.5 g protein) BCAA-matched bolus of mycoprotein. Postulating, that in a within-protein context (mycoprotein-to-mycoprotein) that the matching of leucine, isoleucine and valine content would rescue the muscle protein synthetic response to provide parity to that of a larger bolus.

Again, contrary to our hypothesis, whilst the lower-dose enriched mycoprotein beverage stimulated resting and postexercise muscle protein synthesis rates, even in this within-protein context it did so to a lesser extent compared with the ingestion of the larger bolus. This was observed despite a more rapid and greater increase (both peak magnitude and overall availability) in postprandial plasma concentrations of all 3 BCAAs following the ingestion of the lower-dose enriched mycoprotein beverage. In agreement with the findings described in Chapter 3, the greater postprandial muscle protein synthetic responses to the larger mycoprotein bolus occurred despite lower and slower increases in plasma leucine concentrations, so I would propose that plasma leucine concentrations are only partially responsible for regulating postprandial muscle protein synthesis rates, at least where mycoprotein is concerned. Further, the greater muscle protein synthetic response to the larger mycoprotein bolus adds credence to the previous suggestion of a “whole food” potentiating effect on muscle protein synthesis rates. Equally so, and in caveat, the results could simply be evidence of the greater protein content of the larger mycoprotein bolus stimulating a greater muscle protein synthetic response. This postulation in itself is intriguing, as it would suggest a different mechanism than those accepted to drive the dose-response relationship.

A mycoprotein based high-protein vegan diet supports equivalent daily myofibrillar protein synthesis rates compared with an isonitrogenous omnivorous diet in older adults.

In the third study I broadened the scope of the research to consider the *diet* in free-living conditions, transitioning to investigating older individuals, a population that is of particular interest to researchers given their apparent insidious dysregulation of protein metabolism. I applied an oral deuterated water approach to determine whether a mycoprotein-based high-protein non-animal diet could support rested and exercised daily muscle protein synthesis to the same extent as an isonitrogenous omnivorous protein diet. I hypothesised that in older adults consuming a high-protein diet muscle protein synthesis rates would be similar irrespective of whether dietary protein was obtained primarily from animal or non-animal sources, and that daily resistance exercise would increase daily muscle protein synthesis rates compared with rested muscle.

In line with the stated hypothesis, a single bout of daily unilateral knee extensor resistance exercise modestly potentiated daily myofibrillar protein synthesis rates compared with a rested control leg in older adults consuming a high-protein (1.8 $g \cdot kg\ bm^{-1} \cdot d^{-1}$) diet. Additionally, daily myofibrillar protein synthesis rates were equivalent regardless of whether dietary protein was obtained primarily from animal or exclusively from non-animal sources, in both rested and exercised muscle. Therefore, when consuming a high-protein diet whether said protein comes from animal or non-animal sources does not modulate daily myofibrillar protein synthesis rates, and obtaining dietary protein from animal-derived sources is not an essential prerequisite to support daily myofibrillar protein synthesis rates in older adults. Of note, is that this study is the first to consider the muscle protein synthetic response to a purely non-animal-derived diet.

A non-animal-derived high-protein diet supports resistance training adaptation to the same extent as an isonitrogenous animal-protein based diet.

In the fourth study I broadened the scope further, applying a two-pronged approach to investigate the ability of non-animal-derived protein diets to support daily muscle protein synthesis rates and resistance exercise adaptation, in young healthy individuals. Firstly, I applied an oral deuterated water approach to determine whether a mycoprotein-based, high-protein, non-animal diet could support rested and exercised daily muscle protein synthesis to the same extent as an isonitrogenous omnivorous protein diet, in young healthy adults. Secondly, I applied a 10-week high-intensity, high-frequency, high-volume progressive resistance training regime to determine whether a majority non-animal-derived high-protein diet could support resistance exercise adaptation to the same extent as an omnivorous high-protein diet. Resistance exercise adaptation was characterised by determining changes in lean mass, fat mass, muscle volume, muscle fibre morphology, muscle strength and function. I hypothesised that daily muscle protein synthesis rates would be similar irrespective of whether dietary protein was obtained primarily from animal or non-animal sources, and likewise, that resistance exercise adaptation would be similar irrespective of dietary protein source.

Firstly, in line with the hypothesis, daily myofibrillar protein synthesis rates were equivalent regardless of whether dietary protein was obtained from omnivorous or exclusively from non-animal sources, in both rested and exercised muscle. Therefore, when consuming a high-protein diet, whether protein is obtained from animal or non-animal sources does not modulate daily myofibrillar protein synthesis rates, and obtaining dietary protein from animal-derived sources is not an essential prerequisite to support daily myofibrillar protein synthesis rates in young men and women. Secondly, resistance adaptation was equivalent in all measures, irrespective of whether a majority non-animal or an omnivorous high-protein diet

was consumed. This would suggest that if enough protein (~$2g \cdot kg\ bm^{-1} \cdot day^{-1}$) is consumed, a non-animal-derived diet can support a high degree of resistance training adaptation, equivalent to that of an omnivorous diet. Moreover, consuming protein from animal-derived sources is not an essential prerequisite to support resistance training adaptation. Of note, this is the first study to consider the adaptive remodelling response to what was an almost exclusively non-animal-derived diet

This work directly translates the acute findings on mycoprotein to a protracted context, as the equivalency of the response is directly predicated on a net equivalency in the anabolic response to each meal consumed. The greater anabolic response observed in the initial acute work did not translate into greater daily muscle protein synthesis rates, nor greater adaptive remodelling, perhaps underlying the importance of investigating the anabolic response over acute, intermediate and chronic periods of time in over to deliver a full characterisation.

THE IMPLICATIONS OF THE RESEARCH FINDINGS

Acute Implications

Firstly, the data in Chapters 3 and 4 demonstrate that mycoprotein is capable of robustly stimulating muscle protein synthesis rates, adding to the relatively scant data on non-animal-derived protein sources. Furthermore, the ingestion of mycoprotein stimulated muscle protein synthesis rates to a comparable, and greater, extent as a leucine-matched bolus of milk protein over a four-hour postprandial period. This is an exception when compared to previous investigations of non-animal-derived protein sources, where soy protein, in both young (Tang et al., 2009) and older individuals (Yang et al., 2012b), and wheat protein in older individuals (Gorissen et al., 2016b), proved less anabolic than dairy proteins. We are the first to show parity in the muscle protein synthetic response between comparable doses of an animal and non-animal-derived protein. This is significant, and potentially impactful, as it demonstrates that mycoprotein is a high-quality protein, with a high metabolic value, that might represent a suitable protein to replace some of the meat and dairy in our diets. On the note of protein quality, it could be argued that true "quality" can only be discerned once muscle protein synthesis rates consequent to the ingestion of that protein have been determined. Increasingly so, the term protein quality is semantically conflated with a number of measures, i.e. amino acid composition, DIAAs, PDCAAs and bioavailability, all of which essentially become obsolete, or of lesser meaning, when the true anabolic value of that protein has been quantified. The fact that mycoprotein stimulates a high magnitude of muscle protein synthesis introduces the possibility that other non-animal-derived proteins might achieve a similar result. Given that it is imperative we broaden our evidence base and seek suitable alternative protein sources, this is a particularly encouraging proposition. However, it is worth considering that mycoprotein is a relatively unique "non-animal" whole food, deriving as it does from a fungal source, and it may differ significantly in terms of metabolic handling compared to other non-animal foods. Thus, whilst there are other

differences to the soy and wheat protein investigations listed above, the use of isolated as opposed to whole foods for instance, the contrasting results of these studies may partially reflect the atypical nature of mycoprotein amongst non-animal-derived foods.

The findings illustrated in Chapters 3 and 4 add to an emerging body of evidence that suggests a more complex, and nuanced, role of leucine in the stimulation of postprandial muscle protein synthesis. Leucine content, and postprandial leucine concentrations, have been suggested to be the primary anabolic stimulus, one that can predict postprandial muscle protein synthesis rates (Phillips, 2016, Devries et al., 2018). As alluded in Chapter 1, in studies where isolated protein sources or amino acids are ingested the rate and magnitude of leucinemia does indeed appear to predict subsequent rates of muscle protein synthesis (Koopman et al., 2009a, Tang et al., 2009, Pennings et al., 2011b, Burd et al., 2012, Churchward-Venne et al., 2013). However, this does not appear to hold true in studies where protein is coingested with additional macronutrients (Koopman et al., 2007, Gorissen et al., 2014, Staples et al., 2011, Hamer et al., 2013, Gorissen et al., 2017), where whole foods are consumed (van Vliet et al., 2017), or when the digestion kinetics of the same protein are manipulated (Chan et al., 2019). In the above studies, there is a marked attenuation in the rapidity and magnitude of peak postprandial leucinemia in one condition, but no apparent inhibition of the muscle protein synthetic response. In short, postprandial plasma leucine concentrations do not appear predictive of the postprandial muscle protein synthetic response in all cases, and potentially not within the context of a mixed-meal.

In the current work, the ingestion of leucine matched milk protein and the ingestion of BCAA enriched mycoprotein caused significantly greater leucinemia than the ingestion of mycoprotein alone, yet in neither case did this translate to a greater muscle protein synthetic response, the opposite in fact. In Chapter 3, the ingestion of milk protein, providing 2.5 g of leucine, caused a more rapid leucinemic response that peaked at a significantly higher magnitude than mycoprotein, with the divergence between the two protein sources

particularly apparent during the first hour of the postprandial period. Moreover, total plasma leucine availability was lower following the ingestion of mycoprotein than of milk protein, in contrast to our previous work (Dunlop et al., 2017). This in all likelihood can be ascribed to the slower digestion of mycoprotein, due to its greater energy content and its high fibre content. Muscle protein synthesis rates were similar between mycoprotein and milk protein, although the postprandial increase in muscle protein synthesis was greater following the ingestion of mycoprotein. In Chapter 4, the ingestion of a BCAA enriched lower dose of mycoprotein caused a more rapid and significantly higher peak magnitude of leucinemia than the ingestion of a larger bolus of mycoprotein (an almost twofold greater increase from postabsorptive conditions). This is in line with what we expected, as we chose to enrich the lower mycoprotein dose with crystalline leucine precisely because we wanted to provoke a high magnitude of rapid leucinemia in order to further probe the effect that leucinemia plays in the context of mycoprotein, and to potentially “rescue” the muscle protein synthetic response to the lower ingested dose. However, this is not what was observed, with the lower dose enriched mycoprotein stimulating lower postprandial muscle protein synthesis rates and a less substantial postprandial increase in muscle protein synthesis rates. In both cases, with two different protein sources, a theoretically preferential plasma leucine profile did not beget a greater muscle protein synthetic response. Clearly, postprandial leucinemia is only partially predictive of the subsequent muscle protein synthetic response within the context we investigated.

It is difficult to reconcile our results, and those of the aforementioned studies, with the “leucine threshold” or “leucine trigger” hypothesis; the thesis that a specific threshold for plasma leucinemia is required to optimally stimulate muscle protein synthesis (Phillips, 2016). It might be that the mycoprotein we provided simply surpassed the leucine threshold, despite lower postprandial leucine concentrations. Equally so, it might be that the whole food effect lowers the threshold for postprandial leucine requirements, such that a reduced

postprandial leucine concentration is required to optimally stimulate muscle protein synthesis. This might occur via enhanced extraction of leucine from the interstitium by the muscle cell, through amino acid transporter-mediated mechanisms (Drummond et al., 2011, Drummond et al., 2010), or an increased sensitivity of the cell to the effects of leucine at a signalling level. Either an ability to uptake more amino acids, or an ability to utilise amino acids more effectively, or both. Whilst plasma leucine concentration is undoubtedly an important component of the muscle protein synthetic response, reflective as it is of the availability of leucine in circulation, basing the conceptual framework surrounding leucine purely on plasma concentrations neglects kinetics at the level of the cell. Presumably, any threshold of leucine required to stimulate muscle protein synthesis is an intracellular event, where leucine is sensed by leucine sensors, such as sestrin2 (Wolfson et al., 2016), and there is an upregulation of signalling that accelerates the rate of translation initiation and muscle protein synthesis (Norton and Layman, 2006, Anthony et al., 2000). Whilst high plasma concentrations of leucine appear beneficial, as more leucine is presented to the muscle cell, it is the transit through the interstitium and across the cell membrane that determines entry into the cell to then overcome any obstructive intracellular signalling threshold. A cell that is more capable of extracting leucine, or is more leucine sensitive, might require lower absolute leucine concentrations, whilst also directly effecting plasma leucine concentrations through this greater extraction. Resistance exercise sensitises muscle cells to the effects of plasma amino acids (Burd et al., 2011b, Pennings et al., 2010), and it might well be that nutritional stimuli are also able to “sensitise” the muscle, thereby modulating amino acid extraction, or efficiency of handling. Without a more thorough understanding of this connection between plasma concentration and cellular uptake, we remain somewhat in the dark with regards to how dietary derived leucine is acting upon any apparent leucine threshold at the level of the cell. We are concluding on the predominant importance of a plasma leucine concentration without a thorough appreciation of leucine

within the cell; the location where it would seem to exert its effect upon translation initiation. To provide an analogy, are we measuring what is coming out of the tap, what is in the sink, but not what is going down the plug? In summation, while the plasma leucine threshold appears relevant when considering isolated protein sources, this may be less relevant when considering more complex food ingestion. Further to this, following the ingestion of dietary protein, plasma leucine concentrations may not tell the whole story of any leucine threshold for stimulating muscle protein synthesis, as this fails to account for leucine extraction by the muscle cell, and the utilisation of leucine intracellularly, the locus of regulation.

Within Chapter 3, I carried out a number of measurements to plumb the molecular regulation of the anabolic response to the ingestion of mycoprotein and milk protein, with the intention of gleaning where any difference between the two might be derived from mechanistically. The speculation herein regarding the whole food effect, and the specific role of leucine, emerged largely as a *result* of the findings of that study, and as such the investigatory approach taken was not strategically applied to pick apart the whole food effect nor that of leucine *per se*. Nevertheless, I did investigate both the transcriptional and post-translational response to protein ingestion and exercise, affording the opportunity to explore any mechanistic divergences. Firstly, mTOR phosphorylation did not differ following the ingestion of mycoprotein or milk protein, potentially suggesting a lack of difference in intracellular leucine availability, and anabolic signalling. However, it is also feasible that mTOR phosphorylation may have differed prior to the terminal point of measurement (at the end of a postprandial period) (Churchward-Venne et al., 2012, Drummond et al., 2012, West et al., 2011), that the subcellular location, and therefore influence, of mTOR may have differed between conditions (Sawan et al., 2018, Hodson et al., 2017, Abou Sawan et al., 2018a), that different phosphorylation sites may have yielded differentiation (Figueiredo et al., 2019), that downstream targets may have differed independently of mTOR, or that our dataset was not appropriately powered (due to limited tissue availability) to detect small, but

physiologically relevant differences in phosphorylation status. Differences in the acute transcriptional response to the ingestion of mycoprotein and milk protein were also minimal, with no differences observed what so ever in genes associated with amino acid transport, or the mTOR complex. As such, this fails to shed light on any present whole food effect, and how this effect might regulate a potentiation of the muscle protein synthetic response. It might be that any whole food effect is largely regulated post-translationally (Sawan et al., 2018, Hodson et al., 2017), that we selected genes that were unresponsiveness to this effect, or that the combination of the molecular measurement and statistical analysis lacked the sensitivity to detect any differences.

The data in Chapters 3 and 4 adds credence to the emerging concept that there might be a “whole food” effect. That is, a potentiation of muscle protein synthesis rates that derives from consuming protein within a whole food matrix, that occurs somewhat independently of amino acid derived mechanisms, and in synergy with amino acid derived mechanisms (Burd et al., 2019). “Potentiation” is an important qualification, as all evidence points to a sufficiency of amino acids as forming the backbone of the anabolic response, the action of which might be enhanced by the ancillary effect of other nutrients.

The concept that there might be things other than amino acids playing a role in modulating the postprandial muscle protein synthetic response is not a new one, and researchers have invested considerable effort to investigate the effect of carbohydrate coingestion (Koopman et al., 2007, Hamer et al., 2013, Gorissen et al., 2014, Staples et al., 2011), fat coingestion (Gorissen et al., 2017), and the effect of the food “matrix” (Churchward-Venne et al., 2015), in an attempt to isolate conspicuous ancillary factors that might modulate muscle protein synthesis. None of the factors listed above enhanced the muscle protein synthetic effect compared to protein alone. Subsequent interpretation, in reviews and within the scientific community, is that these factors, therefore, do not affect muscle protein synthesis rates, which I would argue to be potentially misleading, and a perhaps a slight misinterpretation

given the completeness of data available. Whilst these factors did not potentiate muscle protein synthesis, we cannot discount that they may have modulated the postprandial response, and even “rescued” the stimulation of muscle protein synthesis to a degree. In support, for example, Gorissen and colleagues (Gorissen et al., 2014) demonstrated that the coingestion of carbohydrate (60 g) with casein protein (20 g) did not increase postprandial muscle protein synthesis compared to isonitrogenous casein alone, in young or older individuals. However, they demonstrated an equivalency in muscle protein synthesis rates between coingested protein and protein alone *despite* significantly lower aminoacidemia and leucinemia. The extent of this discrepancy was not subtle, either, with protein alone increasing plasma leucine concentrations to a twofold greater extent than coingested protein. As such, viewed from this perspective, it could be interpreted that carbohydrate coingestion *is* in fact exerting an effect on muscle protein synthesis rates, “rescuing” the response or lowering the extent to which plasma aminoacidemia dictates the response. This is consistent with a number of studies investigating the coingestion of carbohydrate and fat have alongside protein (Gorissen et al., 2017, Staples et al., 2011, Koopman et al., 2007, Hamer et al., 2013, Churchward-Venne et al., 2015), and, therefore, whilst coingestion does not appear potent enough to increase muscle protein synthesis rates above that of protein alone, it would appear to be *interacting* with the muscle protein synthetic response at a minimum.

Isolating and manipulating individual independent variables in order to determine their effect on an individual or multiple dependent variables is the crux of the scientific method. However, it also possesses an inherent, and necessary, level of reductionism, in that it usually investigates variables in relative isolation. Within *in vivo* human research, this is not to say that a degree of reductionism is avoidable, simply that it is the product of the incrementality of progress, and an acceptance that such knowledge must be accrued in a piecemeal fashion in order to provide a layered understanding over time. This focused

approach is the best method available to us, however, on occasion, it might cause us to overlook something. The manner in which our knowledge of muscle protein synthesis has developed reflects this. Work over the previous century has imbued us with an excellent foundational understanding of how human muscle protein is regulated. However, in reflection of the desire to isolate individual variables, and exert scientific control, this knowledge has largely developed in isolation from foods and meals. Such that we are now in a position that we have an enormous amount of data concerning isolated protein sources, and infusions of amino acids, but comparably little data concerning foods, or mixed meals, leaving a conspicuous gap in our knowledge. This is problematic as the vast majority of human calories are consumed in foods, and meals, so the considerable knowledge base we possess is somewhat divorced from the realities of human food consumption.

When investigating “whole foods”, we must accept the possibility that we cannot isolate, at the first point of investigation, where an effect on muscle protein synthesis might be derived from. Of course, we can postulate that much of it is derived from the amino acids within that protein, but the complexity and multiplicity of the other nutrients within that food precludes us from being able to ascribe a specific effect to any one of those nutrients. Understandably, this makes it a less appealing proposition for scientists; cause and effect become less specific, and interpretation becomes that much more difficult. As such, the study by van Vliet and colleagues (van Vliet et al., 2017) that considered the effect of egg white (largely protein) compared to whole egg (a whole food) on muscle protein synthesis, was atypical, as it accepted from the point of conception that it might not be able to explain exactly what causative factor was driving an enhanced anabolic response to the whole food, if one were to exist. This approach produced some very interesting results. As it transpires, van Vliet and colleagues demonstrated significantly greater postprandial muscle protein synthesis rates following the ingestion of whole egg than egg white, despite the two being matched on protein content, and the latter provoking greater postprandial leucinemia. The authors

ascribed their results to the presence of a "whole food" effect in the whole egg, with unknown factors in the egg yolk, which is particularly nutrient-dense, potentiating the muscle protein synthetic response. The authors suggest a number of factors as to why this might be the case, including micronutrient content and specific lipid subfractions. The authors subsequently wrote an informative and eloquent review where they elaborate upon this theoretical construct in more detail (Burd et al., 2019). Alongside this new intuition, previous work supports the idea of nutrient density playing a role in modulating the muscle protein synthetic response. An earlier study by Elliot and colleagues demonstrated that the ingestion of whole milk stimulated a more robust anabolic response than skimmed-milk, even when matched on caloric content and providing less protein (Elliot et al., 2006). As the major differing factor between the two is the fat content, it would further point towards a role for specific lipids, alongside a complement of other nutrients, playing a role in modulating muscle protein synthesis. How this differs from milk fat intake *per se*, again investigated by Gorissen and colleagues (Gorissen et al., 2017), is unclear, although the implication would be that it is through wider interactions with a more complete complement of other nutrients.

Our work builds upon the concept of a whole food potentiation of postprandial muscle protein synthesis rates, although, in line with analogous studies, we cannot explain the mechanistic basis that might underlie a potentiating effect on skeletal muscle anabolism. In truth, at present we only have an intimation that such an effect is apparent. Nevertheless, as we see a slightly greater muscle protein synthetic response following the ingestion of mycoprotein than leucine matched milk protein, despite significantly lower magnitude and rapidity of postprandial leucinemia, there is a strong suggestion that something is potentiating the anabolic response to mycoprotein. Rarely in the literature does significantly lower postprandial aminoacidemia and leucinemia beget a *greater* muscle protein synthetic response. The suggestion of such an effect in mycoprotein is intriguing, as mycoprotein is markedly different to whole egg or milk (van Vliet et al., 2017, Elliot et al., 2006). Mycoprotein

does contain a proportion of fat (13%), although this fat is compositionally different to that of animal-derived proteins; it contains no cholesterol (a factor that has been suggested as a potentiating factor (Castellano et al., 2017)) and is low in saturated fat. Mycoprotein is also, quite uniquely for a protein-rich food, high in fibre, two-thirds β glucan and one-third chitin (a major constituent in the exoskeleton of arthropods as well as the cell walls of fungi). Fermentation of dietary fibres leads to the production of short-chain fatty acids (SCFA), primarily acetate, propionate, and butyrate (Cummings et al., 1987), and *in vitro* data from colonic models has shown that the dietary fibre derived from mycoprotein is capable of producing SCFAs (Harris et al., 2019). In turn, the production of SCFAs and propionate has been implicated with a range of metabolic effects (Besten et al., 2013, Wolever et al., 1995, Chen et al., 1984, Chambers et al., 2015, Chambers et al., 2019), and it is not unreasonable that these might extend to anabolism (Ni Lochlainn et al., 2018). As such, mycoprotein may represent a useful tool to investigate whether the fermentation of SCFA does affect muscle tissue anabolism, and, concomitantly, whether this might be responsible for any whole food potentiation from mycoprotein ingestion. Additionally, mycoprotein is micronutrient rich, and there is the suggestion that a rich micronutrient profile may affect intracellular signalling events associated with the upregulation of protein synthesis (Zhao et al., 2016, Salles et al., 2013). The novelty of mycoprotein as a food source, and the consequent difficulties in drawing direct comparison to other foods, presents further challenges when speculating about potential mechanisms through which anabolism might be potentiated.

There are numerous factors that could at least, in theory, potentiate an anabolic response, in mycoprotein and elsewhere, although any suggestion on which of these constituents is having an effect remains tenuous with our current knowledge. Indeed, it may be that a whole food effect is dependent on a wide range of nutrient interactions, that a myriad of nutrients exert an influence, as opposed to a single overriding factor. The whole food “matrix” may exert an influence, although extricating this from a wide array of nutrients is inherently

difficult. Determining what a “whole food” *is,* is somewhat elusive. We can consider whole foods on a spectrum, from hydrolysed amino acids and isolated proteins at one end, through coingestion and then whole foods in the middle, to a nutrient-rich balanced meal at the other end. On a broad level this is a useful framework, but when titrated down to individual foods it is of limited usefulness. How does one determine the “wholeness” of a food, and how does this inform upon its potential capacity to stimulate a muscle protein synthetic response. In this authors opinion, it is perhaps better, at least initially, to consider food from the perspective of nutrient density or nutrient richness. Viewing foods from the perspective of the nutrients they provide gives us a more tangible framework for assessing foods, and it is almost indistinguishable from what we actually mean when discussing “whole foods”. That is to say, that the “whole foods” investigated with regards to their ability to stimulate muscle anabolism (whole eggs, salmon, whole milk, and mycoprotein), were more nutrient-rich than their comparators. Considering food from the perspective of nutrient density better allows us to identify commonalities in the foods we investigate, subsequently aiding us in identifying foods that may exert a potentiating effect, to better understand which nutrients exert an effect.

Transitional Implications

In Chapters 5 and 6 we demonstrate the novel finding that if an adequate quantity of protein is consumed it does not appear to be of great concern whether that protein is derived from an animal, or not. This is of course asserted with the caveat that such non-animal sources that are consumed are of a relatively “high quality”; meaning they possess a suitably high essential amino acid content, and those amino acids become available in circulation following digestion to be incorporated into muscle protein. Granted, we provide data that may be heavily influenced by the amount of mycoprotein in the diet, which has proved capable of stimulating a comparatively large muscle protein synthetic response. At present, given the data available to us, mycoprotein appears as the exception to the rule with regards

to non-animal proteins, and it is conceivable that the equivalency we demonstrate herein is a product of this exceptionalism. Indeed, even at lower intakes we have no reason to believe, based on our acute data, that a mycoprotein rich diet would support a lesser muscle protein synthetic response than an omnivorous one. Nevertheless, our results provide an important proof-of-concept; namely that a non-animal-derived diet is capable of stimulating equivalent daily muscle protein synthesis rates to an animal protein-rich omnivorous diet, in both young and old individuals.

In many ways the fact that a non-animal-derived diet is capable of supporting anabolic equivalency to an omnivorous diet should not be especially surprising. Evidence would dictate that if a diet is able to provide sufficient amino acids, which can be properly digested and absorbed, it will be able to support a high magnitude of muscle protein synthesis. That being said, upon first appearance our results do appear to contrast with some of the thinking within the field, which state a significant advantage of animal-based sources (van Vliet et al., 2015b, Phillips, 2016). The evidence at our disposal clearly draws us to the assertion that in most cases animal-derived proteins are likely to outperform non-animal-derived ones on a gram-for-gram basis when considering acute muscle protein synthesis in a single postprandial period. Although mycoprotein, and potentially other non-animal proteins, may represent exceptions. Twenty grams of whey protein is likely to outperform twenty grams of a given plant protein, and if we extrapolate this to a whole day, we would expect four doses of twenty grams of whey protein to outperform four doses of twenty grams of plant protein. Similar to the effect observed by Oikawa and colleagues (2020b) when investigating whey protein vs collagen protein under isonitrogenous conditions. However, if we consider a *diet*, that incorporates a more complex array of protein sources and nutrients, doses that reach closer to a total of 1.8 $g{\cdot}kg\ bm^{-1}{\cdot}day^{-1}$, and daily muscle protein synthesis rates, I would suggest the picture becomes more complex. Even when consuming a high-protein omnivorous diet, one does not consume animal protein alone; only a proportion of protein is

provided by animal sources, with the remainder derived from plant-based sources. The dichotomy of "animal vs plant" protein is an oversimplification, at least where the diet is concerned, and potentially a legacy of our penchant for investigating isolated animal or plant proteins. The reality, as is often the case, is a much murkier shade of grey, with a polar view of "animal vs plant" diets only truly relevant from a dietary perspective if one were to compare a vegan diet to a carnivorous one. Moreover, within the proportion of animal protein in an omnivorous diet there is likely to be considerable heterogeneity in the nature of the protein foods consumed (meat, milk, cheese, yogurt etc). Protein is generally not consumed in isolation, but alongside other nutrients as part of meals, which may further modulate the anabolic potency of the ingested protein; at the very least it will modulate the digestion of the ingested protein (Gorissen et al., 2017, Gorissen et al., 2014). If such a thing as a whole food effect does exist then it may become significantly more complex, or amplified, when considering the myriad of nutrient interactions in a balanced diet. Hence, if the archetypal 70 kg man were to consume four doses of omnivorous protein averaging slightly above thirty grams, alongside a full complement of nutrients as part of a balanced diet, are we likely to see a discernible difference in daily muscle protein synthesis rates compared to four equivalent doses of non-animal protein, also consumed as part of a balanced diet? Possibly, in certain circumstances, although such a difference is likely to be narrowed compared to an assessment of 20 g isolated animal protein vs 20 g isolated plant protein. This is not to suggest that differences in protein quality are inconsequential, nor downplay the role that small anabolic advantages might play over a prolonged period of time, merely an attempt to illustrate the nuance and indistinctiveness when considering high-protein omnivorous or non-animal *diets* in their entirety.

In practical terms, we are left with the postulation that if enough protein is consumed, non-animal proteins may serve equally as effectively as animal proteins, despite the fact that in some cases, on a gram-for-gram basis, they may prove less stimulatory of muscle protein

synthesis rates. Practically, this is very important as it makes clear that those following a largely, or purely, non-animal-derived diet should look to consume a specific adequacy of protein if they wish to optimise daily muscle protein synthesis rates, and that this should be of particular concern to them as a strategy. Moreover, and conspicuously, it demonstrates that there is not an unavoidable, unsurpassable decrement in muscle tissue anabolism that is concomitant to consuming a non-animal-derived diet. At least, this is the case when consuming a mycoprotein-rich diet. Empirically, it would suggest we need to determine the "cut-off" point at which potential differences in protein source, and protein quality, might make themselves apparent. Certainly, when we consider mycoprotein, and a mycoprotein rich diet, we have no reason to believe that there would be a difference in daily muscle protein synthesis rates compared to an omnivorous diet even at a significantly lower protein intake. Whether this would be the case were mycoprotein to be emitted, is more doubtful. More broadly, we need a larger body of evidence considering daily muscle protein synthesis that inform upon practical questions regarding our dietary intake, not just concerning type, but also concerning dosing and other modulating dietary factors.

Longitudinal Implications

In Chapter 6 we show the novel finding that a majority non-animal-derived diet is capable of supporting resistance adaptation across multiple measures of skeletal muscle hypertrophy and strength. Pertinently, this provides the proof-of-concept that significant resistance training adaptation is feasible irrespective of whether an individual were to obtain protein from omnivorous or non-animal sources. Similarly to that previously discussed, this might not be logically surprising, as any diet that can provide an adequate complement of essential amino acids to the muscle is at least theoretically capable of optimising the hypertrophic response, contingent on the diet also being adequate in energy. Nevertheless, therein we provide proof-of-concept that non-animal-derived proteins can fully support the hypertrophic response, that animal-derived proteins are not an essential prerequisite, and that non-animal

proteins are not grossly inferior when consumed in adequate quantity. This, in turn, suggests that those adhering to a non-animal diet can do so in the knowledge that they may expect an equivalent response to that of an animal-derived diet, contingent on them strategically incorporating high-quality, non-animal-derived proteins, such as mycoprotein into their diet.

We chose to address the question of whether a non-animal-derived diet could support resistance training adaptation in a very practical manner, applying a training and dietary intervention that aimed to strike a balance between the necessary scientific control whilst allowing participants a degree of freedom, thus easing what was a significant burden. This appears to have paid dividends as we were able to show significant increases in all measures of training adaptation pre to post, and we were able to significantly shift participant's diets in a manner that would not have been possible otherwise. Just so, this approach facilitated a relatively extreme intervention in the non-animal group, but one that was both practically feasible and ecologically valid, albeit potentially challenging. We provided participants with advice on how to compose their diet, a number of caloric and protein targets, and access to certain food products. In turn, participants successfully adhered to a high-protein omnivorous diet, or an almost entirely non-animal-derived diet. This is an important distinction from previous studies within the area, as we did not just provide a plant-based supplement (Hartman et al., 2007, Volek et al., 2013, Babault et al., 2015), but a non-animal *diet*. As such, almost all (a conservative estimate of at least 95%) of their protein was derived from non-animal sources, as opposed to only a supplementary protein. Although investigating the response to a plant-based protein supplement is conceptually (and practically) valuable, I would suggest a large proportion of individuals who choose to consume a plant-based supplement are likely to adhere to a plant-based diet, or at least a vegetarian one. Thus, although supplement studies provide an indication of the efficacy of plant-based supplements, they do so in a context that might be vanishingly rare; plant protein supplementation and an omnivorous diet. It is more a comment on the quality

of that particular supplement, than the efficacy of a particular diet, which encompasses a broader outlook. In our work, whilst participants in the non-animal group were afforded a day in which they could consume animal protein if they wished, almost all abstained. Whilst impossible to fully quantify, I suspect that less than 1% of protein in the non-animal group came from animal protein during this period. This affords the study an advantage over previous studies in the realm of adaptation to non-animal proteins, as it more properly informs those adhering to, or considering transitioning to, a non-animal-derived diet. As such the findings are more applicable and more impactful for these individuals, and, accordingly, we add significantly to the literature concerning non-animal-derived *diets* and resistance training adaptation.

There are two substantial caveats that I would place on the findings of this study. Firstly, and along the same lines as previously discussed, whilst we provided a non-animal diet, this diet did contain a high proportion of mycoprotein. As such we cannot assume that the same results would be observed were we to provide a non-animal-derived diet devoid of mycoprotein. The findings, therefore, are contextually specific to that of a high-protein mycoprotein rich non-animal-derived diet. Practically speaking, the mycoprotein products proved highly valuable. Not only can we qualify them as a "high quality" protein, but also they are commercially available, easy to cook, and represent a bridging option for habitual meat-eaters, as they appeal as a somewhat like-for-like exchange. Secondly, an observation based on the wider experience of overseeing the study is that obtaining 2 $g{\cdot}kg\ bm^{-1}{\cdot}day^{-1}$ of protein from non-animal-derived protein sources is significantly more challenging than obtaining a comparable amount from animal proteins (running the study in a more free-living manner afforded the opportunity to make valuable observations such as this). In the second phase of the work described in Chapter 6, participants were largely responsible for structuring their own diet, whilst receiving protein supplements and protein products from the researcher, and it was noticeable how much more difficult it was for them to attain their

protein target. Non-animal-derived foods are typically less protein-rich, being as they are neither muscle nor milk, and one must eat a larger volume of said products to gain the same amount of protein. This becomes even more difficult if one were to discount "meat-replacement" products, such as mycoprotein, which are useful in bolstering the protein content of non-animal diets as they typically contain a higher proportion of protein than is typical of plant-based foods. Noteworthy is that some of these meat replacement products, those constituted from soya beans, do not necessarily possess particularly good sustainability credentials, linked as they are to deforestation, monoculture, and substantial environmental transportation costs. This is coupled with the relatively low anabolic value of soy protein (Yang et al., 2012b, Tang et al., 2009). It is well documented that animal-derived foods typically contain a concomitant quantity of saturated fat, and similarly, plant-derived protein-containing foods typically contain a concomitant amount of carbohydrate. If one wants to obtain a sufficient amount of protein from plant sources, then relatively large volumes need to be consumed, and therefore a significant amount of carbohydrate also. This might present issues with regards to energy balance, and the physical volume needed to obtain an optimal serving of protein may present issues concerning appetite. Just so, a number of participants who were randomised to the non-animal-derived protein group found the volume of food they had to consume to hit their designated protein target particularly challenging. Animal protein, in contrast, can be selected to provide a relative "pure" protein source, which might be useful in situations where appetite is compromised or in situations of deliberate energy deficit. As such, whilst consuming non-animal-derived protein in sufficient amounts to optimise the hypertrophic response is certainly possible, it might be more challenging for some, and may require a more focused strategy on the part of the individual.

The approach that we took towards the resistance training and nutritional intervention also appeared fruitful with regards to the degree of resistance training adaption we were able to

elicit. Indeed, we show a very significant degree of hypertrophy, with a 5.5% increase in lean mass, an 8.1% increase in muscle volume, and a 32% increase in muscle fibre CSA. Satisfying, is the fact that we see a high degree of concurrency between these three measures (r=0.69 for the change in lean mass and whole thigh volume, r=0.60 for lean mass and strength). The findings herein appear in line with those of Nilwik et al. (2013), with regards to the extent to which muscle fibre hypertrophy explains changes in muscle size as derived from imaging. Whilst immunohistochemical analysis is ongoing, initial analysis indicates 33% Type II hypertrophy and an 8% increase in muscle volume, similar to the 24% change in type II fibre size and ~8% change in muscle CSA observed in Nilwik's work. Whether we "maximised" the hypertrophic response, short of supra-physiological exogenous hormone treatment, is unverifiable. Systematic review evidence suggests improvements in lean mass gains of between 1.1 and 1.6 kg with resistance exercise in healthy males (Benito et al., 2020, Morton et al., 2018). More analogously, Snijders et al. (2015) and Hartman et al. (2007) observed increases in lean mass and FBFM of 1.9 kg and 3.9 kg in young men, respectively, after 12 weeks of whole-body resistance training alongside a high-protein diet. In young women, Josse et al., (2010) observed a 1.9 kg increase in lean mass following 12 weeks of whole-body resistance exercise training. We included both males and female participants, who exhibited increases in lean mass of 3.6±0.7 and 2.1±0.4 kg, respectively. It is clear that, therefore, after considering other studies in the field, that we induced a large degree of muscle hypertrophy. Importantly, as this was consistent across both groups it suggests that not only can a non-animal-derived diet support a hypertrophic response, but also that it can do so to a high degree. This distinction is potentially important, as if the degree of hypertrophy was of a lower magnitude it could be argued that a non-animal-derived diet was sufficient to support a moderate response, but insufficient to support very intense training and produce an adaptive response of a very high magnitude. By applying an extremely challenging training programme, with a

large emphasis on progressive overload under conditions of high volume, high frequency, and high intensity, we show this not to be the case.

One semi-anecdotal observation that I made through the course of the resistance exercise training intervention, after observing hundreds of sessions, was that some individuals were able to execute training more proficiently than others, and drive a greater stimulus. Whilst this might seem obvious, it is interesting to consider how this interacts with training responsiveness. There is a tendency to consider an individual's propensity to increase muscle mass as being largely a genetic, intramuscular effect. However, this perhaps ignores the less tangible ability of certain individuals to more thoroughly tax the muscular system through training. Even an individual who possesses a genetic predisposition towards resistance-exercise induced anabolism is unlikely to reach their hypertrophic potential unless this inheritance is combined with an ability to optimally train the muscle. At first glance one might interpret this as "effort", however, I think this would be missing the mark somewhat, and discount what are in fact ultimately important determinants of training responsiveness. This "execution", for want of a better term, represents the combination of control, mobility to train through a full range of motion, technical proficiency, intuitive corrections to place tension on the targeted muscles, the stability of motor programs under fatigue, and the ability to utilise a high relative external load. This ability to control relatively high loads, through a full range of motion, under fatigue, in a mindful manner, potentially means that targeted muscle fibres produce force through their full contractile range with an ensuing high degree of fatigue, causing individual muscle fibres to experience greater mechanical tension, therefore greater resultant anabolism. Such an effect on the potentiation of skeletal muscle anabolism might prove almost imperceptible after a single session, yet is potentially made physiologically meaningful by rote training over time. Whilst these traits are hard to quantifiably measure, indeed, they are hard to eloquently identify and describe, it was seemingly the individuals who possessed the above collection of traits

who were able to increase lean mass to the greatest extent. Just so, three individuals increased their total lean body mass by more than 10%, and I would suggest that these individuals executed training in the most adroit fashion. A commonality they all shared, appeared to be this controlled and mindful execution of training. This ability to execute training in all likelihood has an innate aspect, which can then be improved via learning and practice. In future studies where whole-body resistance training is utilised, it might be worthwhile to consider how well resistance exercise is executed in greater detail, alongside more quantifiable aspects. Admittedly, this speculation is tangential and subjective, but I felt it worthy of mention nonetheless.

It is possible that consuming a non-animal-derived diet may compromise the provision of specific nutrients, which in turn might have a deleterious effect on health, and subsequently training adaptation, over a prolonged period of time. Those adhering to vegetarian diets have lower intramuscular creatine concentrations (Burke et al., 2003, Delanghe et al., 1989), a reduced skeletal muscle carnitine transport capacity (Stephens et al., 2011), lower serum n-3 fatty acid concentrations (Clarys et al., 2014, Dinu et al., 2017, Davey et al., 2003, Rosell et al., 2005), issues with iron and zinc intake/bioavailability (Davey et al., 2003, Hunt, 2002), lower calcium intake (Davey et al., 2003, Janelle and Barr, 1995), and lower vitamin B12 intake (Davey et al., 2003) and concentrations (Gilsing et al., 2010, Pawlak et al., 2014). Indeed, pilot data from the work described in Chapter 6 suggest that those who underwent the non-animal-derived dietary intervention had lower fasting iron, calcium and B12 concentrations than those who adhered to an omnivorous diet. Although these decreases, and any other possible deficiencies, in the non-animal-derived diet did not inhibit adaptation over the period of investigation, we cannot discount that the same would be true over a longer period of time.

AREAS OF FUTURE RESEARCH

Acute

A clear direction for future research is to further elucidate whether a "whole food" potentiation of postprandial muscle protein synthesis rates is a persistent phenomenon; whether "whole foods" consistently outperform isolated protein sources on an isonitrogenous or leucine matched basis. Subsequently, or concurrently, we need to determine the factors that may be responsible for causing this potentiation, and the mechanisms through which they exert an effect. With regards to mycoprotein, the next logical step is to compare the muscle protein synthetic response of "whole" mycoprotein to an isolated form of mycoprotein, one that has been processed to create a less nutritionally complex product that is a higher percentage of protein. Any differences in the muscle protein synthetic response in favour of whole mycoprotein would inform upon whether a whole food effect is responsible for the robust stimulation of muscle protein synthesis that we demonstrate in Chapters 3 and 4. If the whole food potentiation of the muscle protein synthetic response were to hold true, then perhaps a deductive approach is subsequently required. For example, to determine whether the fibre in mycoprotein plays a role in modulating muscle protein synthesis researchers could add a matched amount of the same fibre to an isonitrogenous bolus of another protein source (e.g. milk protein). Similarly, to determine whether micronutrients play a role in the postprandial muscle protein synthetic response perhaps "high" and "low" conditions could be employed, with the "low" condition utilising a micronutrient depleted protein / whole food. The issue with employing a deductive approach is if the whole food potentiation of muscle protein synthesis is reliant upon a multitude of small independent potentiating effects, which in turn may interact or act in a cumulative fashion. The question then arises whether we have the sensitivity to detect these factors independently *in vivo*. A further challenge will be discerning whether there is an effect of the food matrix *per se*, or whether such an effect is more reflective of foods that have a high nutrient density.

If a whole food potentiation of muscle protein synthesis is an enduring phenomenon across multiple investigations, as at this point the evidence remains in relatively short supply, then future work should seek to identify the regulatory junctions which potentiating factors might influence. Muscle protein synthesis is regulated at a finite number of control points or junctions (Figure 1.1), and it stands to reason that a whole food effect must act on one or more of these points of regulation. Firstly, the ingestion of whole foods slows the rate of gastric emptying, and attenuates the rise in circulating plasma amino acids (van Vliet et al., 2017, Monteyne et al., 2020b). Therefore, the muscle itself is not exposed to a greater systemic availability of amino acids, and we can discount a mechanism involving circulating plasma amino acid availability *per se* as a point of potentiation. Sequentially, this would lead us to speculate that when a whole food is consumed the muscle is either able to extract more amino acids from this less abundant plasma supply, or is simply able to utilise them intracellularly with a greater deal of efficiency, or both. Therefore, three points at which nutrients may modify, or potentiate, the muscle protein synthetic response is via enhanced microvascular perfusion (Wagenmakers et al., 2016, Keske et al., 2017), enhanced amino acid transport at the cell membrane (Dickinson and Rasmussen, 2013), or within the cell itself through enhanced signalling mechanisms (Sawan et al., 2018). Indeed, as whole foods and coingested foods do provoke a lower magnitude of aminoacidemia, but equivalent or potentiated muscle protein synthetic responses, there may be indication of enhanced cellular extraction or more efficient utilisation of amino acids. The work of Abou Sawan and colleagues (Sawan et al., 2018, Abou Sawan et al., 2018a) demonstrated that whole egg caused greater mTOR colocalisation with the lysosome than did egg white, demonstrating that non-amino acid derived nutrient factors can modulate the anabolic signalling response. As this coincided with a greater muscle protein synthetic response, we can postulate that this event directly modulated the incorporation of amino acids into tissue (although a dissociation between anabolic signalling events and muscle protein synthesis is regularly

observed, such that greater “signalling” does necessarily beget a greater muscle protein synthetic response (Greenhaff et al., 2008)). It would be intriguing to see if mycoprotein, and other whole foods, had a similar effect on mTOR colocalisation. Further, intracellular signalling events, such as mTOR colocalisation, should be considered alongside membrane-associated amino acid transporter content (Agergaard et al., 2017, Hodson et al., 2018), interstitial amino acid concentrations (Gore et al., 2007, Miller et al., 2004), and intracellular amino acid concentrations (Bergström et al., 1990), in order to build a comprehensive model of the possible nutrient potentiation of the anabolic response.

Transitional

In Chapters 5 and 6 I broadened the scope of my research to consider the effect of non-animal diets as a whole on daily muscle protein synthesis rates, scratching the surface on what could be an impactful area of research. In these studies we set protein intake at a level that can be considered optimal (1.8 $g \cdot kg\ bm^{-1} \cdot day^{-1}$) in both young and old individuals, in order to provide proof-of-concept that if protein was provided in an adequate amount, then the source of such protein, animal or non-animal, would be irrelevant as far as daily muscle protein synthesis rates were concerned.

As this thesis is focused to a large degree on mycoprotein specifically, a significant proportion of the diet that we provided in the studies described was mycoprotein and mycoprotein product-based. Mycoprotein is an unusual protein source in the realm of non-animal protein sources, as being fungal derived it is not technically plant-based, and early indications suggest that it is atypically anabolic. We cannot assume that our results would be replicated with regards to an equivalency in cumulative muscle protein synthesis were we to remove this element from the diet, and replace it with a more typical plant-based protein source. As such it would be intriguing to repeat the study with a more typical plant-

based diet, to thoroughly test the concept that under conditions of an adequacy of protein, source might prove less relevant.

Similarly, our conclusions were drawn on the basis of having provided a high-protein diet, so it would be interesting to investigate the cumulative muscle protein synthetic response to an omnivorous and non-animal diet when providing a lesser amount of protein. Indeed, whilst our work provided a proof-of-concept, the question outlined above is arguably more impactful. When providing protein in a lesser amount any divergences in protein quality between animal and non-animal protein sources might become apparent, with the lower relative essential amino acid content of non-animal sources potentially becoming limiting to the stimulation of muscle protein synthesis rates (Gorissen et al., 2018, van Vliet et al., 2015b). Presumably, there is a threshold for the quantity of essential amino acids required to maximise daily muscle protein synthesis rates, and as protein intake is decreased this cut off is likely to be met sooner when following a non-animal (non-mycoprotein) diet (van Vliet et al., 2015b, Gorissen et al., 2018). This being the case, future research might wish to investigate any possible divergences in muscle protein synthesis rates between animal and non-animal-derived diets when less protein is provided. There have been calls for older individuals to consume ≥1.2 $g \cdot kg\ bm^{-1} \cdot day^{-1}$ protein (Traylor et al., 2018), and evidence would suggest that younger and middle-aged individuals consume around ~1.4 $g \cdot kg\ bm^{-1} \cdot day^{-1}$ (Smeuninx et al., 2020), so perhaps a dietary intake within this range could be applied. Elucidating the amount of non-animal protein required to support optimal muscle protein synthesis rates also has connotations for sustainability. From the perspective of sustainability, the lowest amount of protein required to provide such a response, the point of threshold if you will, is the most desirable as it utilises the smallest amount of protein to greatest effect. However, I appreciate this is a potentially reductive and over-precise supposition given the vagaries of human behaviour when it comes to food intake. To conclude with a slight digression, it would seem productive that we start working towards

ascribing protein sources with a (semi)quantifiable metric that takes into account both the sustainability and anabolic value of that protein. This greater insight might subsequently facilitate a less dichotomous, antagonist view with regards to the dietary choices people make.

Lastly, an area that has received relatively little attention is the interaction between energy availability and muscle protein synthesis. Whilst there are data investigating acute muscle protein synthetic responses to periods of energy deficit, there is a paucity of data examining the interaction between energy availability and muscle protein synthesis over a longer period of time (Hector et al., 2018). Muscle protein synthesis is an energy expensive process, that is therefore at least partially modulated by the prevailing cellular environment (i.e. availability of energy) (Smiles et al., 2016). Accordingly, it is unsurprising that periods of energy deficit appear to reduce postabsorptive (Areta et al., 2014, Pasiakos et al., 2010, Murphy et al., 2015, Hector et al., 2014), and postprandial muscle protein synthesis rates (Murphy et al., 2015, Pasiakos et al., 2013, Hector et al., 2014), although this reduction in muscle protein synthesis can be partially mitigated by the structured resistance exercise (Hector et al., 2018, Murphy et al., 2015, Areta et al., 2014). The heavy water method for measuring cumulative muscle protein synthesis rates would seem well suited to investigating the effects of energy intake, encompassing both postpabsorptive and postprandial muscle protein synthesis, whilst also being easily combined with measures of muscle morphology (e.g. MRI). Hector and colleagues (Hector et al., 2018) utilised a heavy water approach to measure cumulative muscle protein synthesis rates over a more extended period of time, whilst participants consumed 40% below their estimated caloric requirements. They demonstrated that although resistance exercise and a high-protein diet (2.35 $g{\cdot}kg\ bm^{-1}{\cdot}day^{-1}$) mitigated reductions in protein synthesis compared to an energy balanced diet, a "lower protein" (1.2 $g{\cdot}kg\ bm^{-1}{\cdot}day^{-1}$) diet and resistance exercise was insufficient to mitigate a decrease in cumulative muscle protein synthesis rates. Even with this high protein intake,

only in exercised muscle did protein synthesis rates remain comparable to rested muscle in a state of energy balance. Whilst this study is enlightening, it only partially explains the role of energy *per se*, as only in the lower protein condition was protein (roughly) matched between the energy balanced run-in diet and the energy restricted diet, and, therefore, energy intake the only variable manipulated. To more fully characterise the response of the muscle to energy *per se*, it would also be useful to consider the cumulative muscle protein synthetic response to overfeeding, as it stands to reason that an overabundance of energy may be facilitative of an anabolic cellular environ. Anecdotally, it would appear that periods of resistance training in concert with an energy surplus provide more optimal conditions for lean mass gain (alongside some fat mass gain), and multiple nutrition textbooks advocate the application of an energy surplus to facilitate skeletal muscle hypertrophy (Manore et al., 2009, Burke et al., 2000, Karpinski and Rosenbloom, 2017). It would therefore be interesting to investigate this empirically, to elucidate whether there is a direct potentiation of muscle protein synthesis that underpins this effect. Indeed, it would be intriguing to concurrently compare conditions of energy deficit, balance and surplus under isonitrogenous conditions in order to thoroughly investigate the role that energy availability has upon the anabolic response. Whilst potentially interesting with regards to resistance training exercise adaptation, characterising the effect of energy intake on muscle protein synthesis rates may also be of value elsewhere. For example, in clinical populations, where energy intake might be optimally manipulated to negate potential lean mass loss in during periods of disuse or catabolic stress. Additionally, in ageing populations where the mitigation of insidious lean mass loss must be weighed against the negative consequences associated with sarcopenic obesity. As such, investigating the effect of energy availability alongside disuse models, and ageing, may represent a fruitful area of research.

Longitudinal

An obvious area of future work would be to conduct a similar training study to that described in Chapter 5 in an older population. This would determine whether a non-animal-derived high-protein diet is capable of supporting resistance-exercise adaptation in a population that is a deal more protein (in)sensitive. Just so, any significant decrements in the total protein quality of a non-animal diet might be more consequential in this population, and more clearly present themselves as a lesser accrual of protein over time. Moreover, practical concerns might also be more apparent within this population. I earlier discussed the potential issues with the volume of food that had to be consumed by those in the majority non-animal-derived diet group, and how this was a challenge for a proportion of those participants. What was a challenge for these young healthy individuals could potentially be a much more serious issue within an older more appetite compromised population, making the provision of protein more difficult. Moreover, it would be valuable to investigate more “typical” non-animal-derived diets, where there is a lesser preponderance of mycoprotein; which might have been decisive in eliciting the equivalent hypertrophic response that we observed in Chapter 6. This would inform upon the broader context of non-animal-derived diets, and scrutinise the supposition that equivalent hypertrophy might be realised adhering to a non-animal diet as adhering to an omnivorous one.

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