THE RELATIONSHIP OF LEAN BODY MASS AND PROTEIN FEEDING:

THE SCIENCE BEHIND THE PRACTICE

By

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ABSTRACT

The development of lean body mass (LBM) is closely linked to protein feeding. Along with resistance exercise protein feeding, or amino acid provision, stimulate muscle protein synthesis (MPS). Repeated stimulation of MPS above protein breakdown results in lean mass accretion. Many athletes aim to build or maintain LBM. The aim of this thesis was to better understand the relationship between LBM and protein feeding in trained individuals. This aim was studied in the applied setting and at whole body, muscle and molecular level.

Chapter 2 revealed differences in total body mass and LBM between young rugby union players competing at different playing standards. Protein consumption was higher in players that played at a higher standard. The protein consumption of players at both playing standards was higher than current protein recommendations for athletes. The Under 20 (U20) rugby union players in Chapter 3 also consumed more protein than current recommendations state. Their dietary habits changed depending on their environment and they consumed more protein while in Six Nations (6N) camp compared with out of camp. Also, there were changes in dietary habits for individuals, however, those changes did not occur at the group level. Using the camp as an education tool for good nutrition habits could be advantageous.

As a group, rugby union players' body composition did not change from pre to post a 6N tournament. However, there was individual variation, which could be meaningful for the individual players. We provide evidence suggesting that in elite sport, athletes should be considered as individuals as well as part of a group if appropriate.

The protein ribosomal protein S6 kinase 1 (p70S6K1) is part of the mammalian target of rapamycin complex 1 (mTORC1) pathway, which regulates MPS. The response of p70S6K1 activity was 62% greater following resistance exercise coupled with protein feeding compared with protein feeding alone in Chapter 3. P70S6K1 activity explained a small amount of the variation in previously published MPS data. The activity of the signalling protein p70S6K1 was unchanged in response to different doses of whey protein in Chapter 4 and 5. These data suggest that resistance exercise is a larger stimulus of p70S6K1 activity and when manipulating aspects of protein feeding p70S6K1 activation may be a limited measure.

Consumption of 40 g of whey protein stimulated myofibrillar MPS to a greater extent than 20 g after a bout of whole body resistance exercise. The amount of LBM that the trained individual possessed did not influence this observed response. These data suggest that the amount of muscle mass exercised may influence the amount of protein required to increase MPS stimulation. For those engaging in whole body resistance exercise 20 g of protein is not sufficient to maximally stimulate MPS. The athletes in Chapters 2 and 3 of this thesis consumed more protein than current recommendations that do not take into account whole body exercise. Current post-exercise protein recommendations may no longer be optimal given this new information. Future work should directly investigate the MPS response to protein ingestion following resistance exercise engaging different amounts of muscle mass in well trained and elite populations. Identifying the protein dose required for maximal stimulation of MPS following whole body exercise would be an informative area of future research.

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I would firstly like to thank the man that gave me the push I needed to start my Ph.D. journey. It is said that life starts on the edge of your comfort zone and I have had to remind myself of this saying numerous times.

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ABBREVIATIONS

% BF	Percentage body fat
1RM	One repetition maximum
4C	Four compartment
4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
6N	Six Nations
ATP	Adenosine triphosphate
AUC	Area under the curve
BD	Body density
BM	Body mass
BMD	Bone mineral density
CI	Confidence intervals
DEXA	Dual-energy x-ray absorptiometry
DNA	Deoxyribonucleic acid
DRI	Dietary reference values
EAA	Essential amino acids
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FSR	Fractional synthetic rate
HLBM	Higher lean body mass
IC	Intracellular
ISAK	International Society for the Advancement of Kinanthropometry
LBM	Lean body mass
LLBM	Lower lean body mass
LMI	Lean mass index
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribonucleic acid
mTORC1	Mechanistic target of rapamycin complex 1
NBAL	Net muscle protein balance
p70S6K1	Protein ribosomal protein S6 kinase 1
PCA	Perchloric acid
RNI	Reference nutrient intake
SRU	Scottish Rugby Union
U20	Under 20s

LIST OF PUBLICATIONS

Articles published:

Macnaughton LS, Wardle SL, Witard OC, McGlory C, Hamilton DL, Jeromson S, Lawrence CE, Wallis GA, Tipton KD (2016) The response of muscle protein synthesis following whole body resistance exercise is greater following 40 g than 20 g of ingested whey protein. Physiol. Rep. 4, e12893.

Witard OC, Wardle SL, **Macnaughton LS**, Hodgson AB, Tipton KD (2016) Protein considerations for optimising skeletal muscle mass in healthy young and older adults. Nutrients. 8, 4, E181.

Jones, T.W., Smith, A., **Macnaughton, L.S**., French, D.N., 2016. Strength And Conditioning And Concurrent Training Practices In Elite Rugby Union. J. Strength Cond. Res. Epub ahead of print.

McGlory C, Wardle SL, **Macnaughton LS**, Witard OC, Scott F, Dick J, Bell JG, Phillips SM, Galloway SDR, Hamilton DL, Tipton KD (2016) Fish oil supplementation suppresses resistance exercise and feeding-induced increases in anabolic signalling without affecting myofibrillar protein synthesis in young men. Physiol. Rep. 4, e12715.

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CHAPTER 1 General introduction

1.0. Nutrition

Nutrition is an integral part of any athlete's daily life. At the most simple level, athletes, and non-athletes, need to consume food in order to stay alive and at the highest-level, nutrition can be manipulated to help yield world class sporting performances. Three macronutrients make up the majority of humans' nutrient intake: carbohydrate, protein and fat. Additionally, humans require a range of vitamins and minerals in their diet that are essential for normal bodily function. Carbohydrate is the main source of fuel for the brain and an important source of fuel for skeletal muscle (Jeukendrup and Gleeson, 2004). Fat is another essential fuel source, as well as being important for whole body and cellular structure and function. Protein is essential for the growth and repair of tissues within the body (Jeukendrup and Gleeson, 2004). Due to their roles within the body these nutrients are vital for all humans, not just athletes.

In addition to these nutrients being a vital part of the human diet, athletes can strategically manipulate consumption of these nutrients to obtain the best adaptations to their training and to provide the optimum conditions for peak performance. Athletes should consider nutrition on a day-to-day basis to allow their body to function optimally. Furthermore, athletes should optimise their nutrition to fuel and then recover from a training session or competition. Carbohydrate and fat are the main substrates utilised during exercise so are required in the diet to fuel training and competition (Jeukendrup, 2003). If glycogen has been depleted during an exercise bout it must be replaced following exercise (Burke et al., 2004). A minimal amount of protein is used as a substrate during exercise (Jeukendrup and Gleeson, 2004) but is key for the repair and remodelling process that occurs after exercise.

1.1. Protein nutrition

This thesis will focus primarily on protein nutrition and its relationship with lean body mass (LBM). Protein is essential for the growth and repair of tissues and cells and is the main source of amino acids in the diet. The body produces some amino acids, termed non-essential amino acids. However, there are 9 amino acids the body cannot produce and these come from the diet (Widmaier et al., 2008). Amino acids are the constituent part of any protein within the body. Both essential and non-essential amino acids are required for the generation of new proteins. Consequently, dietary amino acids are required for muscle maintenance and growth as well as for the production of nitrogen-containing compounds, *e.g.,* neurotransmitters, DNA, hormones *etc.* (Lodish et al., 2008).

1.1.1. Protein recommendations

Due to the critical role that amino acids play in the human body an important consideration for athletes, and the general population alike, is how much protein to consume. For several reasons, unsurprisingly, there is not a simple answer to this question. First, the amount of protein that an individual needs to consume to maintain health and avoid illness due to deficiency is different from the amount of protein an individual should consume to optimise training adaptations and performance (Macnaughton and Tipton, 2015). Meeting protein requirements is important to maintain an individual's health but considering protein recommendations is important for individuals who wish to support, and gain the maximum adaptations from, training. It is vital that recommendations for athletes should consider the health and wellbeing of the athlete as well as performance.

Protein recommendations for athletes differ from those provided for the general population. The UK reference nutrient intake (RNI) for adults is 0.75 g of protein per kg body mass per day (British Nutrition Foundation 2015). There is a degree of disparity within the literature as to whether the protein requirements for athletes are greater or less than the general population. It has been shown that exercise training increases the utilisation of amino acids as measured by increased nitrogen retention (Hartman et al., 2006; Moore et al., 2007). These data may suggest a reduction in protein requirement because the efficiency with which the protein is used increases with training. However, it has been demonstrated that athletes require more protein because training increases amino acid demand (Tarnopolsky et al., 1988). Overall, current thinking is that athletes require more protein than non-athletes do. Endurance athletes should consume ~1.2-1.4 $g \cdot kgBM^{-1}$ (Tarnopolsky, 2004) and resistance trained athletes should consume 1.2-1.7 $g \cdot kgBM^{-1}$ (Rodriguez et al., 2009) to meet protein requirements. However, it has been suggested that nitrogen balance studies, which are used to determine protein requirements, may not represent protein requirements for gains in LBM (Phillips, 2004). The majority of athletes are likely to consume sufficient protein to meet requirements to remain healthy (Phillips, 2006; Tarnopolsky, 2004; Tipton & Witard, 2007). However, for athletes the goal is to optimise performance while remaining healthy. A vast number of studies exist that have attempted to optimise protein nutrition in order to gain the maximum adaptations to training. The individual's body size and type, type of sport, playing position and even time of the season within the sport all will impact on the protein recommendations provided (Mujika et al., 2014; Phillips, 2004; Tarnopolsky, 2004). Also, the goals of the athlete must be considered carefully. For example, athletes may want to increase LBM or reduce fat mass. These factors illustrate why provision of

blanket protein recommendations is not effective. However, implementation of a number of generic strategies is possible, which could maximise any athlete's training response and induce adaptations above that of training alone. The majority of studies that have attempted to optimise protein nutrition strategies have involved participants – often untrained that are all of a similar body mass and composition. Lean mass, often used as a measure of muscle mass, is an important aspect of body composition. Many athletes, at various standards, are aware of the importance of maintenance or increase in muscle mass and actively aim to achieve an increase in muscle mass through their training and diet.

1.1.2. Measuring the impact of protein nutrition

Much of the work focussed on optimising protein nutrition has measured the building of new muscle proteins over an acute period (muscle protein synthesis or MPS). The type of exercise performed affects the type of proteins within the muscle that are stimulated (Wilkinson et al., 2008). Yet, the magnitude of the response tends to be influenced by the different factors of protein provision in combination with the exercise bout (Phillips & Van Loon, 2011; Tipton & Phillips, 2013). Provision of amino acids results in elevated MPS as the amino acids are incorporated into new muscle proteins (Pennings et al., 2011). Although MPS is not a direct measure of muscle mass accretion, muscle mass would not increase in healthy individuals (at a constant rate of MPB) if MPS was not elevated (Tipton & Wolfe, 2001). The validity of measuring MPS with a view to assessing the potential for an intervention to increase muscle mass is often questioned. Mitchell and colleagues (2014) showed that there was no correlation between myofibrillar MPS, measured 1-6 h post resistance exercise, and resistance training induced hypertrophy. However, recent work by Damas et al.,

(2016) demonstrated that myofibrillar MPS after three and ten weeks of resistance training, correlated strongly with hypertrophy. Therefore, measuring acute changes in MPS to assess the potential effectiveness of an intervention designed to increase muscle anabolism is a useful tool. Ideally, interventions would be assessed using long-term training studies but there are a number of practical difficulties in running these types of studies that limit their use. Controlling total nutritional intake; timing of that intake; physical activity; sleep and compliance with the intervention, are a few of the challenges involved with long-term studies (Atherton et al., 2015). Due to these difficulties, use of acute MPS studies as an alternative approach to assess the effectiveness of an intervention is common.

1.1.3. Protein dose

Protein recommendations to support training adaptions do not simply comprise a daily total amount of protein; there are a number of other aspects to consider. Defining the dose of protein required to stimulate MPS maximally is particularly important in athletic populations. Athletes in power, speed and strength based sports as well as intermittent team sports are, at the very least, aiming to maintain, if not increase, muscle mass. Stimulating the MPS response following exercise is important to allow athletes to achieve body composition goals. Defining the optimal protein dose for maximising MPS will ensure athletes are maximising their training adaptations (Phillips and Van Loon, 2011) while not wasting energy, time or money consuming surplus amounts of protein. Some athletes also may be working within an energy budget so the consumption of the optimal amount of protein is beneficial, in order that their energy intake is no higher than necessary. Cuthbertson et al., (2004) carried out an essential amino acid (EAA) dose-response study in young healthy

males. Calculation of myofibrillar fractional synthetics rate (FSR), one way to measure MPS, occurred over a 3 h post ingestion period following consumption of 0, 2.5, 5, 10 or 20 g of EAA. The 2.5-10 g conditions stimulated FSR in a dose dependent manner but 20 g did not elicit further stimulation of FSR compared with 10 g. Again at rest, Symons, et al., (2009) fed young adults either 113 g or 340 g of lean beef which contained either 30 g or 90 g of protein respectively. Calculation of mixed FSR occurred over a 5 h postprandial period. The investigators found that both doses of beef stimulated FSR significantly compared with basal values, however, no difference in FSR stimulation existed between the doses. At rest, 10 g of EAA or 30 g of protein stimulate FSR to the same extent as double or triple the respective doses. However, both these studies were carried out at rest and the MPS response to amino acid provision following resistance exercise is elevated above that of amino acid provision alone (Biolo et al., 1997).

Two studies have compared the MPS response to different protein doses following resistance exercise. Moore and colleagues (2009) fed young males 0, 5, 10, 20 or 40 g of egg-protein following a bout of lower limb bilateral resistance exercise and measured mixed FSR over a 4 h post ingestion period. The 5 and 10 g conditions stimulated mixed muscle FSR to a greater extent than 0 g with no difference between 5 and 10 g. There was no difference between 20 and 40 g although both these doses stimulated FSR to a greater extent than the 0, 5 and 10 g conditions. A study complementing the results from Moore et al., (2009) was carried out by our research group (Witard et al., 2014). Young males consumed 0, 10, 20 or 40 g of whey protein following a bout of unilateral leg resistance exercise and myofibrillar FSR was calculated over a 4 h period, post protein consumption. Rates of myofibrillar FSR

were greater in the exercised leg compared with the rested leg. FSR was greater following ingestion of 20 and 40 g compared with 0 and 10 g but there was no difference between 20 and 40 g as recorded previously. It was observed that FSR was not different between the 0 and 10 g conditions, a finding not observed in Moore et al., (2009). One explanation for this difference is that the participants in Witard et al., (2014) had consumed a high protein meal 4 h before consuming the whey protein, whereas, Moore et al's (2009) participants consumed the protein following an over-night fast. Atherton et al., (2010) observed that ingestion of 48 g protein resulted in a latent period of ~45 min and then a peak MPS response at 90 min. MPS then returned to basal levels although amino acids were still available. Elevated intracellular and extracellular amino acid concentrations stimulate MPS (Biolo et al., 1995b; Bohé et al., 2003). Therefore, it would appear that the change or rapid elevation in amino acid concentration stimulates MPS. Although plasma amino acid concentrations had returned to baseline pre-drink in Witard et al's (2014) study, concentrations within the muscle may not have reduced to baseline before the drink was consumed. The 10 g protein dose may not have been enough to generate the required change in amino acid concentrations from post breakfast concentrations to stimulate MPS, whereas, the 20 and 40 g doses appeared to elicited sufficient change in amino acid concentrations. Currently, based on the available evidence, it is reasonable to conclude that the optimum dose required for maximal MPS stimulation is 10 g EAA or 20 g of high quality protein (containing ~10 g EAA) for young individuals both at rest and following resistance exercise.

1.1.4. Protein type

The type of protein consumed is another aspect of protein nutrition that could influence the MPS response. Whey protein stimulates MPS to a greater extent than

either soy or casein protein, while soy protein stimulates MPS to a greater extent than casein, both at rest and following resistance exercise (Tang et al., 2009). The difference in MPS, in response to these different types of protein, can be attributed to both the speed of appearance of amino acids in the plasma (West et al., 2011) and the amino acid content of the protein (Tang et al., 2009; Tipton & Phillips, 2013). Rapid availability of amino acids in the plasma increases the rate of amino acid delivery to the skeletal muscle where amino acids are readily utilised. Indeed, Burke et al., (2012) investigated the pattern of appearance of amino acids in plasma following ingestion of a number of protein sources. The liquid sources reached their peak amino acid concentrations in the plasma much faster than the solid proteins, with whey being the fastest. Different types of protein have different amino acid profiles. It is clear that the essential amino acid content of a protein is the main driver of MPS and nonessential amino acids are not in fact required for the stimulation of MPS (Tipton et al., 1999). Of the essential amino acids, leucine appears to be the most significant for stimulation of MPS (Norton and Layman, 2006). Therefore, proteins with high leucine content stimulate MPS to a greater extent than those with lower leucine content (Tang et al., 2009). It is thought there is a leucine threshold that must be reached in order for MPS to be stimulated (Rieu et al., 2006). Churchward-Venne et al., (2012a) observed that whey protein was the best stimulator of MPS when consumed following resistance exercise compared with leucine alone or EAA without leucine. Although leucine alone achieved the same initial MPS stimulation as whey protein, the duration of the stimulation was less (Churchward-Venne et al., 2012a). Though leucine alone initially stimulates MPS, endogenous sources must maintain the pool of other EAA, as those amino acids are required for the construction of new proteins (Figure 1.1). Leucine appears to play a key role in the stimulation of the

mTORC1 pathway that stimulates muscle growth (Moberg et al., 2014). However, the other amino acids contained in the whey protein are still required, not to further increase MPS but to maintain it, as they form the myofibrillar proteins. If individual amino acids are not available and are required within the peptide chain then protein synthesis will stop.

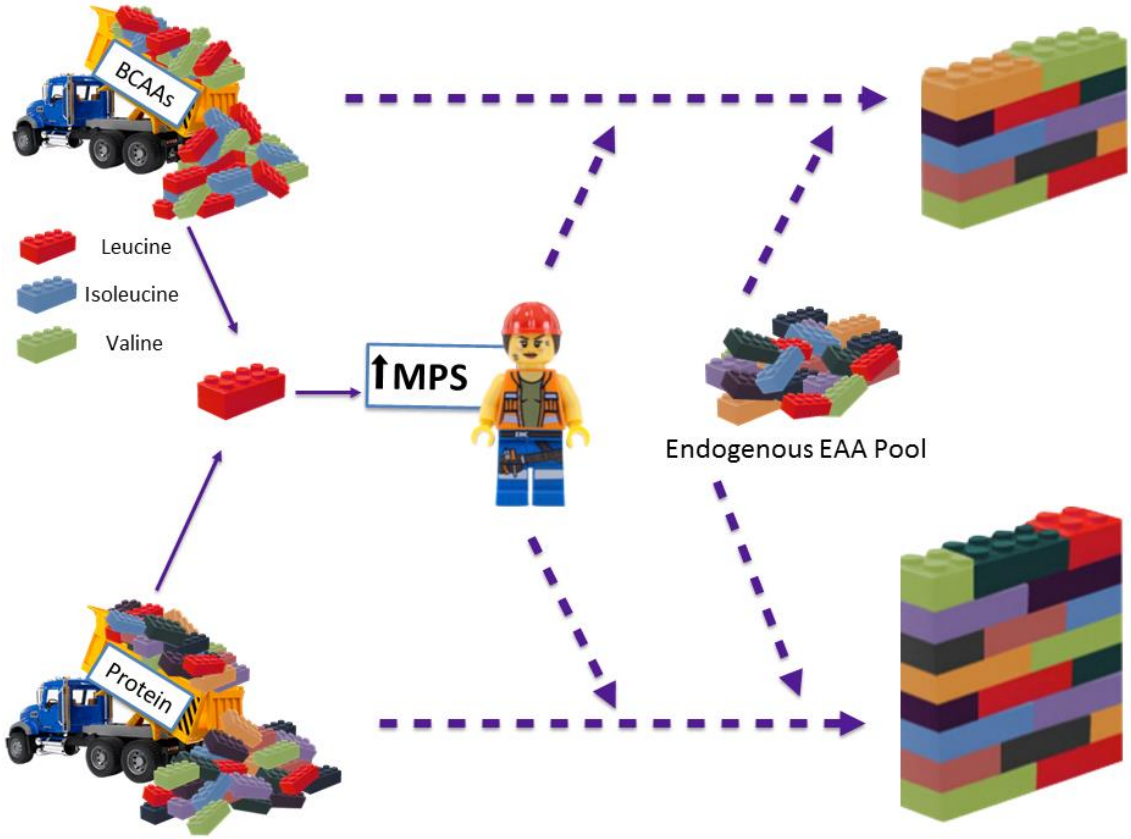


Figure 1.1 – Illustration of the requirement of all essential amino acids for longer stimulation of MPS.

Longer-term training studies also have detailed the influence of protein type, with mixed results. During an 8 wk training study there was no difference in LBM gains following consumption of 48 g of whey or rice protein (Joy et al., 2013). No studies to date have directly compared the acute MPS response to these two proteins following exercise. Two proteins that have been compared directly in an acute setting following exercise are whey and casein. No difference was observed in LBM gains between

whey and casein protein (24 g pre and post each exercise session) over an 8 wk period (Wilborn et al., 2013). However, following resistance exercise whey protein stimulates MPS to a greater extent initially than casein, due to its higher leucine content (Tang et al., 2009) but over a 6 h period there is no difference in MPS stimulation (Reitelseder et al., 2011). Whey stimulates MPS to greater extent than soy protein (Tang et al., 2009) and in a 9 month training study LBM also increased to a greater extent following whey supplementation compared with soy (Volek et al., 2013). It is possible that when protein intake is high or protein consumption is regular the type is less significant. However, type becomes more significant when an individual's intake is restricted. Protein type is likely to be more influential on adaptations when protein intake is inadequate or consumption is less regular. The type of protein consumed may influence the overall MPS and muscle growth response in certain circumstances so should be considered when making protein recommendations.

1.1.5. Protein timing and pattern

The timing of protein intake is another consideration that must be made when protein recommendations are being advised. Timing refers to the timing of intake throughout the day, often referred to as pattern of protein intake, and timing of intake in relation to exercise. Atherton et al., (2010) captured the time-course response of MPS to a single bolus of 48 g whey protein designed to saturate MPS rates. Peak MPS was reached at 90 min, thereafter, MPS rates rapidly returned to basal values although availability of EAA continued. Therefore, considering postprandial MPS rates declined despite the continued availability of exogenous amino acids, the importance of spacing protein feeds sufficiently far apart for the repeated stimulation

of MPS with multiple feeds is clear. Resistance exercise alters the MPS response to protein feeding. Consequently, Areta et al., (2013) investigated various patterns of whey protein ingestion to stimulate MPS 12 h post resistance exercise. Participants ingested the same amount of protein over the 12 h recovery period but in different patterns. Supplementing with 20 g of protein spaced out in four doses over the 12 h recovery period was the most effective strategy to stimulate post exercise MPS. This pattern of ingestion was compared to two 40 g boluses and eight 10 g doses of protein. Furthermore, equal distribution of protein intake throughout the day has been shown to stimulate 24 h MPS to a greater extent than a skewed distribution (Mamerow et al., 2014). From the available evidence, it appears that an even distribution of protein, in ~20 g boluses, is the best strategy to follow to stimulate MPS to the greatest extent.

Regarding protein timing in relation to exercise, it is well accepted that what is known as an 'anabolic window' exists. This period of time refers to the time in which the MPS stimulatory benefits of resistance exercise can be amplified following protein ingestion. During two 10 wk training studies participants were supplemented with protein either pre and post resistance exercise or in the morning and evening (Cribb and Hayes, 2006; Hoffman et al., 2009). One of the studies observed that gains in LBM were greater in the pre and post group compared with morning and evening (Cribb and Hayes, 2006) and the other study observed no difference between supplementation strategies (Hoffman et al., 2009). There is a consensus throughout the field that the 'anabolic window' exists but what is not agreed on is the duration of this effective window. Burd et al., (2011) showed that protein could augment the increase in MPS observed following resistance exercise for up to 24 h. The additional anabolic effect of consuming protein will still occur 24 h post resistance exercise but

there is evidence to suggest that consuming protein immediately post exercise increases protein synthesis to a greater extent than consuming the protein later (Levenhagen et al., 2001). The practicalities of immediate protein consumption must be weighed up against the benefits; consumption of protein within a couple of hours of training will be adequate for most people. Most studies that investigate protein ingestion combined with resistance exercise have their participants ingest the protein immediately after exercise (within a few minutes). This strategy may induce the largest response of MPS to the combined stimuli but it is also practical to save time when it is not necessary to wait to ingest the protein. However, it has been observed that protein ingestion before or during resistance exercise may be just as effective as post consumption (Tipton et al., 2007). Providing protein pre or during a workout could stimulate an increase in MPS earlier than post exercise, resulting in MPS being stimulated for a longer period of time. Also, MPB could be reduced, however, MPB is important for adaptations to resistance exercise and is elevated in response to resistance exercise (Phillips et al., 1997). Proteins damaged during exercise have to be removed or repaired and MPB is essential in that process. More work must be carried out investigating whether consuming protein pre or during a workout is a better strategy for stimulating MPS than consuming protein post exercise.

1.1.6. Protein co-ingestion

Co-ingestion of the other macronutrients with protein is another factor of protein nutrition to consider. However, for the majority of athletes the co-ingestion of all three macronutrients – protein, carbohydrate and fat – typically occurs within a meal. Following resistance exercise, carbohydrate co-ingestion with protein does not increase MPS compared with protein ingestion alone (Staples et al., 2011). An

alternative role for protein is apparent when training in a low carbohydrate state. Co-ingestion of protein with a sub-optimal amount of carbohydrate can increase muscle glycogen synthesis compared with consumption of a suboptimal dose of carbohydrate alone. This combined ingestion results in greater storing of fuel within the muscle for use during exercise (Burke et al., 2011). Fat co-ingestion may alter amino acid digestion and absorption kinetics. It appears concomitant lipid ingestion increases amino acid utilisation of milk proteins following resistance exercise (Elliot et al., 2006). However, only one study to date demonstrates this effect and the mechanism for increased muscle anabolism with concomitant lipid ingestion is not clear. Co-ingestion of protein with other nutrients warrants further investigation given the majority of individuals consume protein within a meal.

There are a number of aspects of protein nutrition for athletes and practitioners to consider. Protein nutrition is not simply how much protein should be consumed in a day. Athletes' protein nutrition strategy will differ depending on their individual goals, sport and training programme. Protein is required in the diet for growth and repair of tissues. Many athletes are attempting to increase or maintain their LBM. Manipulation of protein feeding strategies may help achieve desired body composition.

1.2. Body composition

It is often important for athletes to alter their body composition. Depending on the requirements of the sport, a certain proportion of fat to lean mass is considered optimal (Fleck, 1983). Training and nutrition can manipulate body composition. When the training programme and nutrition strategies complement each other, the

greatest change is likely to be observed. Maintaining a relatively high protein intake whilst trying to reduce body mass should preserve LBM (Mettler et al., 2010). Altering protein nutrition helps to increase lean body mass, in conjunction with the appropriate training. The provision of amino acids along with the stimuli of resistance exercise results in muscle growth.

1.2.1. Measurement of body composition

Body composition can only be estimated in humans, not directly measured. There are a number of methods to estimate body composition but dissection is the only method that would give a true value. This method would clearly not be suitable for athletes so body composition must be estimated. The four-compartment (4C) model is often considered the gold standard model for estimating body composition (Toombs et al., 2012; Van der Ploeg et al., 2003). The four compartments refer to bone, fat mass, fat free mass and body water. Often within the 4C method, body density measured by hydrodensitometry, total body water is calculated using isotopic dilution and bone mineral mass by DEXA. The 4C model requires highly specialised equipment and expertise, furthermore, its use is costly and time consuming. Therefore, more practical methods of measuring body composition are required. These other methods have been validated against the 4C model. Skinfold thickness is a two compartment model (fat mass and fat free mass) that can be used to estimate body composition (Siri, 1961). Practitioners commonly use this method as the equipment required is portable, the process can be done almost anywhere and is not very time consuming. Another method used to estimate body composition is dual-energy x-ray absorptiometry (DEXA) scanning. Originally designed to assess bone health, DEXA scanning has been used to estimate lean mass and fat mass as it can differentiate

between the three different tissue types (three compartment model) (Mazess et al., 1990). There are a number of other methods that estimate body composition but skinfolds and DEXA are the two methods used in this thesis.

1.3. Metabolism

Skeletal muscle is a highly metabolically active tissue. Furthermore, skeletal muscle is the major site of glucose disposal. Consequently, it plays a critical role in glucose control in response to insulin, which can prevent the development of type II diabetes (DeFronzo and Tripathy, 2009). Therefore, the more skeletal muscle an individual has the more energy they expend, which can help to maintain both a healthy body composition and mass. Skeletal muscle mass is crucial for human survival as it supports the skeletal system and is vital for locomotion. Maintaining skeletal muscle mass allows individuals to perform daily tasks and conserves quality of life (Wolfe, 2006). Besides the clear health benefits associated with muscle mass, maintaining or indeed increasing skeletal muscle mass, is important in the context of all sporting performance from elite to recreational level. Depending on the physiological demands of the sport and the energy systems used the appropriate amount of muscle mass for an individual to possess, in order to perform effectively in the sport, will vary. The maintenance of muscle mass is paramount for almost all elite athletes and often gaining muscle mass results in a competitive advantage. For most team sport and strength athletes having a critical muscle mass to deliver the power required to perform the skills involved in their sport is essential (Tipton and Wolfe, 2004).

1.4. Regulation of muscle mass

Skeletal muscle is in constant turnover, as part of a highly dynamic process. The

metabolic basis that underpins the regulation of muscle mass is net muscle protein balance (NBAL). NBAL fluctuates in response to changes in MPS and muscle protein breakdown (MPB). Circulating amino acids, obtained from dietary protein, are transported via amino acid transporters into the cell's free amino acid pool (Palacin et al., 1998). Incorporation of amino acids from this pool generates new proteins (MPS). When proteins are degraded, the amino acids are released into the cell's free amino acid pool (MPB) (Figure 1.2). Maintenance of skeletal muscle mass occurs when MPS is equal to MPB. However, muscle mass will increase over time when an individual is in a state of positive NBAL (Tipton and Ferrando, 2008). This metabolic situation is achieved when MPS exceeds MPB. Conversely, muscle mass is lost when an individual is in negative NBAL which occurs when MPB exceeds MPS (Tipton and Ferrando, 2008). Due to the importance of maintaining or gaining muscle mass there is a large body of work that has investigated various stimuli of MPS. Much of the work has aimed to identify the optimum conditions in which MPS can be maximally stimulated.

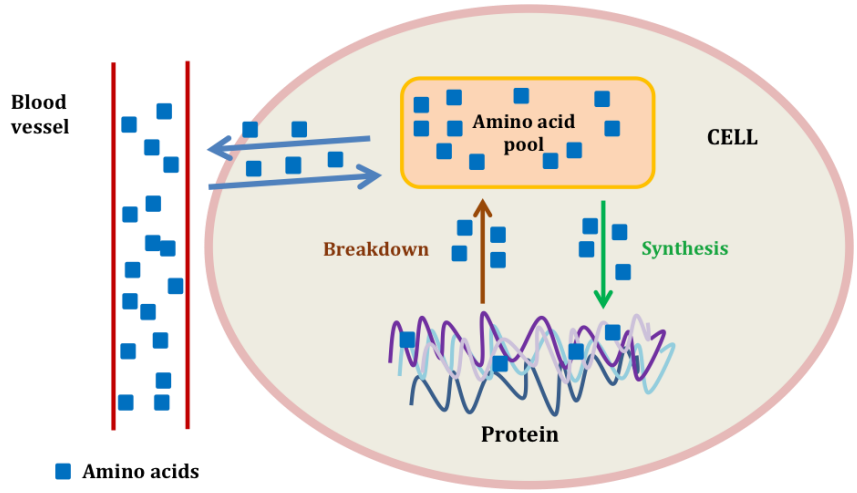


Figure 1.2 – Movement of free amino acids into and out of a cell.
Amino acids move from the blood stream into the free amino acid pool within the cell via amino acid transporters. Amino acids are incorporated from the amino acid pool within the cell into new proteins that are being synthesised. As proteins are broken down the amino acids are released into the amino acid pool and can then move into the blood stream.

Two key stimuli of MPS are resistance exercise and amino acid provision. It is well accepted that MPS increases in response to a bout of resistance exercise. Chronically, repeated bouts of resistance exercise lead to MPS being stimulated over an extended period of time, which leads to skeletal muscle hypertrophy (Phillips, 2000). Work by Kumar et al., (2009) demonstrated no difference in MPS when resistance exercise was performed at intensities between 60-90% of an individual's one repetition maximum (1RM). MPS also is stimulated by the provision of amino acids (Biolo et al., 1997). The greatest stimulation of MPS occurs when resistance exercise is combined with amino acid provision (Biolo et al., 1997; Witard et al., 2014). Therefore, to maximise skeletal muscle hypertrophy resistance exercise should be coupled with amino acid provision.

1.5. Molecular control

The mechanistic target of rapamycin complex 1 (mTORC1) pathway is central to cell growth and has been identified, along with its downstream target proteins, as a key regulator of MPS (Kimball et al., 2002; Philp et al., 2011). mTORC1 is a protein complex made up of five of regulatory proteins. Downstream targets of mTORC1 include p70S6K1 and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1)(Kimball and Jefferson, 2010). Regulation of MPS occurs at the level of translation initiation as mTORC1 and subsequently p70S6K1 activate translation initiation factors. Both mTOR and p70S6K1 are up-regulated in response to resistance exercise and protein feeding (Glover et al., 2008; Karlsson et al., 2004; Moore et al., 2011). P70S6K1 phosphorylation/activity is used as a readout of anabolic signalling activity (McGlory et al., 2014) and is often measured in MPS studies in an attempt to gain a better understanding of the signalling processes that leads to changes in MPS.

1.6. Aims and objectives

There are a number of factors to consider for optimising protein nutrition to support training adaptions. All of the factors related to protein nutrition discussed in this chapter interact and influence each other. Muscle mass is usually measured and referred to as LBM and the development of this tissue is critical to athletes. One sport that high LBM is particularly relevant for is rugby union. Rugby union is a collision sport and high LBM confers an advantage (Bell, 1979). The field of nutrition is becoming more and more relevant within the context of daily living, at the top level of performance sport and everything in between. The hunger for knowledge drives the availability of nutritional information and with such high accessibility on the internet there is much information in the public domain. Some of this information is accurate, some is not and some needs the skill of a trained individual to interpret the information. This availability of large amounts of information leads to people implementing, practicing or following strategies that may, or may not, have any scientific basis. It is the aim of this thesis to better understand the relationship between LBM and protein nutrition in trained individuals. We aim to assess the protein feeding practices of athletes in 'the real world', assess the impact of protein feeding at the molecular level and assess the effect of protein feeding directly on the building of new muscle proteins. The aims of this thesis will be addressed by successful completion of the following objectives:

i. To assess and compare current nutritional practices and body composition of young rugby union players at different playing standards (Chapter 2 and 3).
ii. To investigate whether dietary habits of young rugby union players change in different environments and whether body composition changes during a period of international competition (Chapter 3).

iii. To examine the signalling response that underpins the MPS response to protein feeding (Chapter 4 and 5).

iv. To evaluate the influence of LBM on the MPS response to protein dose following whole body resistance exercise (Chapter 5).

CHAPTER 2 Body composition and dietary habits differ between young Scottish elite and amateur rugby union players

Macnaughton LS, Hardie E, Chessor R, Tipton KD

2.0. Abstract

Rugby union is an intermittent team sport played worldwide that combines high-intensity running with physical collisions. The aim of the current observational study was to characterise differences in body composition and dietary habits between young elite and amateur rugby union players and to examine any differences between forwards (tend to be involved in more collisions) and backs (tend to do more running). Body composition measurements were taken from twenty-eight rugby union players (elite n=26; amateur n=25) during a rugby season. A subset of players kept a 3 d food diary during the regular season (elite n=19; amateur n=20).

There was no difference in the age of the players (elite forwards 18.9 ± 1.0 y, elite backs 20.0 ± 2.7 y, amateur forwards 19.1 ± 2.9 y, amateur backs 19.7 ± 2.7 y). Elite forwards (191.6 ± 6.8 cm) were significantly taller ($p=0.031$) than elite backs, amateur forwards and amateur backs (181.6 ± 5.7 cm, 181.3 ± 8.4 cm, 179.5 ± 4.0 cm respectively). Elite players (forwards 105.4 ± 7.4 kg, backs 88.5 ± 5.3 kg) were significantly heavier ($p<0.001$) than amateur players in equivalent positions (forwards 93.1 ± 11.2 kg, backs 78.9 ± 7.6 kg) and forwards were significantly heavier ($p<0.001$) than backs. The difference in body mass between elite and amateur players was attributed to differences in lean mass but between forwards and backs this difference was attributed to both fat and lean mass differences equally.

Elite players consumed significantly more ($p=0.046$) protein per day (201.8 ± 65.1 g) than amateur players (163.0 ± 60.7 g). Daily protein intake was skewed towards the noon and evening segments of the day ($p=0.005$ effect of segment) (morning ~25%; noon ~34%; evening ~41% of total protein intake). Elite backs consumed

significantly more (p=0.035) meat and protein supplements (p=0.021) than amateur players. There were no differences in energy or macronutrient intake between forwards and backs. In summary, the differences in playing standard are characterised by differences in physical characteristics between young elite and amateur rugby union players. Also, various aspects of each group's diet differ. There are variances in the physical characteristics of forwards and backs that are most likely linked to the difference in demands of their playing position.

2.1. Introduction

Rugby union is a sport that is growing worldwide and World Rugby (formally the International Rugby Board or IRB) Chairman, Bernard Lapasset, said:

> "Rugby World Cup 2015 will be remembered as the biggest tournament to date"

with the largest attendances so far (World Rugby 2015). The introduction of the smaller-sided version of the game, rugby 7s, to the Olympics in Rio 2016, for both men and women, also has raised the profile of the game. During a game of rugby union 15 players from each side attempt to score a 'try' over the opposition's line by passing, backwards only, and kicking the ball. The opposition must attempt to stop a try from being scored by tackling their opponents and regaining possession of the ball. A full game is played for 80 min, made up of two 40 min halves, and movement patterns are intermittent in nature. During a match, players will spend periods moving at various speeds from standing to sprinting and several speeds in between (Roberts et al., 2008). Physical contact is a large component of rugby union and comes in a number of forms including tackling, rucking, mauling and scrummaging. Tackling, rucking and mauling occur in open play and are performed by all players while scrummaging and line-outs are ways to restart the game and conventionally

only are performed by certain players. Players are divided into two broad categories based on their roles and responsibilities within the team.

Players that are typically part of the scrums and line-outs are called forwards and are numbered 1 to 8. The players that are not directly part of the scrum are referred to as backs and are numbered 9 to 15. Forwards spend more time performing high-intensity activities and are involved in more high-intensity bouts than backs (Roberts et al., 2008). This difference in high-intensity activity is due to forwards being involved in more rucks and mauls and performing more tackles than backs (Roberts et al., 2008). Although backs do not participate in these activities as much as forwards, backs do spend more time performing high-intensity running and sprinting than forwards (Duthie et al., 2006a). Also, backs covered around 550 m more than forwards during a match (Roberts et al., 2008). Physical characteristics of rugby union players have been well documented (Bradley et al., 2015a; Duthie 2003; Fontana et al., 2015; Nicholas 1997). Forwards generally are heavier, taller and not as lean as backs (Nicholas 1997; Duthie et al., 2003; Fontana et al., 2015; Bradley et al., 2015a). These differences in physical characteristics exist due to the varying demands placed on forwards and backs.

Physical characteristics also differ between players at various playing standards. Players at a higher playing standard tend to be heavier (Quarrie et al., 1995; Olds 2001; Fontana et al., 2015) and taller (Fontana et al., 2015; Olds 2001; Quarrie et al., 1995). Although some physical characteristics cannot be changed *e.g.,* height, some can be manipulated, *e.g.,* body mass and body composition. Players that play at a higher standard also tend to possess more fat free mass and have lower percentage

body fat (%BF) (Fontana et al., 2015). Identifying which physical characteristics appear to be linked to playing standard would be useful for players attempting to play at a higher level. The physical characteristics of players have changed over the past several decades as players have got taller and heavier (Olds, 2001). These changes are thought to be due, in part, to the advent of professionalism (Duthie et al., 2003; Quarrie & Hopkins 2007). Consequently, less recent literature has limited application for the present day. More recent data comparing elite and amateur rugby union players would be valuable to coaches, support staff and players alike.

The manipulation of modifiable physical characteristics can be achieved through training and nutrition. Understanding the physical and dietary differences between elite and amateur rugby union players could help inform the goals of players who aspire to play at a higher standard. Thus far no comparison of diet has been made between elite and amateur rugby union players. Only one study has compared the diet of forwards and backs 'in-season'. The authors observed a similar relative macronutrient intake between forwards and backs, but forwards had a higher energy intake than backs (Bradley et al., 2015b). Two previous studies have investigated the dietary intake of rugby union players in a 'pre-season' period (Bradley et al., 2015a; MacKenzie et al., 2015). The energy and macronutrient intake were similar in both studies. Carbohydrate intake was identified as being below recommendations (Burke et al., 2011) while protein intake was above recommendations (Rodriguez et al., 2009; Tipton & Wolfe, 2004). Knowledge of rugby union players' nutritional practices could help to inform practice and manage body composition.

Therefore, the primary aim of the current study was to investigate differences in physical characteristics and dietary habits of young Scottish rugby union players at elite and amateur levels. A secondary aim was to assess differences in physical characteristics and dietary habits between forwards and backs.

2.2. Methods

2.2.1. Participants and ethical approval

Twenty-eight male rugby union players were recruited via their involvement in the Scottish Rugby Union (SRU) development squads of two professional clubs, Edinburgh Rugby and Glasgow Warriors (Elite n=26) or from amateur clubs in Central Scotland (Amateur n=25). Ethical approval for the study was granted by the NHS Scotland A Research Ethics Committee (REC number 14/SS/1095) and the study conformed to the standards set out in the latest version of the Declaration of Helsinki (2013). Players provided written informed consent before participating in the study.

2.2.2. Study design

In an observational study to assess differences in body composition and diet between young elite and amateur rugby union players DEXA scans, skinfold measurements and dietary analysis were carried out on both groups of players during the rugby season (October-February).

2.2.3. Body composition

A whole-body DEXA scan (Lunar iDEXA, GE Healthcare, Hertfordshire, UK) was performed on participants in a fasted (>3 h), rested state (no exercise on the day of the scan). Participants were asked to drink 500 mL of water 2 h before attending the

laboratory to ensure euhydration (Rodriguez-Sanchez and Galloway, 2015). Upon arrival participants were asked to void their bladder before their height and body mass were measured. Participants were then scanned in the standardised supine position (Nana et al., 2012) wearing only underwear. The DEXA scanner was calibrated to the manufacturer's guidelines using a standard calibration block. The same trained investigator conducted all scans. The digital image of the player was partitioned into the anatomical regions of the head, trunk, arms, legs, android (area between the ribs and the pelvis) and gynoid (hips and upper thigh) for segmental body composition analysis. The CV for this particular model of DEXA is 0.4-0.5% for LBM, 0.7-1% for fat mass and 0.6-0.9% for %BF (Toombs et al., 2012).

Following the DEXA scan participants had further anthropometric measurements taken by an ISAK accredited anthropometrist. Measurements of the elite players were part of a series of measurements by the SRU to monitor changes over a season and, as such, two different anthropometrists measured the elite and amateur players. Measurements were taken with Harpenden callipers on the right side of the body following ISAK protocol and included skinfolds at eight sites (triceps, subscapular, biceps, iliac crest, supraspinale, abdominal, front thigh, medial calf; intra technical error of measurement 2.4 ± 0.6%).

2.2.4. Calculations

The sum of 6 skinfolds was calculated by summing the values measured at the following skinfold sites: triceps, subscapular, supraspinale, abdominal, front thigh and medial calf. The sum of 7 skinfolds was calculated by summing the values

measured at the following skinfold sites: triceps, subscapular, biceps, supraspinale, abdominal, front thigh and medial calf.

Body density was calculated in the following manner (Withers et al., 1996):

Body density = 1.0988-(0.0004*sum of 7 skinfolds)

Percentage body fat was calculated using the Siri equation (Siri, 1961) where BD is body density:

% body fat = (495/BD)-450

Lean mass index (LMI) was calculated as follows where the exponent is 0.14 for forwards and 0.13 for backs (Slater et al., 2006) and BM is body mass:

LMI = BM/ (sum of 7 skinfolds^fwd/back exponent)

2.2.5. Dietary analysis

Participants completed a 3 d food diary during their regular playing season. The same investigator performed dietary analysis using Wisp Version 4.0 (Tinuviel Software, Anglesey, UK). Mean daily energy and nutrient intake were calculated from the 3 d dietary recording. Players did not consistently eat the same meals as each other so analysing protein timing and pattern of intake was challenging. Sectioning intake over the day allowed, to some extent, the assessment of protein intake pattern. Within the dietary records, players self-reported breakfast, lunch, dinner and snacks but not the exact timing of each meal. We partitioned the day so that the 1st segment of the day was from breakfast to before lunch (morning), the 2nd segment was lunch and anything before dinner (noon) and the 3rd segment was dinner and the rest of the day (evening). Protein type was quantified by counting the number of self-recorded eating occasions in which one of the following types of protein was consumed: dairy, meat, eggs, fish, and supplements.

2.2.6. Statistical analysis

Data are presented as mean ± SD unless otherwise stated. Statistical analysis was carried out in Minitab Version 17.0 (Minitab Statistical Software, Coventry, UK). Significance was set at the 95% confidence level ($p<0.05$). Where data did not follow a normal distribution pattern, box cox transformations were performed. Following transformation if data still were not normally distributed (training (h), alcohol (g), dairy and supplements) the Kruskal-Wallis test was performed for group and position. Two-way ANOVAs were run with group (2 levels - elite and amateur) and position (2 levels - forwards and backs) as factors for all descriptive characteristics, body composition outcomes, energy intake and nutrient intake. A further factor of segment (3 levels – morning, noon and evening) was introduced to assess protein distribution. Tukey's post-hoc test was used to assess where differences existed, if significance was detected. Paired t-tests were run to assess whether outcomes from the DEXA were significantly different from outcomes calculated from skinfold measurements. Pearson's Product correlations were performed in GraphPad Prism 6 (GraphPad Software Inc, California, USA) and r was reported.

2.3. Results

2.3.1. Descriptive characteristics and whole body composition

The age of the players in the current study did not differ between groups. There was no difference in the number of years players had spent playing rugby (Table 2.1). Elite players trained for around 15 $h \cdot wk^{-1}$ more than the amateur players ($p<0.001$) but there was no difference in training hours based on playing position. Elite players were ~12 kg heavier ($p<0.001$) and ~0.70 m taller ($p<0.001$) on average than amateur players. Forwards were heavier ($p<0.001$) and taller ($p<0.001$) on average

than backs. Elite forwards were significantly taller than all other groups (group × position interaction; p=0.031). BMI was greater for forwards compared with backs (p<0.001) but there were no differences between groups.

Table 2.1- Descriptive and whole body characteristics of young elite and amateur rugby union players.

	Elite (n=26)		Amateur (n=25)	
	Forwards (n=15)	Backs (n=11)	Forwards (n=15)	Backs (n=12)
Age (y)	18.9 ± 1.0	20.0 ± 2.7	19.1 ± 2.9	19.7 ± 2.7
Time spent playing (y)	9.7 ± 3.6	7.3 ± 2.9	8.2 ± 4.3	8.0 ± 3.9
Training (h·wk) *	24.6 ± 3.6	23.0 ± 3.2	10.3 ± 6.9	8.2 ± 1.8
Height (cm) * † ‡	191.6 ± 6.8 [a]	181.6 ± 5.7 [b]	181.3 ± 8.4 [b]	179.5 ± 4.0 [b]
Body mass (kg) * †	105.4 ± 7.4	88.5 ± 5.3	93.1 ± 11.2	78.9 ± 7.6
Body mass index ($kg \cdot m^2$) †	28.8 ± 2.5	26.8 ± 1.2	28.4 ± 3.3	24.5 ± 2.4
Bone mineral density ($g \cdot cm^2$) * †	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1

Values are presented as means ± SD. * denotes significant difference between group; † denotes significant difference between positions; ‡ denotes significant interaction; means with different letters are different from each other.

Body composition outcomes are displayed in Table 2.2. Due to a measurement error four participants from the elite forwards group were removed from the whole body DEXA measurements, giving a n=12 in that group for total LBM, fat mass and bone mineral density (BMD). However, the results (outcomes from statistical testing) were the same with these four players included and with them removed. LBM measured by DEXA was, on average, ~10 kg greater in the elite group compared with the amateur

group ($p<0.001$) and ~15 kg greater when lean mass was calculated from skinfold measurements ($p<0.001$). The forwards had, on average, ~7 kg more LBM than the backs when calculated using DEXA and ~10 kg more when calculated using skinfolds (both $p<0.001$). There was a strong correlation between LBM measured by DEXA and skinfolds ($r=0.976$; $p<0.001$), however the values calculated from skinfold measurements were significantly greater than those calculated from the DEXA ($p<0.001$).

Fat mass did not differ between groups but forwards had, on average, ~7 kg more fat mass than backs calculated by DEXA ($p<0.001$) and ~6 kg more fat mass when calculated using skinfold measurements. There was a significant correlation between fat mass measured by DEXA and skinfolds ($r=0.931$; $p<0.001$). However, the values calculated from skinfold measurements were significantly lower than those calculated from DEXA ($p<0.001$). Percentage BF measured by DEXA tended to be lower in the elite group compared with the amateur group ($p=0.073$). Forwards had a greater percentage body fat as measured by DEXA than backs ($p<0.001$). Percentage BF calculated using skinfolds was lower in the elite group compared with the amateur group ($p=0.036$) and greater in forwards than backs ($p<0.001$). Percentage BF from DEXA correlated well with % BF calculated from skinfolds ($r=0.904$; $p<0.0001$) but values calculated from skinfolds were significantly lower than those from DEXA ($p<0.001$). Sum of 8 skinfolds had the strongest correlation ($r=0.934$; $p<0.0001$) with % BF from DEXA followed by sum of 6 ($r=0.928$; $p<0.0001$) and 7 ($r= 0.903$; $p<0.0001$) respectively.

Table 2.2 – Body composition of young elite and amateur rugby union players measured using DEXA and skinfold thickness with the Siri equation.

	Elite (n=26)		Amateur (n=25)	
	Forwards (n=15)	Backs (n=11)	Forwards (n=15)	Backs (n=12)
Lean body mass (kg) (DEXA) * †	81.8 ± 5.6	72.4 ± 4.8	69.1 ± 6.6	63.5 ± 6.6
Lean body mass (kg) (Skinfolds) * †	92.4 ± 5.4	80.0 ± 4.5	76.8 ± 7.0	70.4 ± 6.5
Fat mass (kg) (DEXA) †	18.7 ± 4.2	12.1 ± 2.4	20.2 ± 7.7	12.1 ± 3.4
Fat mass (kg) (Skinfolds) †	13.0 ± 3.6	8.5 ± 1.8	16.3 ± 7.3	8.5 ± 3.0
% body fat (DEXA) †	17.7 ± 3.0	13.7 ± 2.6	21.3 ± 6.3	15.3 ± 3.9
% body fat (Skinfolds) * †	12.2 ± 2.9	9.6 ± 1.8	17.0 ± 6.3	10.7 ± 3.3

Values are presented as means ± SD. * denotes significant difference between group; † denotes significant difference between positions.

2.3.2. Regional body composition

Regional body composition results from DEXA scanning are displayed in Table 2.3. The lean mass of the arms was greater in the elite forward group compared with all other groups and the elite backs had more lean mass in the arm region than amateur backs (group × position interaction; $p=0.036$). Leg and trunk lean mass were significantly greater in the elite group compared with the amateur group ($p<0.001$) and forwards had greater leg and trunk lean mass than backs ($p<0.001$). Arm, leg and trunk fat mass did not differ between groups but was greater for forwards compared with backs ($p<0.001$). BMD was greater in the elite group compared with the amateur group ($p<0.001$) and was greater for forwards compared with backs ($p=0.004$). Arm, leg and trunk BMD was greater in the elite group than the amateur group ($p<0.001$)

and greater in forwards compared with backs (p=0.002, 0.015 and 0.004 respectively).

Table 2.3 - Regional body composition from DEXA measures of young elite and amateur union rugby players.

		Elite (n=26)		Amateur (n=25)	
		Forwards (n=15)	Backs (n=11)	Forwards (n=15)	Backs (n=12)
Lean body mass (kg)	Arms * † ‡	11.6 ± 1.2 [a]	10.0 ± 0.8 [b]	9.1 ± 1.0 [b c]	8.7 ± 1.2 [c]
	Legs * †	28.3 ± 2.0	24.7 ± 2.1	24.1 ± 2.4	21.9 ± 2.5
	Trunk * †	38.2 ± 2.4	34.1 ±2.5	32.2 ± 3.1	29.4 ± 3.2
Fat mass (kg)	Arms †	2.2 ± 0.6	1.4 ± 0.4	2.2 ± 0.7	1.2 ± 0.4
	Legs †	7.0 ± 1.2	4.5 ± 1.0	7.6 ± 2.8	4.7 ± 1.1
	Trunk †	8.7 ± 2.4	5.3 ± 1.3	9.4 ± 4.5	5.3 ± 2.1
Bone mineral density ($g \cdot cm^2$)	Arms * †	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
	Legs * †	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
	Trunk * †	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1

Values are presented as means ± SD. * denotes significant difference between group; † denotes significant difference between positions; ‡ denotes significant interaction; means with different letters are different from each other.

2.3.3. Skinfold thickness

Skinfold thickness measurements, including LMI, are displayed in Table 2.4. Sum of 6, 7 and 8 skinfolds were lower in the elite group compared with the amateur group (p=0.015; 0.038; 0.018 respectively) and were higher for forwards compared with backs (all p<0.001). LMI was greater in the elite group compared with the amateur group (p<0.001) and was greater for forwards than for backs (p<0.001). LBM as

measured by DEXA and skinfolds correlated significantly with LMI (r=0.946; p<0.0001 and r=0.979; p<0.0001 respectively).

Table 2.4 - Measurements of skinfolds and lean mass index of young elite and amateur rugby union players.

	Elite (n=26)		Amateur (n=25)	
	Forwards (n=15)	Backs (n=11)	Forwards (n=15)	Backs (n=12)
Sum of 6 (mm) * †	64.3 ± 16.8	48.2 ± 7.4	90.7 ± 33.2	56.8 ± 18.6
Sum of 7 (mm) * †	69.7 ± 16.8	54.2 ± 10.6	96.8 ± 35.4	60.6 ± 19.0
Sum of 8 (mm) * †	83.1 ± 23.2	61.9 ± 10.3	115.4 ± 42.0	73.0 ± 24.7
Lean mass index * †	60.9 ± 3.4	50.7 ± 2.8	51.7 ± 4.8	44.7 ± 4.1

Values are presented as means ± SD. * denotes significant difference between group; † denotes significant difference between positions.

2.3.4. Energy, macronutrient and micronutrient intake

Macronutrient intake is displayed in Figure 2.1. The elite players consumed on average ~40 g more protein than the amateur players (p=0.046). There was no difference in total carbohydrate and total fat intake between elite and amateur player or between backs and forwards. However, forwards consumed less carbohydrate than backs when intake was calculated relative to body mass (p=0.025) (Table 2.5).

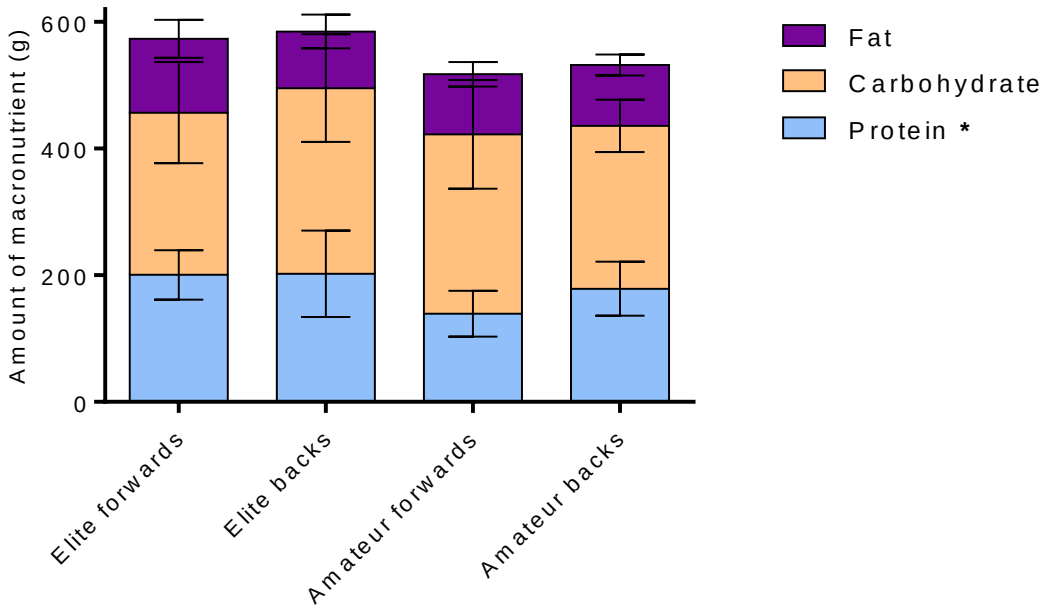


Figure 2.1 – Amount of each macronutrient consumed by young elite and amateur rugby players. Data presented as mean ± 95% CI. * denotes main effect of group.

Energy, a breakdown of macronutrient intake and micronutrient intake are reported in Table 2.5. There were no differences in energy intake, There was no difference in protein intake between backs and forwards. The elite group consumed more zinc ($p=0.044$), carotene ($p=0.013$), thiamine ($p=0.037$), riboflavin ($p=0.029$), niacin ($p=0.010$), vitamin B6 ($p=0.014$), folate ($p<0.001$) and pantothenate ($p=0.041$) than the amateur group. Forwards consumed more potassium ($p=0.045$), calcium ($p=0.026$), phosphorous ($p=0.034$), zinc ($p=0.004$), riboflavin ($p=0.006$) and vitamin B12 ($p=0.021$) than backs.

Table 2.5 - Energy, a break down of macronutrient and micronutrient intake of young elite and amateur rugby union players.

	Elite (n=19)		Amateur (n=20)	
	Forwards (n=11)	Backs (n=8)	Forwards (n=12)	Backs (n=8)
Energy (MJ)	11.1 ± 3.6	10.4 ± 2.9	9.9 ± 2.3	9.3 ± 3.3
Sugars (g)	101.4 ± 49.4	88.4 ± 32.7	103.6 ± 58.2	88.6 ± 56.9
Starch (g)	142.7 ± 73.0	191.2 ± 68.9	135.8 ± 55.1	168.6 ± 58.6
Saturates (g)	44.4 ± 16.8	32.2 ± 13.6	34.5 ± 13.9	33.4 ± 13.9
Monounsaturates (g)	39.4 ± 15.8	26.9 ± 6.7	30.4 ± 10.1	28.6 ± 9.3
Polyunsaturates (g)	15.5 ± 6.7	11.8 ± 3.4	13.4 ± 6.3	12.1 ± 4.3
Protein ($g \cdot kg^{-1}$)	1.9 ± 0.6	2.2 ± 0.9	2.0 ± 0.8	1.8 ± 0.5
Carbohydrate ($g \cdot kg^{-1}$) †	2.4 ± 1.2	3.3 ± 1.0	2.8 ± 0.9	3.7 ± 1.2
Fat ($g \cdot kg^{-1}$)	1.0 ± 0.5	1.0 ± 0.3	1.1 ± 0.4	1.2 ± 0.3
Fibre AOAC (g)	23.7 ± 10.3	26.0 ± 10.4	21.7 ± 7.8	20.8 ± 11.0
NMES (g)	18.6 ± 15.1	17.6 ± 13.7	28.6 ± 52.7	37.7 ± 28.2

Alcohol (g)	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 10.7	2.5 ± 5.7
Sodium (mg)	3833.9 ± 1474.7	3988.9 ± 1018.5	4028.7 ± 1284.0	3611.0 ± 1570.5
Potassium (mg) †	4838.4 ± 1261.4	4255.9 ± 1160.6	4368.2 ± 1440.5	3120.4 ± 1511.5
Calcium (mg) †	1314.0 ± 448.8	1070.6 ± 349.9	1286.4 ± 644.2	865.8 ± 418.9
Magnesium (mg)	438.5 ± 127.3	407.8 ± 145.1	392.9 ± 131.2	306.8 ± 146.6
Phosphorus (mg) †	2594.3 ± 686.9	2226.8 ± 680.2	2307.7 ± 704.6	1668.1 ± 728.6
Iron (mg)	18.9 ± 4.9	16.5 ± 3.8	14.5 ± 4.4	15.0 ± 4.8
Copper (mg)	1.4 ± 0.5	1.4 ± 0.5	1.7 ± 1.3	1.5 ± 1.2
Zinc (mg) * †	17.7 ± 5.2	13.5 ± 3.3	15.1 ± 5.1	11.0 ± 4.2
Chloride (mg)	5493.4 ± 2383.1	5348.6 ± 1147.7	5612.8 ± 2074.5	4664.8 ± 2271.4
Manganese (mg)	4.0 ± 1.8	4.1 ± 1.5	3.5 ± 1.5	3.4 ± 1.4
Selenium (µg)	102.3 ± 37.2	108.8 ± 46.3	99.0 ± 48.3	80.6 ± 41.4
Iodine (µg)	292.4 ± 96.6	223.6 ± 119.3	221.8 ± 128.3	166.3 ± 85.6
Retinol (µg)	698.9 ± 249.9	426.9 ± 316.6	452.6 ± 266.9	426.9 ± 298.8

Carotene (µg) *	7403.6 ± 4348.1	6209.0 ± 6552.5	2991.1 ± 2498.1	3628.8 ± 2886.0
Vitamin D (µg)	6.4 ± 2.8	4.6 ± 2.9	4.1 ± 2.7	4.4 ± 3.3
Vitamin E (mg)	10.0 ± 4.3	7.6 ± 2.5	7.3 ± 3.3	8.3 ± 4.9
Thiamin (mg) *	2.9 ± 1.2	2.6 ± 0.9	2.4 ± 0.7	1.7 ± 0.9
Riboflavin (mg) * †	3.4 ± 0.8	2.5 ± 0.9	3.1 ± 2.1	1.9 ± 0.8
Niacin (mg) *	53.1 ± 15.4	55.4 ± 25.0	43.7 ± 11.0	34.5 ± 17.6
Vitamin B6 (mg) *	3.9 ± 0.8	3.6 ± 1.1	3.3 ± 1.0	2.5 ± 1.0
Vitamin B12 (mg) †	11.8 ± 3.0	8.3 ± 4.0	9.1 ± 4.6	6.8 ± 2.6
Folate (µg) *	424.3 ± 105.9	332.3 ± 79.1	311.3 ± 125.1	274.8 ± 114.0
Pantothenate (mg) *	11.9 ± 2.7	9.9 ± 3.3	9.8 ± 4.1	7.6 ± 2.1
Biotin (µg)	65.8 ± 18.4	50.3 ± 24.6	66.3 ± 66.3	43.8 ± 15.3
Vitamin C (mg)	146.6 ± 70.2	124.5 ± 97.4	121.9 ± 69.9	94.6 ± 70.4

Values are presented as means ± SD. * denotes significant difference between group; † denotes significant difference between positions.

2.3.5. Protein feeding

Protein intake is displayed in Table 5. The difference in protein intake between elite and amateur players also was observed when protein intake was divided into morning, noon and evening segments of the day (p=0.005) (Figure 1). There also was a significant difference between the amount of protein consumed in the morning compared with both noon and evening. On average, players consumed ~20 g and ~30 g less protein in the morning segment compared with noon and evening segments of the day, respectively.

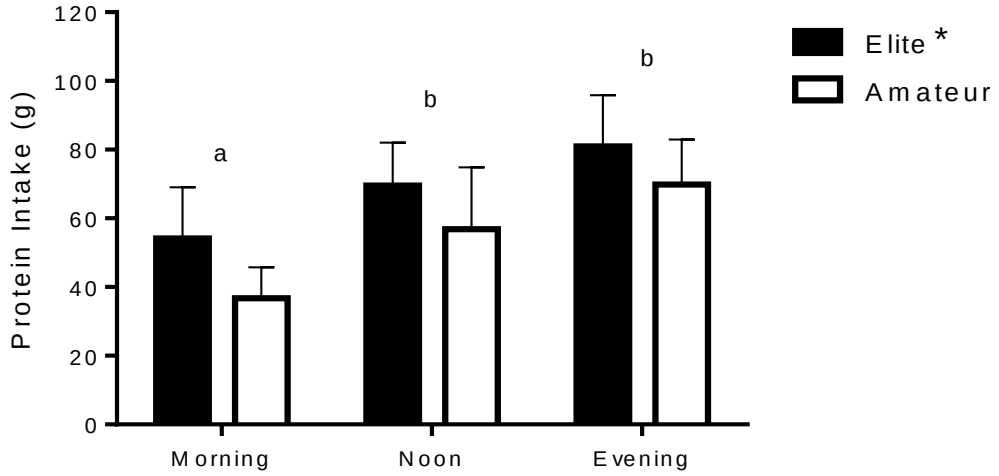


Figure 2.2- Distribution of protein intake of elite and amateur rugby union players across a day. Data are presented as mean ± 95% CI. * denotes main effect of group; segments of the day with different letters are significantly different from each other.

There was a significant, but weak, correlation between LBM and total daily protein intake (r=0.336; p=0.042) (Figure 2.2).

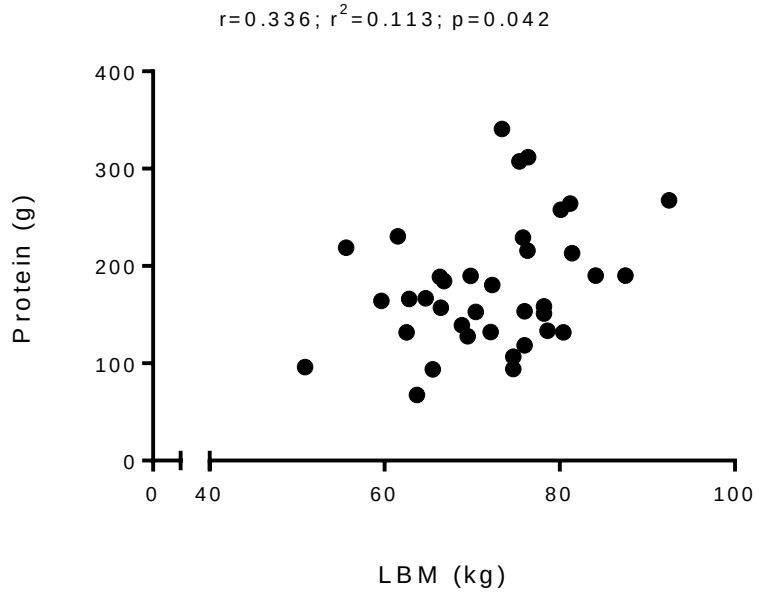


Figure 2.3 - Correlation between lean body mass and daily total protein intake.

The types of protein that players consumed at each eating occasion are displayed in Table 2.6. Elite backs consumed more meat than all amateur players and elite forwards consumed more meat than amateur backs (group × position interaction; p=0.035). Elite players consumed significantly more protein supplements than amateur players (p=0.021).

Table 2.6 - Types of protein consumed at each eating occasion by young elite and amateur rugby union players .

	Elite (n=19)		Amateur (n=20)	
Protein type	Forwards (n=11)	Backs (n=8)	Forwards (n=12)	Backs (n=8)
Dairy	2.2 ± 1.3	1.4 ± 0.7	1.4 ± 0.5	1.3 ± 1.0
Meat * ‡	2.4 ± 0.3 [a b]	2.7 ± 0.7 [a]	2.0 ± 0.5 [b c]	1.6 ± 0.6 [c]
Egg	0.8 ± 0.4	0.5 ± 0.5	0.5 ± 0.5	0.6 ± 0.4

Fish	0.2 ± 0.4	0.3 ± 0.2	0.2 ± 0.2	0.4 ± 0.4
Supplements *	0.8 ± 0.9	0.9 ± 0.8	0.3 ± 0.3	0.4 ± 0.6

Values are presented as means ± SD. * denotes significant difference between group; ‡ denotes significant interaction; means with different letters are different from each other.

2.4. Discussion

The aim of the current study was to describe and compare the anthropometric characteristics and dietary habits of young elite and amateur Scottish rugby union players. We also aimed to assess whether differences in body composition and dietary habits existed between forwards and backs. We observed that elite players were heavier due to greater LBM compared with amateur players. Forwards were taller, heavier and possessed more fat mass than backs. No differences in energy intake or total carbohydrate and fat intake existed between groups or playing position. Protein intake was greater in the elite group compared with the amateur group.

Identifying differences between elite and amateur players could inform future study of rugby union players. The elite group trained for almost three times as long per week compared with the amateur players. Although, we can speculate that elite players are likely to have trained for more hours per week as a result of being involved in a professional environment. All the players in the current study were of similar age (16-24 y) and the number of years they had been playing rugby for did not differ between groups. These results are consistent with a study of senior Italian rugby players (Fontana et al., 2015). It would appear that age or number of years spent playing do not differ between playing standard in the current populations.

There were significant differences in the anthropometric characteristics of elite and amateur groups in the current study. The elite forwards in the current study were taller than all the other groups. Fontana et al., (2015) observed a similar pattern, with players in higher leagues being taller than those in lower leagues. Similarly, Ross et al., (2015) observed that international rugby sevens players were significantly taller than provincial players. Our observation that elite players were heavier than amateur players is consistent with Fontana et al., (2015) and Ross et al., (2015) who both observed that international players were heavier than first/second division and provincial players respectively. The difference in body mass and height observed between forwards and backs in the current study is consistent with previous work at junior (Delahunt et al., 2013; Lombard et al., 2015) and senior level (Fontana et al., 2015; Zemski et al., 2015). Rugby union players have increased in height and body mass over the last two decades (Duthie et al., 2003; Olds 2001). Furthermore, similar changes have been observed in South African U20 rugby players over the last 13 y (Lombard et al., 2015). Duthie et al., (2003) propose that the comparison of literature that is 10 or more years old, including from the pre professional era, has reduced relevance compared with more up to date or current findings, as presented herein. However, the differences in height and body mass we observed in our elite and amateur groups and between forwards and backs also are supported by contemporary literature. Sedeaud et al., (2012) suggest that teams with taller backs and heavier forwards and backs are more successful at Rugby World Cups. It is likely that players that are taller and heavier may possess an advantage in jumping, tackling, rucking, mauling and competition for the ball. Players that are naturally taller and heavier may be more likely to reach an elite standard; however, it will be dependent also on the players' technical and tactical ability.

It would appear that higher LBM is a characteristic of players at the elite level. Elite players in the current study had more total lean mass than amateur players. These data support findings from Fontana et al., (2015) who observed that fat free mass increased as the playing standard increased. Unlike height, body mass and composition can be altered through diet and training. Arguably, not all mass is equal and the desired body composition for many athletes including rugby players is high lean mass and lower fat mass. Gabbett et al., (2011) postulate that in another code of rugby, rugby league, leaner players have improved tackling ability and that higher fat mass decreases performance. This reduction in performance is linked to reduced power to mass ratio and decreased aerobic capacity (Withers et al., 1987). The higher lean mass in the elite group was detected in all regions of the body (arms, legs and trunk). Interestingly, elite forwards had more lean mass on their arms than all the other groups and elite backs had more lean mass on their arms than amateur backs. Although we have no training programme data for the players in the current study, elite rugby union practitioners report use of a number of upper body exercises including bench press and push press (Jones et al., 2016). Supporting previous findings, forwards in the current study had more lean mass than backs (Delahunt et al., 2013; Fontana et al., 2015; Zemski et al., 2015). However, fat mass was not different between elite and amateur players and to our knowledge this finding is novel. This absence of difference in fat mass demonstrates that the majority of the difference in total body mass is attributed to lean mass. Forwards possessed more fat mass than backs in the current study, a finding that has been previously reported (Delahunt et al., 2013; Zemski et al., 2015). The difference in mass between elite and amateur players was almost exclusively due to a difference in lean mass rather than

fat mass. Conversely, the difference in mass between forwards and backs was due to fat mass and lean mass in almost equal contributions.

Statistically significant differences existed between body composition outcomes from DEXA compared with skinfold measurements. LBM was greater when measured using skinfolds compared with DEXA whereas fat mass and % BF were lower when measured using skinfold thickness compared with DEXA scanning. These differences did not affect the statistical outcomes for LBM and fat mass but for % BF there was a difference in the results. Percentage body fat measured by skinfolds and DEXA was significantly greater in forwards compared with backs. However, when playing standard was examined % BF measured by DEXA was not different between groups, whereas, elite players had significantly lower % BF as estimated from skinfold thickness compared with amateur players. Our results align with previous studies that found forwards had a higher % BF than backs when estimated using DEXA (Delahunt et al., 2013) and skinfolds (Fontana et al., 2015). Fontana et al., (2015) observed also that higher-standard players had lower % BF estimated from skinfolds compared with players in lower leagues. The body composition measures from DEXA and skinfold thickness correlate strongly but there are significant differences between the results.

The strongest correlations between measures of body composition were observed between measures of LBM rather than fat mass. One of the measures that quantified LBM was the rugby specific LMI, which is calculated from the sum of 7 skinfolds (Duthie et al., 2006a; Slater et al., 2006). An exponent is included in the equation to account for whether a player is a forward or a back. LMI was higher in elite players

compared with amateur players and forwards compared with backs. This study is the first, to our knowledge, to compare the LMI of a group of elite and amateur players. Duthie et al., (2006a) and Zemski et al., (2015) observed also that LMI was higher in forwards compared with backs. Unsurprisingly due to its development for elite rugby players, the LMI correlated strongly with LBM (measured by DEXA) – a stronger correlation than any of the correlations observed between measures of fat mass or % BF calculated by skinfolds and DEXA. The LMI appears to represent LBM (as measured by DEXA) well and should be considered in future anthropometric studies to allow comparisons of LBM between populations. Sum of 8 skinfolds was the most commonly reported method of monitoring body composition by practitioners working in professional rugby union (Jones et al., 2016). DEXA was used by only two practitioners compared with twenty-two that used the sum of 8 skinfolds. Routinely incorporating the LMI into the anthropometric profile of rugby players would provide more information than calculating % BF, fat mass and LBM from skinfolds. The skinfold measurements in the current study appear, overall, to correlate well with measurements obtained from the DEXA but the values obtained are significantly different. The use of sum of 8 skinfolds to monitor changes in fat and LMI to monitor changes in LBM would be a practical and useful combination. Avoidance of comparisons of % BF, LBM, and fat mass calculated using different equations or between skinfolds and DEXA would be achieved. When access to DEXA is not possible due to cost or practical limitations practitioners should use sum of 8 skinfolds including the LMI to measure body composition. This study is the first we are aware of that measures body composition of young elite and amateur rugby union players using both DEXA and skinfolds. Further research is required to quantify the reliability of both methods for measuring body composition in rugby union athletes.

The current study is the first to compare the diet of elite and amateur rugby union players. Resistance exercise and protein feeding drive the development of lean mass (Tipton & Wolfe, 2004). A significant but weak correlation existed between protein intake and LBM in the current study. The link between protein intake and the amount of LBM an individual, in the current study, possessed may be related to factors outwith total protein intake. Considering the significant difference in LBM between elite and amateur players, we were particularly interested in the players' protein nutrition. Total daily protein intake was greater in elite players compared with amateur players. Since energy intake did not differ between groups, the difference in protein intake is not simply a result of elite players consuming a greater daily energy intake. The % of energy intake from protein differs between groups, demonstrating that elite players were consuming more protein than amateur players. However, when protein intake was calculated relative to body mass, there was no longer a difference between the groups. This finding suggests that although elite players consume more protein in total, there is no difference in protein intake when we accounted for their greater body mass. It is generally thought that larger athletes require more protein than smaller athletes to meet the demand for amino acids induced by resistance exercise (Churchward-Venne et al., 2012b). Consciously or not, larger players were on average consuming more protein than smaller players. Consequently, when protein intake was calculated relative to body mass this difference in intake was no longer observed. The protein intake of the players was slightly less than previously observed in rugby players who consumed 2.2-2.7 $g \cdot kgBM^{-1}$ (Bradley et al., 2015a; Bradley et al., 2015b; MacKenzie et al., 2015). Protein recommendations for strength athletes are 1.2-1.8 $g \cdot kgBM^{-1}$ (Rodriguez et al., 2009;

Tipton & Wolfe, 2004) and all the groups in the current study, and indeed in previous studies, exceed these recommendations. A protein intake higher than recommendations observed in rugby players could be related to the importance of building lean mass and its perceived link to success.

Total amount of protein consumed is not the only factor thought to influence muscle anabolism. It is thought that the distribution of protein throughout the day influences muscle anabolism due the 'muscle full effect'. The 'muscle full effect' is a concept whereby, under conditions of amino acid availability, muscles have a limited capacity to produce new muscle proteins. Amino acid delivery to the muscle increases but a point is reached at which MPS fails to increase (Atherton et al., 2010; Bohé et al., 2001). The body can only use a maximum amount of protein at any one time due to the 'muscle full effect'. By reaching the muscle's capacity several times in a day, it is thought that muscle anabolism can be increased. Mamerow et al., (2014) suggest that an even distribution of protein throughout the day is more beneficial for increasing MPS and muscle anabolism than a skewed distribution of protein intake. It is currently thought that amounts higher than 20 g of protein (Moore et al., 2009; Witard et al., 2014) or 0.24 $g \cdot kg^{-1}$ (Moore et al., 2014) do not offer additional benefit and increase amino acid oxidation. Further to this Areta et al., (2013) demonstrated that provision of protein in 4 × 20 g doses increased 12 h MPS post-exercise to a greater extent than 8 × 10 g or 2 × 40 g. Recently it has been demonstrated that altering the number of eating occasions, that included 20 g of protein, from 4 to 6 did not increase LBM during a pre-season period (MacKenzie-Shalders et al., 2016). Players in the current study consumed more protein in the noon and evening segments of the day than in the morning segment. This pattern of protein intake did

not differ between groups or between positions and is typical of a skewed protein distribution. MacKenzie et al., (2015) recorded the number of eating occasions (defined as 30 minutes between consumption) in which players ate 20 g of protein or more. They observed that players had 3.8 eating occasions that contained more than 20 g. Unfortunately, we did not have enough information about the timing of consumption to carry out this analysis. However, we do know that at least 20 g of protein was consumed in each segment of the day for the majority of players (except for one elite and four amateur players). From previous literature it seems that four eating occasions containing ≥ 20 g of protein is sufficient for muscle anabolism (Areta et al., 2013; MacKenzie-Shalders et al., 2016). Increasing the number of times protein is consumed in a day above four does not lead to improved lean mass development (MacKenzie-Shalders et al., 2016). It may be that as long as there is sufficient protein in each eating occasion the distribution is irrelevant. However, if one eating occasion or part of the day has less than the optimal dose of protein the protein nutrition is not ideal to support training or aid lean mass development. Further research is needed in athlete groups to try and determine the optimal pattern and distribution of protein intake that best support training and competition.

The type of protein consumed is another factor that could alter muscle anabolism (Hartman et al., 2007; Tang et al., 2009; Volek et al., 2013). Different types of protein differ in their amino acid composition and digestion and absorption kinetics (Boirie et al., 1997). Proteins with a high leucine content that can be digested and absorbed rapidly are thought to be the most anabolic (Tang et al., 2009). However, most studies have only used liquid proteins and the response to whole meals are rarely studied. We recorded the type of protein consumed during each self-reported eating occasion

in the current study. Meat consumption was significantly higher in the elite group compared with the amateur group and elite backs ate more meat than either amateur group. As there are no published data that focus on the types of protein consumed by rugby players we cannot draw comparisons with existing literature. Furthermore, there is little research comparing the anabolic effects of meat to other types of proteins, however, it is know that beef, as a solid, does not cause hyperaminoacidemia to the same extent as liquid forms of protein like milk and a supplement beverage (Burke et al., 2012). Supplement use, defined as the number of times supplements were consumed in one eating occasion, was greater in the elite group than the amateur group. This finding is unsurprising since some supplements are provided to elite players if they wish to use them whereas amateur players may have to buy them themselves. Due to the greater amount of hours spent training by the elite players we speculate that protein supplements were used for convenience and to ensure players were consuming sufficient energy and protein to meet their training demands. Alterations in LBM may not be influenced by a player's total protein intake alone.

The current study examined energy intake and the intake of the other two macronutrients, carbohydrate and fat. There were no differences in energy intake between forwards and backs in the current study. Conversely, Bradley et al., (2015b) observed that during an in-season period the energy intake of forwards was significantly greater than backs. The daily total amount of carbohydrate consumption in the current study did not differ between forwards and backs, however, backs consumed more carbohydrate relative to body mass. Despite the lack of training data we can assume that players were participating in at least moderate, if not higher,

intensity training programme. Previous carbohydrate guidelines suggested that 5-7 $g \cdot kg^{-1}$ per day was required for athletes engaged in a moderate training programme (Burke et al., 2011). However, these guidelines are now slightly out-dated and more current recommendations state that carbohydrate intake should be adjusted according to the fuel costs of training and competition to provide high carbohydrate availability (Fédération International de Natation (FINA) Expert Panel 2014). Players consumed 2.4-3.7 $g \cdot kg^{-1}$ of carbohydrate in the current study, which almost meets the recommendations for individuals in a light training programme (Burke et al., 2011). The intermittent nature of rugby union may reduce the requirements of carbohydrate intake to some extent. It is surprising that given the 15 h difference in hours spent training between elite and amateur that no difference existed in carbohydrate intake and indeed energy intake. Elite players trained more but consumed the same amount of energy and carbohydrate.

There were no differences in energy, carbohydrate or fat intake between elite and amateur players in the current study. No previous study has examined and compared the diets of elite and amateur players, however, previous studies have characterised the diet of elite players (Bradley et al., 2015a; Bradley et al., 2015b) so we are able to draw comparisons with these studies and our own. Energy intake in the current study was lower than previously reported, 9.3-11.1 MJ compared with 14.8-16.6 MJ (Bradley et al., 2015a; Bradley et al., 2015b; MacKenzie et al., 2015). Carbohydrate intake of the players in the current study also was slightly lower than previously observed with values ranging from 3.4-4.1 $g \cdot kg^{-1}$ (Bradley et al., 2015a; Bradley et al., 2015b; MacKenzie et al., 2015). There was no difference in daily fat intake between any of the groups in the current study and the daily amounts consumed are similar to

previously observed values of 0.95-1.4 $g \cdot kg^{-1}$ (Bradley et al., 2015a; Bradley et al., 2015b; MacKenzie et al., 2015). The range of values for carbohydrate and fat from previous studies was likely influenced by the time at which the diets were recorded. Previous studies measured dietary intake during a pre-season period (Bradley et al., 2015a; MacKenzie et al., 2015) when players are probably trying to reduce fat mass and increase lean mass. It is probable that their training would have been more intense and their diet altered. The findings in the current study of no differences in fat or protein intakes between backs and forwards agree with observations by Bradley et al., (2015b). Energy and carbohydrate intake in the current study were slightly lower than previous studies while fat intake was similar.

Micronutrient intake varied between groups and positions in the current study. Furthermore, recommendations for physically active individuals (Whiting and Barabash, 2006) were not met for all micronutrients measured. Elite players consumed significantly more zinc, carotene, thiamin, riboflavin, niacin, vitamin B6, folate and pantothenate than amateur players. Zinc is known to be involved in protein synthesis (Prasad, 1995) and folate is important for amino acid utilisation (Bailey and Gregory, 1999) so both these micronutrients are important in the development of lean mass. Since energy intake did not differ between the groups there must be another explanation for the difference in micronutrient intake. Elite players may have better knowledge about what to eat to meet micronutrient requirements since they are likely to have had access to nutritional information/education. Meat is a good source of a number of the micronutrients mentioned above. Since elite players consumed more meat than the amateur players it may have contributed to the difference in micronutrient intake.

Forwards consumed significantly more potassium, calcium, phosphorus, zinc, riboflavin and vitamin B12 than backs. It is unclear why these differences exist between forwards and backs given there were no differences in energy or macronutrient intake. Whiting & Barabash, (2006) propose that the micronutrient requirements for those that are physically active differ from those for general health. We compared the intakes of the players in the current study to those for physically active individuals (Whiting and Barabash, 2006) and for general health (British Nutrition Foundation 2015). None of the groups met the recommendations for the physically active for vitamin E (15 mg) but did meet recommendations for general health (4 mg). The elite backs and both amateur groups did not meet recommendations for the physically active for potassium (4.7 g), folate (400 μg) and vitamin D (5 μg). Furthermore, the amateur backs did not meet the recommendations for general health for potassium (3500 mg). Amateur players did not meet recommendations for the physically active for magnesium (400 mg). Finally, amateur backs failed to meet the recommendations for the physically active for calcium (1000 mg). All other micronutrient recommendations for general health and physical activity were met. Players should ensure that they meet all the micronutrient recommendations so they remain healthy and can perform to the best of their ability.

In summary, on average young elite rugby union players possess grater total body mass and LBM than amateur players, which confers a potential advantage during competition. This difference in LBM could be attributed to the number of hours spent training and to protein nutrition. In young Scottish rugby union players the general consensus that forwards and backs differ in their physical characteristics is upheld.

Therefore, attaining a high LBM may be more important at the elite standard rather than attaining a low fat mass. Optimising nutrition to support training is key to maximising training adaptations. Consuming sufficient protein at each eating occasion is important and developing an even protein distribution throughout the day could be beneficial. Carbohydrate intake must meet the metabolic demands of exercise. Furthermore, players should ensure their micronutrient intake meets their requirements. We conclude that the LMI is an effective tool to monitor changes in LBM if DEXA scanning is not available. Although the assessment of LMI does not give a value of LBM it correlates well with values of LBM from DEXA. Furthermore, we conclude that clear differences exist in the physical characteristics and dietary habits of young elite rugby union athletes compared with amateur rugby union athletes. These differences may be a result of improved performance behaviours and time to train as well as nutrition resources to draw from.

2.5. Practical Applications

Based on the results from the current study and available evidence rugby players, regardless of the level they play at should ensure they meet protein recommendations for strength athletes (1.2-1.8 g·kg BM^{-1}). Players may be at risk of skewing their protein intake toward the later part of the day and should include some of their daily protein intake in the morning. Rugby players should be aware that their carbohydrate requirement is likely to be lower than other athletes in moderate to intense training programmes and should adjust their carbohydrate intake based on the demands of their training. The micronutrient requirements for athletes are thought to be greater than for sedentary individuals. Rugby players should consider

this increased requirement and consuming a balanced and varied diet will help rugby players reach their micronutrient requirements.

CHAPTER 3 Impact of a Six Nations rugby campaign on body composition and diet

Macnaughton LS, Hardie E, Chessor R, Tipton KD

3.0. Abstract

Rugby union is a physically demanding sport of an intermittent nature. Nutrition is a vital tool for maximising training-induced adaptations and helping to improve rugby performance and body composition. Domestic leagues run throughout an ~35 week season but there are periods of international fixtures – the principal Northern Hemisphere competition being the Six Nations (6N) tournament. The under 20s (U20) version of the 6N consists of five games over seven weeks during which players spend five to six weeks 'in camp' (camp) where all meals and snacks are provided. Differences in dietary intake of elite rugby players 'at home' (home) *vs.* camp have not previously been investigated.

Body composition of Scottish U20 rugby union players was measured using DEXA before and after the 6N (n=8) in a cross-sectional study. Five of these players recorded their home diet during the domestic season and camp diet during the 6N. Dietary records were analysed using WISP Version 4.0. There were minimal group changes in body composition. However, individual responses varied.

There were no differences in energy, carbohydrate and fat intake between the camp and home environments. Players consumed on average 2.3 ± 0.6 g·kg BM^{-1} of protein per day in camp compared with 1.8 ± 0.6 g·kg BM^{-1} of protein at home (p=0.04). Consumption of vitamin D and B12 increased significantly in camp. Vitamin D intake was below recommendations for the physically active (5.0 μg) in the home diet (4.8 ± 2.1 μg) but not in camp (8.2 ± 3.8 μg; p=0.04). Vitamin A and E intake fell below the recommendations both at home and in camp. Despite minimal group changes in body composition over the 6N, there may be value in analysing individual changes in this

high performance environment. Clear differences exist between home and camp dietary intakes of young male elite rugby union players. Use of an 'in camp' diet as a platform to educate players about performance nutrition could be effective.

3.1. Introduction

For Scottish rugby union players the most important annual competition is the Six Nations (6N). One pathway of development for senior rugby union players is through national age grade teams. The 6N is held in Europe towards the end of each winter. There is a senior competition, an under 20s (U20) competition and a women's competition. As part of the U20 competition, young players are exposed to a similar environment to senior players and learn and develop through structured pathways. Scotland, England, Wales, Ireland, France and Italy are the nations that take part in the competition over seven weeks. Each team plays every other team once and players spend five to six weeks in camp where all of their meals and snacks are provided. In season training demands change as players spend less time doing conditioning work (Jones et al., 2016) and more time doing field-based training. Whilst no evidence exists, the playing intensity during the 6N is likely to be increased compared with games during players' regular season because throughout the 6N they are playing at international standard. During the 6N players experience a change in environment, training and standard of playing.

The results from the previous chapter highlight the differences in body composition between elite and amateur players. Predominantly, elite players are heavier, have more lean mass and lower percentage body fat. Higher LBM confers a number of advantages including increased power to mass ratio (Withers et al., 1987) and

improved tackling ability (Gabbett et al., 2011). Players try to achieve a body composition that is effective for their position and playing style. The athlete support team will set limits within which the players' body composition parameters must aim for. These parameters will include total body mass, LBM, fat mass, %BF or skinfold thickness (Jones et al., 2016). The players will then work, mostly during the pre-season period (Jones et al., 2016; Bradley et al., 2015; Argus et al., 2010) to attain their desired body composition which can be achieved through diet and training. Improvements in body composition usually consist of an increase in lean mass and a decrease in fat mass (Argus et al., 2010). It is beneficial to maintain this favourable body composition throughout the playing season. Due to the changes in training demands and environment it is possible that body composition may change over the course of the 6N. There has been no analysis of the impact of a period of international competition on body composition among elite rugby union players.

Nutrition, as well as training, can be manipulated to alter body composition. Also, nutrition is important to augment adaptations and support the demands of training and competition. Athletes must be sufficiently fuelled to prevent any decline in performance during training or competition (Mujika and Burke, 2011). Players in the 6N camp have all of their food and snacks provided and a qualified nutritionist designs the menus. When players are not in camp they are in a home environment and likely make many of their own food choices. Previously, Bradley et al., (2015b) quantified the diet of elite rugby union players during an in-season period and Bradley et al., (2015a) and MacKenzie et al., (2015) analysed diet during a pre-season period in senior and development players respectively. However, the dietary habits of elite rugby union players at home and in camp have never been compared. Therefore,

the aims of the current study were to assess whether body composition changed from pre to post 6N and identify whether any differences in the dietary habits of young elite rugby union players existed between home and camp environments.

3.2. Methods

3.2.1. Participants and ethical approval

Eight U20 Scottish male rugby union players were recruited via their involvement in the SRU development squads of Edinburgh and Glasgow. Ethical approval of the study was granted by the NHS Scotland A Research Ethics Committee (REC number 14/SS/1095) and conformed to the standards set out in the latest version of the Declaration of Helsinki (2013). Players provided written informed consent before participating in the study.

3.2.2. Study design

In a cross-sectional study, body composition was measured pre and post U20 6N using DEXA scans to assess the impact of an intense rugby union competition at age-grade international standard. Five of the eight players kept two 3 d food diaries, one in a free living 'at home' environment (home) and one in a controlled 'in camp' environment (camp), to assess whether players' dietary intake and composition varied between the two living environments. Dietary intake was recorded during their regular playing season (January/February) and again while players were in camp (February/March).

3.2.3. Body composition

A whole-body DEXA scan was performed on participants in a rested state (no exercise that day) following an over-night fast. Participants were asked to drink 500 mL of water ~2 h before attending the laboratory to ensure euhydration (Rodriguez-Sanchez and Galloway, 2015). Upon arrival participants were asked to void their bladder, before their height and body mass were measured. Participants were then scanned in the standardised supine position (Nana et al., 2012) wearing only their underwear. The Lunar iDXA scanner (GE Healthcare Systems, Hertfordshire) was calibrated to the manufacturer's guidelines using a standard calibration block. The same trained investigator conducted all scans. Participants too tall to fit in the scanning area had the top of their head omitted from the scanning area to allow the rest of their body to fit, as multiple scans were not possible. Players were placed in the same position for their repeat scans.

3.2.4. Dietary analysis

Participants completed a 3 d food diary in the middle of their regular playing season and another 3 d food diary during their time in 6N camp. The same investigator performed the dietary intake analysis using Wisp Version 4.0 (Tinuviel Software Systems, Anglesey, UK). An average across the three days was used to calculate mean daily energy and nutrient intake. Dietary protein intake was divided into three sections throughout the day to assess the pattern of protein intake. The 1st segment of the day was from breakfast to before lunch (morning), the 2nd segment was lunch and anything before dinner (noon) and the 3rd segment was dinner and the rest of the day (evening). Protein type was quantified by counting the number of self-recorded

eating occasions in which one of the following types of protein was consumed: dairy, meat, eggs, fish, and supplements.

3.2.5. Statistical analysis

Data are presented as mean ± SD unless otherwise stated. Statistical analysis was carried out in Minitab Version 17 (Minitab Software Systems, Coventry, UK). Significance was set at the 95% confidence level ($p<0.05$). All data followed a pattern of normal distribution. Paired t-tests were used to assess changes from pre to post 6N in all body composition outcomes and to assess differences between home and camp dietary intakes. A two-way ANOVA was performed to analyse whether protein intake varied across the segments of the day (intake; 2 levels; home and camp and segment; 3 levels; morning, noon and evening). Effect sizes are presented as Cohen's d with CI and the pooled standard deviation was used in the effect size calculations. Effect sizes of 0.2 are considered small, 0.5 considered medium and >0.8 are considered large (Cohen, 1969). If 0 is not contained within the confidence intervals for the effect size the effect is deemed significant.

3.3. Results

3.3.1. Body composition

Body mass ($p=0.318$; $d=-0.06$; CI=-1.04 to 0.92), fat mass ($p=0.070$; $d=-0.13$; CI=-1.11 to 0.85) and lean mass ($p=0.846$; $d=0.01$; CI=-0.97 to 0.99) did not change from pre to post 6N (Figure 3.1A-C). Individual changes in body mass ranged from + 3.1 kg to - 1.9 kg. The range of change in fat mass was + 1.9 kg to - 0.9 kg. The change in lean mass ranged from + 1.2 kg to - 2.1 kg. These individual changes are expressed as percentage difference in Figure 3.2.

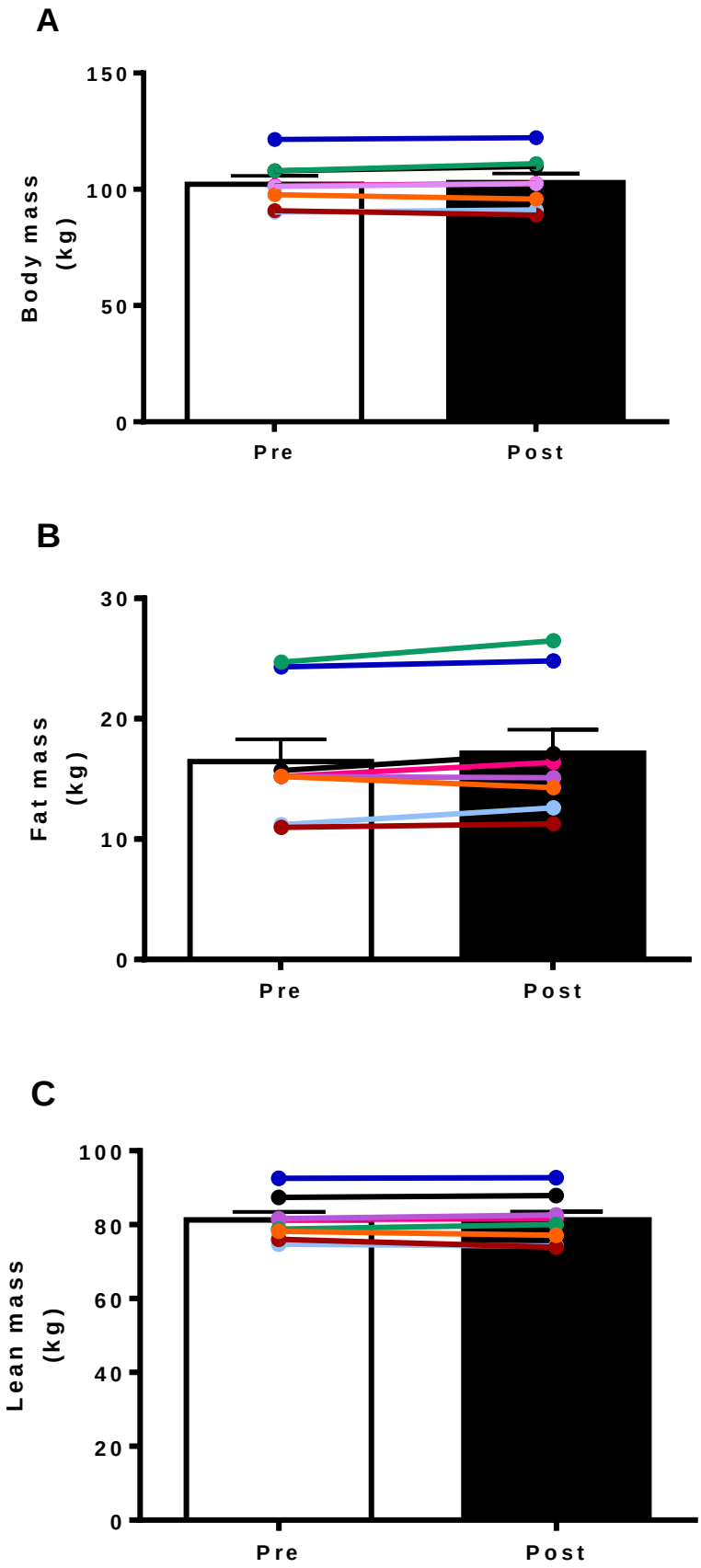


Figure 3.1 - Body mass (A), fat mass (B) and lean mass (C) of elite rugby union players (n=8) pre and post U20 Six Nations. Data are presented as mean values in bars with 95% CI and as individual values shown with the coloured lines. No significant differences were observed.

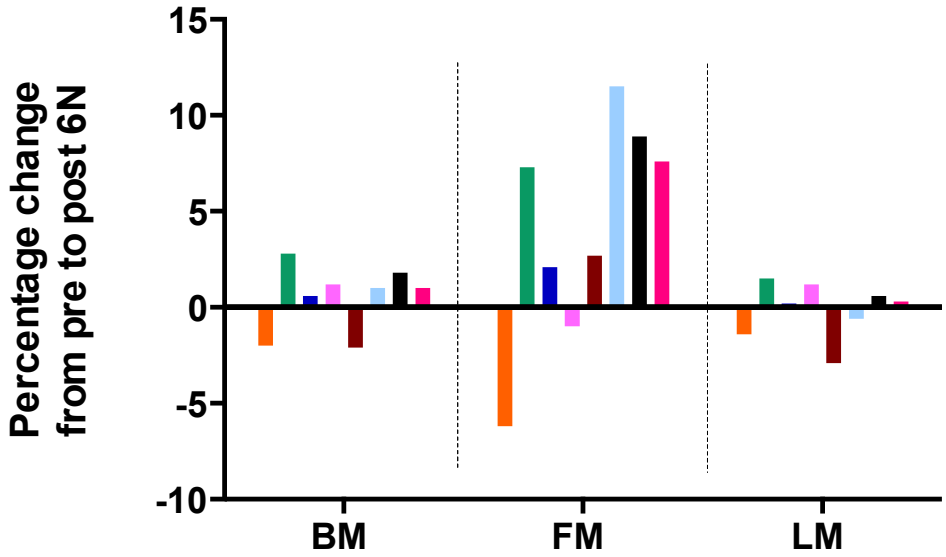


Figure 3.2 – Individual (coloured bars) percentage changes in body mass, fat mass and lean mass of elite rugby union players (n=8) from pre to post U20 Six Nations. Data are expressed as percentage change from pre to post 6N.

3.3.2. Energy and macronutrient intake

Energy intake did not differ between home and camp environments (Figure 3.3) (p=0.612; d=-0.18; CI=-1.42 to 1.07). There was an average increase of ~0.5 MJ when the players were in camp compared with at home. The maximum individual increase in energy intake was 2.1 MJ compared with a maximum decrease of 2.4 MJ.

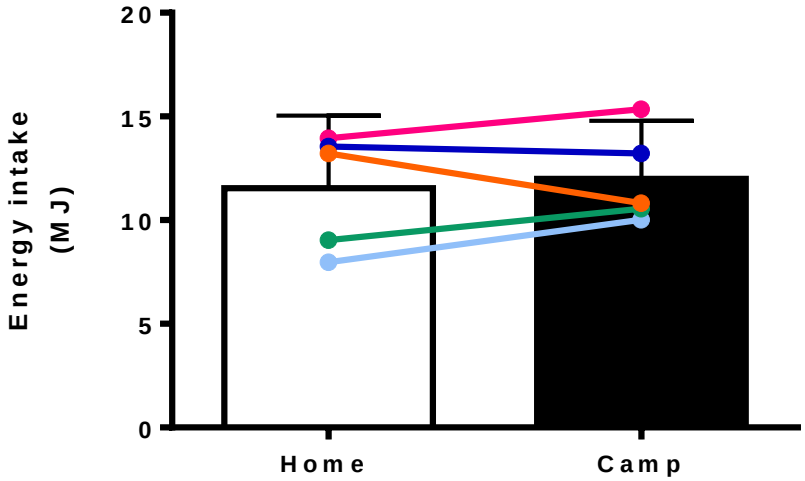


Figure 3.3 - Energy intake of elite rugby union players (n=5) in their home environment and at an U20 Six Nations camp. Data are presented as mean values in bars with 95% CI and as individual values shown with coloured lines. No differences existed between home and camp.

Table 3.1 – Daily dietary macro and micro- nutrient intake of elite rugby union players (n=5) in a home environment (home) and at an U20 Six Nations camp (camp).

Dietary outcome	Home	Camp	Difference	Effect size
Protein (g) *	191.2 ± 67.5	245.3 ± 83.2	-54.1	0.25
Carbohydrate (g)	304.9 ± 77.5	284.6 ± 83.2	20.3	0.17
Sugars (g)	114.7 ± 62.7	105.6 ± 42.7	9.0	0.56
Starch (g)	175.4 ± 37.4	148.5 ± 56.4	26.9	-0.50
Total Fat (g)	113.1 ± 39.6	130.2 ± 27.9	-17.0	-0.21
Saturates (g)	42.4 ± 15.9	45.5 ± 13.3	-3.1	-0.56
Monounsaturates (g) *	36.4 ± 14.7	44.0 ± 12.1	-7.6	-0.99
Polyunsaturates (g)	13.8 ± 4.8	18.4 ± 4.5	-4.6	-1.10
Fibre (g)	23.0 ± 5.2	26.2 ± 6.2	-3.1	-0.23
Non milk extrinsic sugars (g)	12.6 ± 7.2	15.8 ± 18.4	-3.2	-0.42
Sodium (g)	3.9 ± 1.2	4.4 ± 1.4	-0.5	-0.39
Potassium (g)	4.8 ± 1.9	5.4 ± 1.1	-0.6	0.64
Calcium (g)	1.5 ± 0.5	1.2 ± 0.4	0.3	-0.02
Magnesium (mg)	463.6 ± 161.5	466.6 ± 90.8	-3.0	-0.11
Phosphorus (g)	2.7 ± 0.9	2.8 ± 0.7	-0.1	-0.11
Iron (mg)	18.7 ± 6.2	19.4 ± 7.0	-0.7	-0.67
Copper (mg)	1.5 ± 0.4	2.0 ± 1.0	-0.5	-0.94
Zinc (mg)	16.3 ± 2.9	19.8 ± 4.4	-3.5	-0.33
Chloride (g)	5.7 ± 1.8	6.3 ± 1.7	-0.6	0.54
Manganese (mg)	4.6 ± 1.2	4.0 ± 0.8	0.6	-0.74
Selenium (µg)	83.6 ± 28.0	107.8 ± 36.9	-24.2	0.68
Iodine (µg)	328.8 ± 150.5	245.2 ± 89.5	83.6	0.23
Retinol (µg)	673.0 ± 311.0	613.2 ± 189.8	59.8	-1.79
Carotene (mg)	4.0 ± 1.8	9.9 ± 4.3	-5.9	-1.08
Vitamin D (µg) *	4.8 ± 2.1	8.2 ± 3.8	-3.3	-0.54

Vitamin E (mg)	8.7 ± 3.8	10.5 ± 2.5	-1.8	0.42
Thiamin (mg)	3.5 ± 1.2	2.9 ± 1.5	0.6	0.08
Riboflavin (mg)	3.7 ± 1.4	3.6 ± 1.5	0.1	-0.32
Niacin (mg)	52.9 ± 21.2	61.3 ± 29.8	-8.4	-0.48
Vitamin B6 (mg)	3.4 ± 1.4	4.9 ± 1.9	-1.5	-0.89
Vitamin B12 (mg) *	8.8 ± 3.8	14.1 ± 6.1	-5.3	-1.06
Folate (µg)	401.4 ± 129.9	411.0 ± 157.2	-9.6	-0.07
Pantothenate (mg)	11.2 ± 4.4	13.7 ± 5.1	-2.5	-0.52
Biotin (µg)	66.9 ± 26.9	64.2 ± 13.0	2.7	0.13
Vitamin C (mg)	75.8 ± 49.8	173.6 ± 105.9	-97.8	-1.18

Values for home and camp are means ± SD.* denotes significant difference between home and camp ($p<0.050$). Average intake calculated over a 3 d period. Absolute difference calculated by subtracting camp intake from home intake. Effect sizes from home (mean 1) to camp (mean 2) are displayed.

Total macro and micro- nutrient intake are displayed in Table 3.1; difference is calculated by subtracting camp intake from home intake. Both carbohydrate ($p=0.697$; $d=0.25$; CI=-0.99 to 1.50) and fat intake ($p=0.114$; $d=-0.5$; CI= -1.76 to 0.76) (Figure 3.4A&B) was not significantly different from home to camp when expressed relative to body mass. Carbohydrate intake ranged from 2.0 to 4.0 g·kg BM^{-1} per day at home and 1.8 to 3.9 g·kg BM^{-1} in camp. The maximum decrease was 1.3 g·kg BM^{-1} (124 g) and maximum increase was 1.1 g·kg BM^{-1} (114 g). Fat intake ranged from 0.7 to 1.4 g·kg BM^{-1} at home and 0.8 to 1.4 g·kg BM^{-1} in camp. Protein intake was significantly greater in camp compared with at home ($p=0.040$; $d=-0.87$; CI=-2.16 to 0.43) with an average increase from 1.8 ± 0.6 to 2.3 ± 0.3 g·kg BM^{-1} (0.5 g·kg BM^{-1} or 54 g per day) (Figure 3.4C). The individual changes from home to camp are expressed as percentages in Figure 3.5. Numerically, carbohydrate intake was lower in camp compared with at home for most players whereas fat and protein intakes were greater in camp.

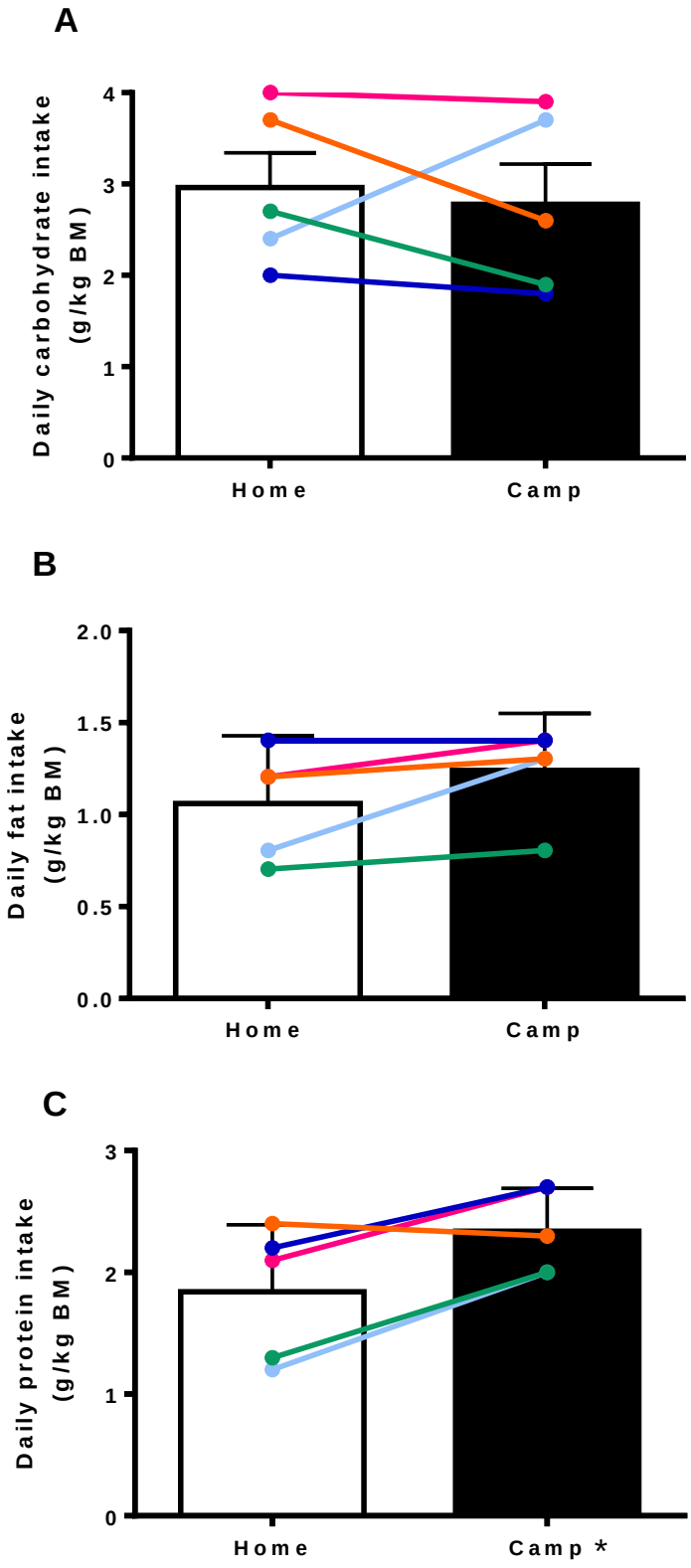


Figure 3.4 - Macronutrient intake of elite rugby union players in their home environment and at an U20 Six Nations camp. Data presented relative to body mass as mean values in bars with 95% CI and as individual values (coloured lines). No differences existed between home and camp for carbohydrate (A) and fat (B), *significant difference between home and camp for protein intake (C) (p=0.040).

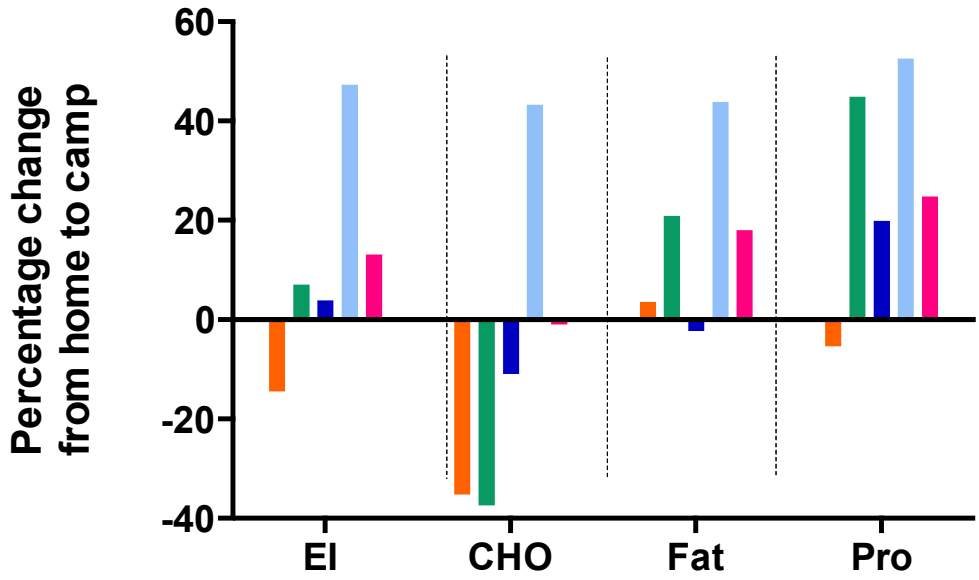


Figure 3.5 - Percentage change in energy (EI) and macronutrient intake (carbohydrate (CHO), fat and protein (Pro)) of elite rugby union players in their home environment and at an U20 Six Nations camp. Data expressed as percentage change from home to camp.

3.3.3. Protein intake

Protein distribution did not differ between home and camp environments (no interaction; p=0.07) but overall more protein was consumed during the noon and evening segments of the day compared with morning (main effect of segment; p=0.002) (Figure 3.6). A large effect size (d=-1.56; CI=-2.97 to -0.14) was observed between home and camp for the noon segment of the day but this difference did not reach statistical significance.

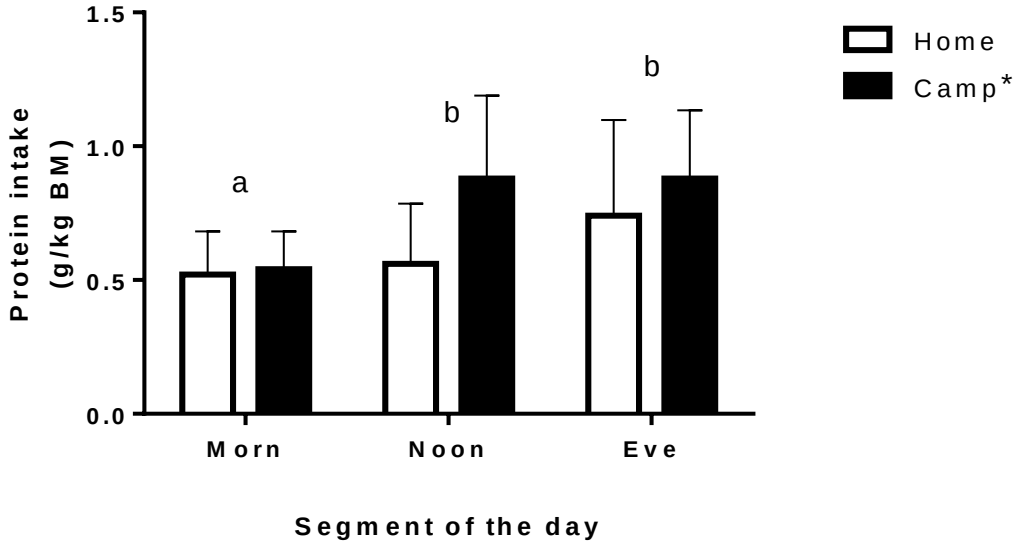


Figure 3.6 - Protein intake of elite rugby union players across the day in their home environment and at an U20 Six Nations camp. Distribution across morning (Morn), noon and evening (Eve) segments of the day. Data presented as mean with 95% CI. * denotes significant difference between home and camp (p=0.04). Significant main effect of segment of the day (p=0.002), means with a different letter are significantly different from each other.

The number of eating occasions that contained eggs, fish or dairy did not differ between home and camp. There was a significant increase in the number of meals that included meat in camp compared with home (p=0.020; d=-1.92; CI=-3.41 to -0.42) (Figure 3.7). There was no difference in the frequency of supplementation consumption from home to camp (p=0.39; d=-0.40; CI=-1.65 to 0.85). The amount of protein obtained from supplements did not differ between home and camp (p=0.424; d=-0.46; CI=-1.67 to 0.84).

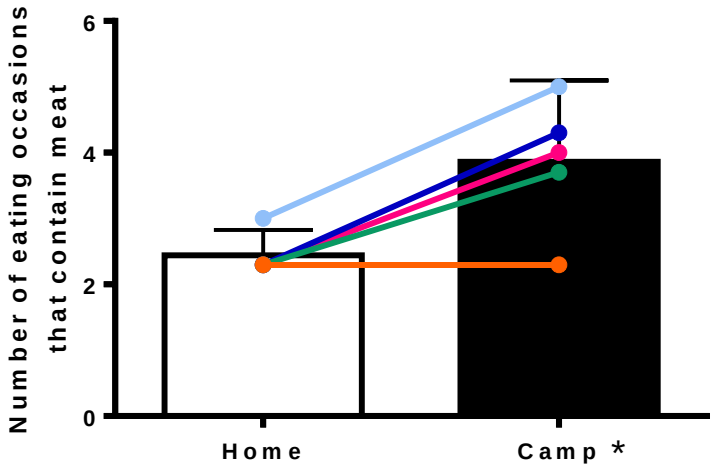


Figure 3.7 - Number of self-reported eating occasions that contained meat by elite rugby union players in their home environment and at an U20 Six Nations camp. Data presented as mean values in bars with 95% CI and as individual values (coloured lines). * denotes significant difference between home and camp (p=0.02).

3.3.4. Micronutrient intake

Dietary vitamin D intake was significantly higher in camp compared with at home (p=0.038; d=-1.08; CI=-2.41 to 0.25) (Table 3.1). Vitamin B12 intake was significantly higher also in camp compared with at home (p=0.012; d=-1.06; CI=-2.38 to 0.27).

3.4. Discussion

There were two main aims of the present study. First, we intended to assess whether body composition changed following a 6N rugby union campaign. Second, we aimed to characterise the diet of elite U20 rugby union players while identifying any differences that existed in the dietary habits of players at home compared with in camp. We observed no significant difference in measures of body composition from pre to post 6N. However, dietary habits of the players in the current study differed between the two environments examined.

Research monitoring body composition changes of a group of elite rugby union players over a period of time is scarce. Our study is the first, to our knowledge, that

has measured body composition using DEXA pre and post an extended period of international rugby union competition. However, there are three previous investigations in rugby union that measured body composition before and after a pre-season period (Bradley et al., 2015a; Argus et al., 2010; Slater et al., 2006). Pre-season is an opportunity for players to improve their physical condition and this improvement is usually achieved through a high-volume, high-intensity training programme (Argus et al., 2010). Improvements in strength and power, aerobic and anaerobic fitness, speed and body composition are all expected as a result of pre-season training (Argus et al., 2010). Furthermore, during a period of competition players aim to maintain the body composition they have achieved during pre-season because it is considered favourable for their playing position and role. During the season rugby union players spend less time performing conditioning work (Jones et al., 2016) and likely more time on game specific training. The metabolic demands placed on the body are altered as a result of the shift in training and these alterations could lead to a change in body composition. A decrease in lean mass and increase in fat mass, for example, would be considered unfavourable and would not be considered a physically advantageous competition condition. Accordingly, the preference of staff and players would be to avoid this situation. There were no statistically significant differences from pre to post 6N in total body mass, LBM and fat mass in the current investigation. This suggests that the training and/or diet of the players are likely appropriate to maintain the group's body composition from pre competition. Since there were no changes in body composition for the group, the structures in place for training and nutrition were likely adequate to maintain body composition throughout the 6N. However, fat mass did increase and although this increase in the current study was not significant, it was similar in magnitude to

changes observed by Bradley et al., (2015a). All previous studies monitoring body composition of elite rugby union players over a period of time, as previously discussed, have only been carried out during a pre-season training period and using anthropometry (Bradley et al., 2015a; Argus et al., 2010; Slater et al., 2006). Consequently, due to the nature and goals of pre-season caution must be exercised when making comparisons between data sets. Hence, we compared the magnitude of change and not the direction of change. Whereas we observed no statistically significant group changes in the current study, mean value changes were of similar magnitude to previous findings indicating that the lack of statistical significance could be due to the lower number of participants in the current study.

The group of players in the current study did not change their body composition from pre to post 6N. However, in elite sport the response of individual athletes is important to consider and can add useful information to the literature. Closer analysis of the individual data revealed a negligible change in body composition in some players. However, there are a number of players who increased fat/lean mass or decreased fat/lean mass. For any given individual those changes could be meaningful within the context of each player's position-specific performance. We believe, therefore, that the individual responses in the current study should be examined. There are two main considerations to be made. First, the physiological relevance of any observed changes in body composition must be established. Changes of 0.575 kg for LBM and 0.465 kg for fat mass are required to be outwith the variability of measurement (Buehring et al., 2014). Considering these values, the LBM changes of five players would be relevant and the fat mass changes of six players would be outwith the variability of the measurement. Second, we only have two time

points to compare and have no indication of how these changes, or lack thereof, correspond to the rest of each player's season. For example, if monitored over a season one player's LBM may not vary much but another's could hypothetically deviate by 2-4 kg across a season. A loss of 1.9 kg LBM would be meaningful to the first player but perhaps not the second. Building up a database or profile for each player over the season and years could be a useful tool for monitoring body composition changes and put any changes observed into perspective. The best use of DEXA for measuring body composition is considered to be for monitoring longitudinal changes (Buehring et al., 2014; Toombs et al., 2012). Therefore, it could be recommended that routinely scanning elite rugby union players at meaningful times of the season would build a useful and reliable database of information.

Protein plays a key role in the developing LBM (Tipton and Wolfe, 2004) making it a nutrient of particular importance and interest to rugby players. Daily protein intake increased from home to camp in the current study from 1.8 to 2.3 g·kg BM^{-1}. This increase equates to around 54 g of protein per day and is equivalent to two additional servings of the recommended maximally stimulatory dose of protein for MPS (Moore et al., 2009; Witard et al., 2014). The protein recommendations for athletes are to consume 1.2 to 1.7 g·kg BM^{-1} of protein each day (Rodriguez et al., 2009). The intakes in the current study are slightly higher than these recommendations. However, Tipton & Wolfe (2004) discuss that there is little evidence to limit protein intake, at least within reasonable limits, and that higher protein intakes might be beneficial for strength and power based athletes. Bradley et al., (2015a; 2015b) reported values for daily protein intake higher than in the current study with 2.7 g·kg BM^{-1} being consumed in-season and 2.5 and 2.6 g·kg BM^{-1} being consumed during pre-season by

forwards and backs respectively. MacKenzie et al., (2015) reported daily protein intake values of 2.2 g·kg BM^{-1} that are very similar to the intakes we observed. Similar to previous studies, it would appear that rugby union players, particularly the elite group, consume more protein than is currently commonly recommended for athletes.

The amount of protein consumed may not be the only contributing factor of protein nutrition to gaining or maintaining muscle mass. The previous chapter revealed that the protein distribution of players at both elite and amateur level is skewed towards the noon and evening portions of the day. We examined the pattern or distribution of protein intake in the current study. The players consumed significantly less protein in the morning segment of the day compared with the noon and evening segments but this observation did not differ between camp and home. Although not significantly different, the amount of protein in the noon segment of the day was greater in camp than at home and increased on average from ~26% to 36% of total protein intake. It is unclear why there is an increase in protein intake during this segment of the day. This segment of the day could contain the main training session or afternoon matches and we can speculate that protein intake may be increased around those times given the perceived importance of the 'anabolic window'. There was a significant increase in meat consumption from home to camp. Our study is the first to investigate the type of protein that elite rugby union players consume so we do not know if the meat consumption is typical of all rugby players. The amount of protein consumed in supplement form did not differ between home and in camp but was lower than previously reported in elite rugby union players (Bradley et al., 2015a). Furthermore, there was no difference in the frequency of supplementation from home to in camp. These results indicate that supplement use did not change overall in response to

environment and could suggest that some habits do not change depending on environment. Other factors that could influence the effect of protein on LBM are timing of intake and macronutrient co-ingestion (Witard et al., 2016). Unfortunately, we did not have sufficient information to explore protein timing. Regarding macronutrient co-ingestion, almost all of the protein was consumed with both carbohydrate and fat. The type of protein, timing of intake and macronutrient co-ingestion may all contribute to the influence of protein on LBM. Future research should examine these factors and try to assess their impact on athletes' performance and goals.

Each rugby union player must meet the energy demands of their training and competition schedule to maximise adaptations and performance. We did not record training or measure energy expenditure in the current study. However, the average daily energy expenditure of elite players in-season has been recorded as 15.9 ± 0.5 MJ for forwards and 14.0 ± 0.5 MJ for backs (Bradley et al., 2015b). Energy intake in the current study was 12.3 MJ at home and 13.6 MJ in camp. Both of these values are lower than has been previously recorded during a season; 16.6 MJ for forwards and 14.2 MJ for backs (Bradley et al., 2015b). The values observed when the players were at home, during the season, in the current study were lower also than values obtained during pre-season; 14.8 MJ for forwards and 13.3 MJ for backs (Bradley et al., 2015a) and 13.6 MJ for a group of developing elite players (MacKenzie et al., 2015). However, the energy intake of the players in the current study in camp was almost exactly the same as the players in previous studies during pre-season. Although there is no statistically significant difference in energy intake between home and camp, there appeared to be a slight numerical increase in energy intake. Furthermore, there was a

range in the individual changes observed from home to camp (range=-1.92 MJ to +5.02 MJ). All except one player increased their energy intake when in camp compared with at home. The individual increases observed could indicate a change in training demands resulting in the players increasing their energy intake to meet higher energy expenditure demands. Without any energy expenditure or training data this suggestion is speculative. We do not know whether energy intake was optimal for performance but as the body composition of the group did not change we can assume that energy intake was close to energy expenditure at least for the duration of the competition.

Carbohydrate is the predominant fuel used in intermittent team sports and performance is impaired if carbohydrate needs do not meet the demands of training and competition (Mujika and Burke, 2011). Carbohydrate intake in the current study was on average ~3.0 g·kg BM^{-1} at home and ~2.8 g·kg BM^{-1} in camp. The carbohydrate intake in the current study, both at home and in camp, was lower than previously reported during a pre-season period (3.3-4.1 g·kg BM^{-1}) (Bradley et al., 2015a; MacKenzie et al., 2015) and an in-season period (3.4-3.5 g·kg BM^{-1}) (Bradley et al., 2015b). All the carbohydrate intake values reported for elite rugby union players correspond to recommendations for individuals engaged in low intensity or skill based activities (Burke et al., 2011). The players involved are likely to have been engaged in at least moderate if not high intensity training depending on the session. Based on these widely accepted recommendations, the players are failing to meet their carbohydrate needs. However, more recent guidelines, rather than provide a value, simply state that carbohydrate intake should meet the fuel requirements of an individual's training programme (FINA Expert Panel 2014). Similar to energy intake,

the change in carbohydrate intake varied greatly for individuals. The greatest individual increase in carbohydrate intake was ~1 g·kg^{-1} and the greatest decrease was ~1 g·kg^{-1}. Carbohydrate may be kept low to maintain energy balance across the week (Bradley et al., 2015b) but without measures of muscle glycogen concentration it is unclear whether the carbohydrate intake was sufficient for training. Furthermore, previous work demonstrates that players periodise their carbohydrate intake depending on when their game is (Bradley et al., 2015b), so the carbohydrate intake reported could be skewed depending on when the next game was. Carbohydrate intake in the current study did not change from home to camp and values were slightly lower than those previously observed but we do not have training intensity data to make between study comparisons.

Dietary fat is crucial for the normal functioning of cells and is an important part of an athlete's diet. Fat intake in the current study did not significantly change between home and camp. At home on average fat intake was ~1.1 g·kg BM^{-1} and in camp it was ~1.3 g·kg BM^{-1}. These intakes are slightly lower than those observed during an in-season period (1.4 g·kg BM^{-1}) (Bradley et al., 2015b). However, daily fat intake was similar to elite development players during a pre-season period (1.1 g·kg BM^{-1}) (MacKenzie et al., 2015) and slightly higher than the intake of senior elite players during a pre-season period (1.0 g·kg BM^{-1} for forwards and 0.95 g·kg BM^{-1} for backs) (Bradley et al., 2015a). The values observed in the current study were close to those recommended for athletes (Bishop et al., 1999). The dietary fat intake in the current study is similar to values previously reported and meets the recommendations for athletes.

Players' micronutrient intake must be sufficient for general health (British Nutrition Foundation 2015). Players in the current study met micronutrient dietary reference intakes (DRI) for general health (British Nutrition Foundation 2015), as previously reported in elite rugby union players (Bradley et al., 2015b). However, Whiting & Barabash (2006) propose that the DRI for physically active individuals are greater than for general health. Dietary intakes of Vitamin D and C at home were lower than the DRIs (5 μg and 90 mg respectively) but were above the DRI in camp. Furthermore, vitamin D intake was significantly greater in camp than at home. Vitamin A and E intakes were below the DRI (900 μg and 15 mg respectively) both at home and in camp. All other micronutrients met the recommendations for physically active individuals. Vitamin B12 intake was greater in camp than at home which is probably a direct result of the increased meat consumption. There were no other significant differences in micronutrient intake between home and camp.

The dietary habits of the elite U20 rugby union players in the current study changed between home and camp environments. Regarding protein and micronutrient intake it could be argued that dietary habits improved. National players are educated about nutrition to support their training and competition demands. However, the time and attention of the support staff is often stretched in elite team sports and exploring different ways to educate athletes about their nutritional practices is important. Time in camp may present an opportunity to educate young players about the food choices they should be making to meet their energy, macro and micronutrient requirements. We propose that using the in camp diet to educate the players about the types of foods they should be eating at what times could be an effective educational tool. The use of an in camp diet as an educational tool would target and reach a lot of athletes

at once. The current study would have benefitted enormously from having another home dietary assessment after the players had been exposed to the in camp diet. We could then have explored whether players reverted to their previous home dietary habits or whether the in camp diet changed their behaviour at home. The assessment of an in camp diet as an educational tool for elite rugby players, at different age groups, would be an interesting area of further study.

In conclusion, the training and nutritional strategies in place to support the U20 around 6N were appropriate for maintaining the group's body composition. Monitoring players' body composition over a season or several seasons is likely to provide more valuable information than one-off scans. The dietary habits of elite U20 rugby union players differ between home and camp environments. We suggest that a camp environment could be used as a platform to educate players about what and when they should be eating to optimise training and performance.

3.5. Practical Applications

The data in the current study demonstrate that U20s rugby union players change their eating habits when they go into camp, compared with their normal environment. Players should attempt to develop consistent habits between home and camp. Energy requirements are likely to change at several points during the season and players must make sure that their energy and carbohydrate intake reflects and supports these changes. Meeting training and playing demands is important to prevent detrimental effects on performance. No changes in body composition suggest that intake was sufficient to meet but not exceed energy demands. Protein intake should remain stable from home to camp unless players are trying specifically to gain

lean mass or lose fat mass. Protein intake should be spread throughout the day and should be included in each meal and snack. Regarding micronutrient intake, players must ensure they meet requirements both in camp and in their normal environment. If micronutrient requirements are not met, players could become ill or their performance could suffer.

CHAPTER 4 Amount of protein ingested post resistance exercise does not influence p70S6K1 activity but resistance exercise sustains p70S6K1 activity in response to protein ingestion

Macnaughton LS, Jeromson S, Witard OC, Jackman SR, Hamilton DL, Tipton KD

4.0 Abstract

The additive effect of protein ingestion and resistance exercise on the stimulation of MPS is well established. However, to date no study has directly measured the activity of the cell signalling protein p70S6K1 in response to increasing doses of ingested protein. The primary aim of the study was to investigate the dose-response of p70S6K1 activity to whey protein ingestion at rest and following resistance exercise. The secondary aim of this study was to compare the response of p70S6K1 activity to protein ingestion alone *vs.* protein ingestion combined with resistance exercise.

In a cross-sectional study, thirty resistance-trained males consumed a high protein breakfast before resting for 3 h. Following a bout of unilateral resistance exercise (8 × 10 leg press and leg extension exercises; 80% 1 RM), participants consumed 0, 10, 20 or 40 g of whey protein isolate. The activity of p70S6K1 was measured at 0 and 4 h post drink ingestion in rested and exercised legs using a validated [γ-32P] adenosine triphosphate (ATP) kinase assay.

There was no difference in p70S6K1 activity between protein doses. The fold change in p70S6K1 activity in response to protein ingestion from 0-4 h was 62% higher (p=0.035; d=0.61; CI=0.14 to 1.08) in the exercised leg (1.8 ± 1.3 fold; mean ± SD) compared with the rested leg (1.1 ± 0.8 fold). Correlation analysis revealed a significant weak-moderate association between p70S6K1 activity and myofibrillar MPS after drink ingestion (p=0.0097; r=0.472). Resistance exercise enhanced the response of p70S6K1 activity to protein ingestion and likely contributes to the enhanced response of MPS when protein feeding and resistance exercise are combined. Conversely, protein dose does not modulate p70S6K1 activity, which could

indicate that the stimulatory effect of resistance exercise alone is sufficient to drive an increase in p70S6K1 activation.

4.1 Introduction

The mTORC1 pathway plays a central role in the molecular control of MPS. A key step in the mTORC1 pathway involves the activation of P70S6K1, which regulates the rate-limiting translation initiation step of MPS. P70S6K1 directly phosphorylates the initiation factor eIF4B (Holz et al., 2005) that initiates the unwinding of the 5'UTR (five prime untranslated region) of mRNA (messenger ribonucleic acid) allowing ribosome binding to mRNA (Gingras et al., 1999). Additionally, p70S6K1 phosphorylates the elongation factor eEF2K allowing the activation (removal of inhibition) of another elongation factor, eEF2, which is involved in ribosome translocation (Wang et al., 2001). The removal or inhibition of p70S6K1 has an impact on growth and MPS. Previous work in p70S6K1 knockout mice demonstrates a ~15% difference in body mass compared to wild-type (Shima et al., 1998a). Furthermore, blocking the mTORC1 signalling pathway in humans through administration of rapamycin prevents an increase in MPS for 2 h following resistance exercise (Drummond et al., 2009) and EAA provision (Dickinson et al., 2011). The control groups in these studies (no rapamycin treatment) experienced a 40% and 60% increase in MPS from basal, respectively. Consequently, the mTORC1 signalling cascade is key for the initial stimulation of MPS in response to resistance exercise and amino acid provision. P70S6K1 Thr^{389} phosphorylation correlates strongly with increases in LBM (Terzis et al., 2008). However, there is some discordance between p70S6K1 phosphorylation and MPS (Atherton et al., 2010; Dreyer et al., 2006) and as such the relationship between p70S6K1 activity and MPS remains somewhat unclear.

P70S6K1 phosphorylation at Thr^{389} is considered a key readout of mTORC1 phosphorylation and is indicative of the anabolic signalling response to a given intervention (McGlory et al., 2014). Information regarding the cell signalling response to an intervention or set of conditions could develop understanding of physiological processes involved in regulating muscle mass. Previous work has shown p70S6K1 Thr^{389} phosphorylation to be elevated following amino acid provision at 1 (Fujita et al., 2007) and 3 h (Cuthbertson et al., 2004) but not at 6 h (Glover et al., 2008). Additionally, resistance exercise has been shown to elicit an increase in p70S6K1 Thr^{389} phosphorylation at 1 (Moore et al., 2011), 1.5, 3 (Apró et al., 2015a) and 6 h (Glover et al., 2008) and p70S6K1 activity at 1.5 and 3 h (Apró et al., 2015a). Conversely, previous studies have reported no increase in p70S6K1 Thr^{389} phosphorylation 1 or 2 h following resistance exercise (Apró and Blomstrand, 2010; Karlsson et al., 2004). Such inconsistencies in the signalling response to resistance exercise could be explained by differences in exercise volume between studies. The exercise volume in Glover et al., (2008) and Moore et al., (2011) (4-5 sets of 10 reps) differed from Apró & Blomstrand (2010) and Karlsson et al., (2004) (8-10 sets of 10-15 reps). However, Apró et al., (2015a) observed increased p70S6K1 Thr^{389} phosphorylation and p70S6K1 activity following 10 sets of 8-10 reps. The combination of resistance exercise and amino acid provision has been shown to increase p70S6K1 Thr^{389} phosphorylation to a greater extent than either stimulus alone (Apró and Blomstrand, 2010; Glover et al., 2008; Karlsson et al., 2004; Moore et al., 2011). The measurement of p70S6K1 phosphorylation is indicative of the protein's activity but is not a direct measure of activation. McGlory et al., (2014), in our laboratory, demonstrated that p70S6K1 activity (direct measure of activity)

increased in response to resistance exercise and amino acid ingestion at 1 and 3 h post resistance exercise whereas the phosphorylation status of p70S6K1 Thr^{389} remained unchanged. Therefore, the measurement of p70S6K1 signalling to anabolic stimuli does not appear to be consistent between methods and studies. The use of phosphorylation as a proxy measure of activity could be introducing further variation.

The measurement of p70S6K1 activity could be beneficial when attempting to detect subtle changes in the cell signalling response to anabolic stimuli. These more subtle changes may occur when interventions or conditions focus on optimising conditions rather than inducing a large anabolic response. Optimising the conditions or aspects of resistance exercise and amino acid ingestion to gain the greatest MPS response is likely to be beneficial for muscle remodelling, growth and maintenance. For example, provision of 20 g of protein compared with 10 g following resistance exercise increases MPS to a greater extent (Moore et al., 2009) and this can be detected due to the sensitivity of the MPS measurement. However, in the same study there was no difference in p70S6K1 Thr^{389} phosphorylation between protein doses (Moore et al., 2009). The western blotting technique, which is most often used to measure p70S6K1 phosphorylation, is a semi-quantitative method, whereas, the kinase assay used to measure the activity of p70S6K1 activity is fully-quantitative. Furthermore, the kinase assay measures the specific activity of the protein whereas the western blotting technique measures phosphorylation, which does not always translate to increased activity. It is of course possible that there is no difference in the signalling response and the stimulation of p70S6K1 Thr^{389} phosphorylation from the resistance exercise was sufficient for p70S6K1 activation irrespective of how much protein was

ingested. However, a difference between protein doses in the p70S6K1 signalling response may have been present but just not detected with a semi-quantitative method but may be detected when p70S6K1 activity is measured rather than phosphorylation.

The primary aim of this chapter was to investigate the dose-response of p70S6K1 activity to ingested protein in resistance-trained young males. Secondary aims were to compare the response of p70S6K1 activity to protein ingestion alone and protein ingestion following resistance exercise, and to examine the relationship between myofibrillar MPS and p70S6K1 activity in response to resistance exercise and protein feeding.

4.2 Methods

4.2.1. Participant characteristics and ethical approval

Muscle tissue samples collected in a previously published cross-sectional study (Witard et al., 2014) were used in the following analysis. Subsequent molecular analysis on previously collected muscle tissue was approved by the National Research Ethics Service Ethics Board, Black Country, Birmingham (REC number 08/H1202/131).

Forty-eight males were recruited for the original study but due to tissue availability samples from thirty healthy resistance trained males (≥6 m recreational weight lifting experience) were analysed. Participant characteristics are displayed in Table 4.1.

Table 4.1 - Participant characteristics.

	0 g (n=7)	10 g (n=12)	20 g (n=8)	40 g (n=3)
Age (y)	22.6 ± 3.0 [a]	20.3 ± 1.1 [b]	20.9 ± 1.0	19.7 ± 1.2
Height (cm)	180.6 ± 6.4	179.1 ± 4.9	182.7 ± 5.7	177.7 ± 6.4
Body mass (kg)	85.8 ± 14.5	84.4 ± 6.1	85.8 ± 6.4	72.5 ± 8.4
Body fat (%)	14.8 ± 5.3	15.7 ± 3.6	15.3 ± 2.8	13.0 ± 5.3

Values are mean ± SD. Means with different letters are significantly different from each other (p=0.045).

4.2.2. Study design

In a cross-sectional study, each participant completed a 1RM testing session for leg press and leg extension prior to the experimental trial. Food packages were provided for 48 h before the experimental trial and reflected the habitual dietary intake of each participant. Participants were assigned to one of four groups; each group received a different dose of whey protein (0 g (n=7); 10 g (n=12); 20 g (n=8); 40 g (n=3)).

4.2.3. Experimental trial

Participants were instructed to refrain from eating after 2000 h the night before the experimental trial. The following morning participants arrived at the laboratory, height and body mass were recorded before a cannula was inserted into the forearm vein and a basal blood sample was collected. Participants then consumed a high protein breakfast (30% EI) and rested for 1 h 45 min before a primed constant infusion of L-[ring- $^{13}C_6$] phenylalanine (prime 2.0 μmol·kg^{-1}; infusion 0.05 μmol·min^{-1}·kg^{-1}) was started and continued for the remainder of the trial. After 3 h resting in a supine position, participants performed an intense bout of unilateral resistance exercise (8 x 10 leg presses and leg extensions; 80% 1 RM) before a skeletal muscle biopsy was collected from the *vastus lateralis* of the rested and exercised legs. Within

10 min of exercise cessation, participants ingested a drink containing 0, 10, 20 or 40 g of whey protein isolate. All protein drinks were enriched to 6% with L-[ring-$^{13}C_6$] phenylalanine. Subsequent muscle biopsies were taken from the rested and exercised legs at 4 h post drink ingestion. Blood samples were collected from the forearm vein periodically during the phenylalanine infusion. The infusion was stopped following collection of the final biopsy and blood samples at 4 h post drink ingestion.

4.2.4. Muscle sampling

Muscle biopsies were collected using a 5 mm Bergström needle modified for manual suction. Prior to the exercise bout, a small incision was made in the lateral portion of the *vastus lateralis* of both legs under local anaesthesia. Immediately after exercise, a muscle biopsy was collected from each leg through incisions made before exercise. New incisions, ~1-2 cm away from the original incisions, were prepared for the muscle biopsies collected 4 h post drink ingestion. Muscle tissue was immediately rinsed with ice-cold saline before any visible fat and connective tissue was removed. Samples were then snap frozen in liquid nitrogen and stored at -80° C until analysis. Myofibrillar FSR was calculated in rested and exercised legs over the 4 h post drink period. The authors granted permission to report the previously published MPS data in this thesis.

4.2.5. Muscle tissue processing

Muscle tissue (~30-50 mg) was homogenised in a 10-fold volume of homogenisation buffer (50 mM TrisHCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (v/v) TritonX-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% β-mercaptoehtanol, 1 mM $Na_3(OV)_4$ and 1 complete protease inhibitor tablet (Roche Diagnostics Ltd, Sussex, UK) per 10 mL)

using dounce homogenisation. Samples were clarified by centrifugation at 4° C for 45 min at 14,800 rpm. The protein concentration of each sample was quantified using the DC protein assay (BioRad, Hertfordshire, UK) and Gen 5 software (BioTek, Vermont, US). A volume of lysate containing 300 μg protein was aliquoted off and snap frozen before being stored at -80° C until further analysis.

4.2.6. P70S6K1 activity assay

P70S6K1 kinase assays were carried out by immunoprecipitation (4 μg of p70S6K1 antibody (Santa Cruz Biotechnology Inc, Heidelberg, Germany)). P70S6K1 was immunoprecipitated from lysate containing 300 μg protein for 2 h at 4° C in homogenisation buffer (as detailed above). Protein G sepharose (2.5 μL per immunoprecipitation) was used to precipitate the immune-complexes. Immune-complexes were washed twice in assay specific high salt washes (homogenisation buffer as above with 0.5 M NaCl added) followed by one wash in assay buffer (50 mM TrisHCl at pH 7.4, 0.03% Brij35, 0.1% β-mercaptoethanol). Prior to carrying out the activity assay, the immune-bead-complex was suspended in a total of 10 μl of assay buffer; assays were carried out in a 50 μl reaction. Assays were started every 20 s by the addition of 40 μL hot assay mix which consisted of assay buffer, ATP-$MgCl_2$ (100 μM ATP + 10 mM $MgCl_2$), $^{32}\gamma$-ATP (1 x 10^6 cpm·$nmol^{-1}$) and finally synthetic peptide substrates ("S6tide" KRRRLASLR at 30 μM). Assays were run for 90 min and were stopped at 20 s intervals by spotting onto squares of p81 chromatography paper (Whatman, GE Healthcare, UK) and immersing in 75 mM phosphoric acid. P81 papers were washed 3 x 5 min in 75 mM phosphoric acid and 1 x 5 min in acetone. The papers were then dried, immersed in Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies Ltd, Chesterfield, UK) and counted in a United Technologies Packard

2200CA TriCarb scintillation counter. Assay results were quantified in fmol·min^{-1}·mg^{-1} (mU·mg^{-1}). Blanks for background subtractions were carried out with immunoprecipitated p70S6K1 with no peptide included in the assay reaction.

4.2.7. Statistical analysis

P70S6K1 activity was measured in muscle tissue samples that remained following initial MPS analysis. The fold change in p70S6K1 activity from immediately before protein ingestion (0 h) to 4 h post protein ingestion (4 h) was calculated for rested and exercised legs. We obtained 30 complete data sets (rest and exercise at 0 and 4 h) for p70S6K1 activity analysis within the following doses of protein: 0 g n=7; 10 g n=12; 20 g n=8; 40 g n=3. P70S6K1 activity data were log transformed to ensure normal distribution. Due to the low number in the 40 g group, these participants were removed from the dose analysis and the analysis was run on the 0, 10 and 20 g doses. A two-way ANOVA was run on fold change in p70S6K1 activity data with dose as a between factor and condition as a within factor (Minitab Statistical Software, Version 17, Coventry, UK). The protein doses were then pooled (10, 20 and 40 g) to compare the effect of resistance exercise and protein feeding with protein feeding alone and an independent Student's t-test was carried out. A Pearson's Product correlation was run on the fold change in p70S6K1 activity between rest and exercised legs at 4 h and the fold change in myofibrillar-MPS between rested and exercised legs (GraphPad Prism v6, GraphPad, CA, US). Data are presented as mean ± 95% confidence intervals (CI). Significance was accepted at the 95% level ($p<0.05$) and effect sizes are presented as Cohen's d with CI. The pooled standard deviation was used in the effect size calculations. Effect sizes of 0.2 are considered small, 0.5 considered medium and

>0.8 are considered large (Cohen, 1969). If 0 is not contained within the confidence intervals for the effect size the effect is deemed significant.

4.3. Results

4.3.1 P70S6K1 activity

There was no interaction between protein dose and condition (p=0.888). Also, there was no effect of protein dose ingested on p70S6K1 activity (p=0.990) (Figure 4.1).

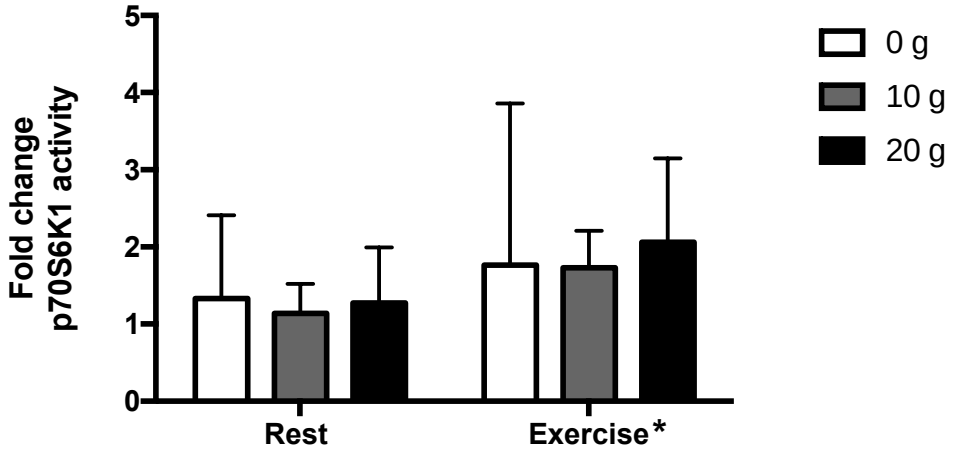


Figure 4.1 - Fold change in p70S6K1 activity from 0-4 h in the rested and exercised legs following ingestion of 0, 10 or 20 g of whey protein (n=27). Data expressed as mean with 95 % confidence intervals. Significant main effect of condition (p=0.035), * significantly greater than rested leg.

Overall p70S6K1 activity was significantly greater in the exercised leg compared with the rested leg (main effect of condition; p=0.035). The fold change in p70S6K1 activity from 0-4 h was 62% greater (p=0.004; d=0.61; CI=0.14 to 1.08) in the exercised leg (1.8 ± 1.3) compared with the rested leg (1.1 ± 0.8) when doses were pooled (Figure 4.2).

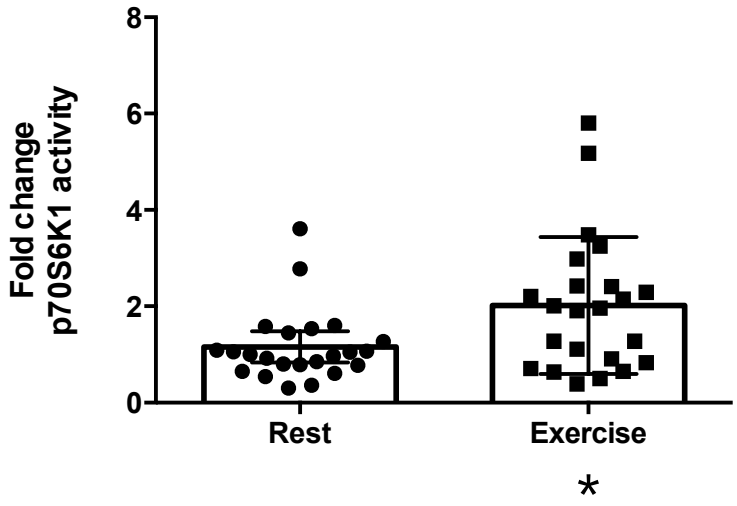


Figure 4.2 - Fold change in p70S6K1 activity between 0-4 h in the rested and exercised legs with the protein doses pooled (n=23). Data expressed as mean with 95 % confidence intervals. Significant difference between conditions (p=0.035), * significantly greater than rested leg.

4.3.2. P70S6K1 activity and MPS

The MPS dataset has been published previously, therefore, results are reported only briefly. Myofibrillar FSR was greater following ingestion of 20 and 40 g compared with both 0 and 10 g. MPS was greater in the exercised leg compared with the rested leg (1.5 ± 0.4 fold; d=1.48; CI=0.85 to 1.98) following protein feeding. The fold change in FSR between the rested and exercised legs was calculated and correlated with the fold change in p70S6K1 activity between the rested and exercised legs at the 4 h time point. Pearson's product correlation revealed a statistically significant relationship (p=0.0097) with an r value of 0.472 (Figure 4.3).

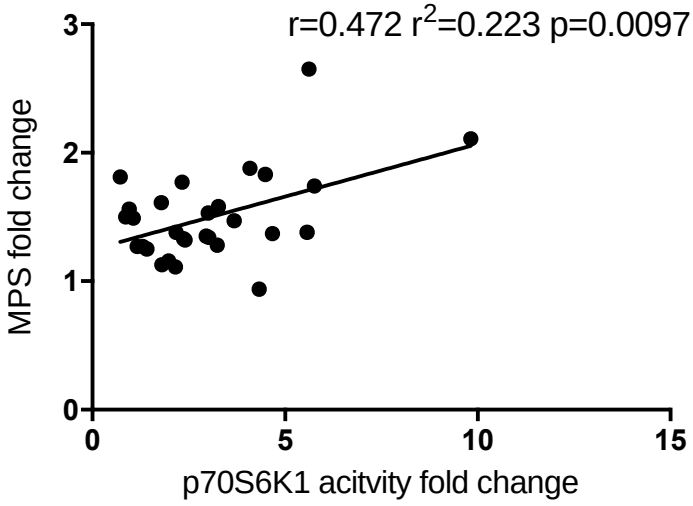


Figure 4.3 - Correlation of fold change of p70S6K1 activity and MPS (n=29). Fold change of p70S6K1 activity between rest and exercise at 4 h plotted on the x-axis and the fold change between rest and exercise in myofibrillar muscle protein synthesis rate plotted on the y-axis.

4.4. Discussion

The primary aim of this study was to compare the response of p70S6K1 activity to ingestion of different doses of whey protein following resistance exercise. Ingestion of increasing protein dose did not alter the activity of p70S6K1. A secondary aim was to compare the response of p70S6K1 activity to protein ingestion alone compared with protein ingestion following resistance exercise. Consistent with the previously reported enhanced stimulation of MPS in the exercised leg compared with the rested leg, the present study revealed increased p70S6K1 activity following resistance exercise and protein ingestion compared with protein ingestion alone. Correlation analysis revealed a significant, weak to moderate relationship between p70S6K1 activity and MPS following resistance exercise and protein ingestion.

The combination of protein ingestion and resistance exercise has previously been shown to stimulate p70S6K1 phosphorylation to a greater extent than protein feeding alone (Apró and Blomstrand, 2010; Glover et al., 2008; Moore et al., 2011). Our

p70S6K1 activity data support these previous results. However, expanding these data, we report no influence of protein dose on p70S6K1 activity despite increased MPS with higher doses up to 20 g of ingested protein. Our results are similar to those of Moore et al., (2009) who observed no statistically significant difference in p70S6K1 Thr^{389} phosphorylation between protein doses at 1 or 4 h post protein ingestion. Interestingly, past (Moore et al., 2009) and the present study included a 0 g protein dose equivalent to an exercise only condition. It is possible that the bout of resistance exercise activated p70S6K1 to such an extent that further stimulation from protein provision did not occur. However, Karlsson et al., (2004) showed that resistance exercise combined with amino acid provision stimulated p70S6K1 Thr^{389} phosphorylation to a greater extent than resistance exercise alone. Irrespective of whether p70S6K1 activation is measured by p70S6K1 Thr^{389} phosphorylation or by p70S6K1 activity, protein dose plays a relatively minor role in stimulation of the p70S6K1 pathway compared with resistance exercise. Moore et al., (2009) recorded no pre exercise and feeding baseline or rest control in the study making it difficult to know whether phosphorylation of p70S6K1 changed in response to resistance exercise. The influence of resistance exercise and protein ingestion on the response of p70S6K1 activity appears to be greater than the influence of protein ingestion alone (Churchward-Venne et al., 2012a; Moore et al., 2011). Our data expand these previous findings by demonstrating that p70S6K1 activity, a direct measurement of p70S6K1 activation status, is stimulated to a greater extent following resistance exercise and protein ingestion compared with protein ingestion alone. Taken together, these data suggest that resistance exercise may be a more potent stimulator of p70S6K1 activity than protein ingestion.

The measurement of p70S6K1 activity following resistance exercise and/or protein ingestion in human muscle is a relatively novel technique that was recently validated in our laboratory (McGlory et al., 2014). McGlory et al., (2014) observed that p70S6K1 activity was elevated from pre-resistance exercise at 1 and 3 h post exercise and ingestion of 20 g egg protein. However, the phosphorylation of p70S6K1 at Thr^{389} was unchanged. Work carried out by Apró et al., (2015a) demonstrated that p70S6K1 activity and phosphorylation of p70S6K1 at Thr^{389} were both elevated at 1.5 and 3 h post resistance exercise without feeding. The responses of p70S6K1 activity and phosphorylation of p70S6K1 Thr^{389} were similar also when participants consumed amino acid containing beverages following resistance exercise (Apró et al., 2015b). Additionally, a significant correlation was observed between p70S6K1 activity and phosphorylation of p70S6K1 at Thr^{389} ($r=0.72$; $p<0.05$). The biopsy collection time points in our study and previous studies (Moore et al., 2011; Churchward-Venne et al., 2012a; McGlory et al., 2014; Apró et al., 2015a; Apró et al., 2015b) are not directly comparable. However, our 4 h measurement falls between 3 and 5 h when elevations of p70S6K1 Thr^{389} phosphorylation or activity have been observed. Given the similar response to resistance exercise and protein ingestion and the correlation of both measures, we can be confident that p70S6K1 activity is greater following resistance exercise and feeding compared with protein feeding alone.

Although the dose of ingested protein had no impact on p70S6K1 activity in the present study, there was a difference in the MPS response between various protein doses. Specifically, myofibrillar MPS was higher in the 20 g group compared with the 0 and 10 g groups. P70S6K1 activation is important for the stimulation of MPS (Dickinson et al., 2011; Drummond et al., 2009; Shima et al., 1998b). However, the

extent to which p70S6K1 controls MPS in humans following resistance exercise and protein feeding remains unclear (Atherton et al., 2010; Dreyer et al., 2006). We observed that the difference in p70S6K1 activity between rest and exercise explained only ~20% of the variation in the difference in MPS between rested and exercised conditions. Similar to our findings, Burd et al., (2010) reported a similar but lower correlation between p70S6K1 Thr^{389} phosphorylation and MPS ($r=0.338$; $p=0.033$). Moore et al., (2011) reported that MPS and p70S6K1 Thr^{389} phosphorylation follow a similar pattern following resistance exercise and protein ingestion. However, there appears to be some disconnect between MPS and p70S6K1 Thr^{389} phosphorylation (Atherton et al., 2010; Dreyer et al., 2006). Although Atherton et al., (2010) observed that following protein ingestion, the phosphorylation of p70S6K1 and MPS initially followed the same pattern (elevated at 1.5 h), at 3 h, phosphorylation of p70S6K1 remained elevated while MPS returned to baseline levels. Furthermore, following resistance exercise only, Dreyer et al., (2006) demonstrated that MPS was elevated above basal levels at 1 and 2 h whereas the phosphorylation status of p70S6K1 Thr^{389} was only elevated above basal levels at 2 h. These data support our finding that only a small proportion of p70S6K1 activity accounts for the variation in MPS. Additionally, provision of whey protein has resulted in MPS being elevated at 1-3 h compared with MPS at the fasted (baseline) and the 3-5 h time points (Churchward-Venne et al., 2012a). However, phosphorylation of p70S6K1 Thr^{389} was not elevated at 1 h but was elevated above fasting at 3 and 5 h. These results provide further evidence that a disconnect exists between the stimulation of MPS and activation of p70S6K1.

This lack of agreement between MPS and p70S6K1 activation could be due to the time course of the response of each measure. Although p70S6K1 activation influences MPS,

the time course of p70S6K1 and MPS stimulation is not necessarily the same. We know that p70S6K1 activation is required for translation to occur (Holz et al., 2005). Accordingly, we would expect the signalling to precede an increase in MPS. Activation of p70S6K1, whether measured as phosphorylation or kinase activity, is measured at a singular time point. Meanwhile MPS, as measured by FSR, is calculated as a rate over a period of time. However, without taking multiple biopsies it is impossible to characterise the time course of p70S6K1 activation and identify the time lag between signalling and an increase in MPS. The lack of time course information could help to explain the discordance between signalling and MPS. Furthermore, as p70S6K1 only accounts for the ~20% of the variation in MPS a number of other factors must contribute to the variation in MPS. The discordance observed between p70S6K1 signalling is most likely because MPS is controlled only to some extent by p70S6K1. Changes in other factors that influence MPS would vary the MPS response without any alterations in p70S6K1 activation, resulting in the observed discordance that occurs between MPS and p70S6K1 signalling.

From our current results, we can conclude that resistance exercise appears to be a greater stimulator of p70S6K1 activity than protein feeding. We have demonstrated that following resistance exercise there is no further increase in p70S6K1 activity when protein is provided. Furthermore, p70S6K1 activity is greater following resistance exercise and protein feeding compared with protein feeding alone. Although, this result has previously been demonstrated for phosphorylation of p70S6K1, until now it has never been demonstrated directly measuring the activity of this particular signalling protein. P70S6K1 controls the MPS response only to a certain extent, the lack of agreement between the two measures is most likely due to

the timing of each response to anabolic stimuli, and that other factors control MPS. It would appear that regardless of how p70S6K1 activation is measured, it only explains a small part of any changes observed in MPS. Future work should critically examine what valuable information is provided when measuring p70S6K1 activation with regard to understanding changes in MPS.

CHAPTER 5 The response of MPS following whole body resistance exercise is greater following ingestion of 40 g compared with 20 g whey protein and is not influenced by LBM

Macnaughton LS, Wardle SL, Witard OC, McGlory C, Hamilton DL, Jeromson S, Lawrence CE, Wallis GA, Tipton KD.

5.0. Abstract

Strategies that help increase or maintain skeletal muscle mass are of interest to many. Ingestion of amino acids following a bout of resistance exercise is the main stimulus for synthesis of new muscle proteins. There appears to be an upper limit or maximal rate at which new contractile muscle proteins can be generated (MPS). 20 – 25 g of protein is currently accepted as the amount of protein required to achieve maximal stimulation of MPS. However, this amount is for average-sized, young males (80-85 kg). Due to a larger capacity and/or demand, it is possible that those with greater LBM will require more than 20 g of protein to maximally stimulate MPS following resistance exercise. Therefore, our aim was to assess the influence of LBM on the response of myofibrillar MPS following a bout of whole body resistance exercise and ingestion of either 20 or 40 g of whey protein.

In a randomised parallel cross-over design, thirty young, resistance trained, healthy males were divided into two even groups based on their LBM (≥ 70 kg or ≤ 65 kg). A bout of whole body resistance exercise was performed 180 min after ingestion of a high protein breakfast. Immediately post exercise, participants consumed either 20 or 40 g of whey protein. MPS was calculated following the infusion of labelled phenylalanine tracer and collection of skeletal muscle biopsy samples from the *vastus lateralis*. Overall, we observed that MPS was stimulated to a greater extent following ingestion of 40 g whey protein (0.059 ± 0.017%·h^{-1}) compared with 20 g (0.049 ± 0.016%·h^{-1}) in the 300 min recovery period from an intense bout of whole body resistance exercise. No difference in the MPS response was observed between groups following ingestion of either dose of protein. Both previous studies of this kind used either lower limb bilateral (Moore et al., 2009) or unilateral (Witard et al., 2014)

resistance exercise. The difference in the amount of exercised muscle between studies is the most likely explanation for the discord in results between previous and the current study.

In summary, following whole body resistance exercise 40 g of whey protein stimulates MPS to a greater extent than 20 g. Moreover, LBM does not appear to influence the MPS response following a bout of whole body resistance exercise and ingestion of 20 or 40 g of whey protein.

5.1. Introduction

The stimulatory effects of resistance exercise and amino acid provision on MPS are well documented (Biolo et al., 1997; Tipton & Wolfe 2004). Amino acid provision from feeding stimulates MPS above basal rates (Witard et al., 2014). However, the combination of amino acid provision and resistance exercise results in greater stimulation of MPS compared with amino acid provision alone (Biolo et al., 1997). Stimulation of MPS is important for the maintenance and growth of skeletal muscle mass. A concept introduced by Bohé et al., (2001) and later termed the 'muscle full effect' by Atherton et al., (2010) suggests that an upper limit of the MPS response to increasing amounts of amino acid provision exists. At this threshold, further provision of amino acids will not result in increased MPS and the unused amino acids undergo other fates, in particular oxidation (Witard et al., 2014). Therefore, it is important to establish a dose of protein that will achieve the maximal stimulation of MPS while limiting significant amino acid oxidation.

Based on available evidence, 20-25 g of high quality protein is considered sufficient to

maximally stimulate MPS post resistance exercise in young adults (Morton et al., 2015; Witard et al., 2016). Results from seminal work by Moore et al., (2009) demonstrated that ingestion of 40 g egg protein following bilateral-leg resistance exercise stimulated a similar MPS response compared with 20 g of egg protein. A more recent study from Witard et al., (2014) replicated these findings. No statistically significant difference was observed in the response of MPS to unilateral resistance exercise after ingesting 20 or 40 g of whey protein. Taken together, these data support the view that ingesting ~20 g of high-quality protein after exercise is sufficient to maximally stimulate MPS during recovery. However, the characteristics of the participants in these studies were similar, *i.e.,* young, trained and of similar size and body composition on average. Therefore, it is unknown whether the optimal dose of protein is the same for individuals from different populations and/or body composition and mass.

It has long been suggested that larger athletes require more protein than smaller athletes (Churchward-Venne et al., 2012a; Witard et al., 2014; Morton et al., 2015) to achieve greater MPS stimulation following resistance exercise, but to our knowledge this notion has not been directly investigated under controlled conditions. Interestingly, studies by Moore et al., (2009) and Witard et al., (2014) reported a mean difference, albeit not statistically significant, of ~10% in post exercise MPS to ingestion of 20 or 40 g of protein. Further unpublished analysis of data generated by Witard et al., (2014) reported a statistically significant positive correlation between the post-prandial MPS response and the amount of ingested protein, expressed relative to total LBM ($r=0.543$; $p=0.001$). Indeed, measured rates of MPS increased as the dose of protein, expressed relative to LBM, increased. One possible explanation

for this correlation is an increased demand for amino acids by skeletal muscle following resistance exercise. It is well established that amino acid provision increases amino acid transport into both resting and exercised muscle (Biolo et al., 1997). Therefore, the availability of, not only endogenous, but also exogenous, amino acids for incorporation into new muscle protein (Pennings et al., 2011a) ultimately limits increases in MPS. Consequently, it seems intuitive to propose that the uptake of amino acids by a greater amount of muscle mass may be limited by a given amount of ingested protein. Therefore, individuals with greater muscle mass (measured as LBM) may be required to ingest greater amounts of protein to achieve greater MPS stimulation. Although protein recommendations are conventionally expressed on a relative g/kg body mass basis, no direct comparisons of the absolute protein dose required for individuals of varying body mass or LBM have been made. Therefore, the primary aim of the present study was to investigate the influence of individual LBM on the post exercise response of MPS to two doses of protein.

The primary aim of the present study was to investigate the influence of the amount of LBM on the MPS response to two doses of whey protein (20 or 40 g) following a bout of whole body resistance exercise. We hypothesised that the group with more LBM would require more protein for greater stimulation of MPS compared with the group with lower LBM.

5.2. Methods

5.2.1. Participants and ethical approval

Thirty healthy, resistance-trained (≥ 2 sessions per week for previous 6 months) males participated in the present study and were grouped according to LBM. Fifty-six

participants were recruited with those that possessed LBM ≤ 65 kg assigned to the lower lean body mass (LLBM) group (n=15) and those with a LBM ≥ 70 kg assigned to the higher lean body mass (HLBM) group (n=15). Volunteers with a LBM between these values were not eligible to participate in the study (n=16) (Figure 5.1). Participant characteristics are displayed in Table 5.1. The current study conformed to the standards of the latest version of the Declaration of Helsinki (2013) and the NHS West of Scotland Ethics Committee approved the study (REC 12/WS/0316). The nature of the study and its associated risks were explained to the participants in lay terms before their informed written consent was obtained.

Table 5.1 - Characteristics of all participants.

	LLBM (≤ 65 kg lean mass)	HLBM (≥ 70 kg lean mass)
Age (y)	21.3 ± 2.2	23.2 ± 3.5
Body mass (kg)	76.8 ± 4.8	98 ± 7.8*
Height (m)	1.78 ± 0.05	1.84 ± 0.05*
Lean body mass (kg)	59.3 ± 3.9 (Range = 51-64.4)	76.9 ± 4.3* (Range = 70.7-83.9)
Fat mass (kg)	14 ± 3.3	17 ± 5.8
Lean mass (%)	77.7 ± 3.6	78.4 ± 4.7
Fat mass (%)	18.8 ± 3.7	17.3 ± 4.9
Appendicular lean mass (kg)	28.12 ± 2.1	37.4 ± 2.3*
1 RM Leg press unilateral (kg)		
Right	126 ± 21.8	159 ± 29.5*
Left	123.6 ± 23.9	158.7 ± 29.1*

Values are means ± SD. LLBM – lower lean body mass group, HLBM – higher lean body mass group. * Significantly different from LLBM ($p < 0.05$).

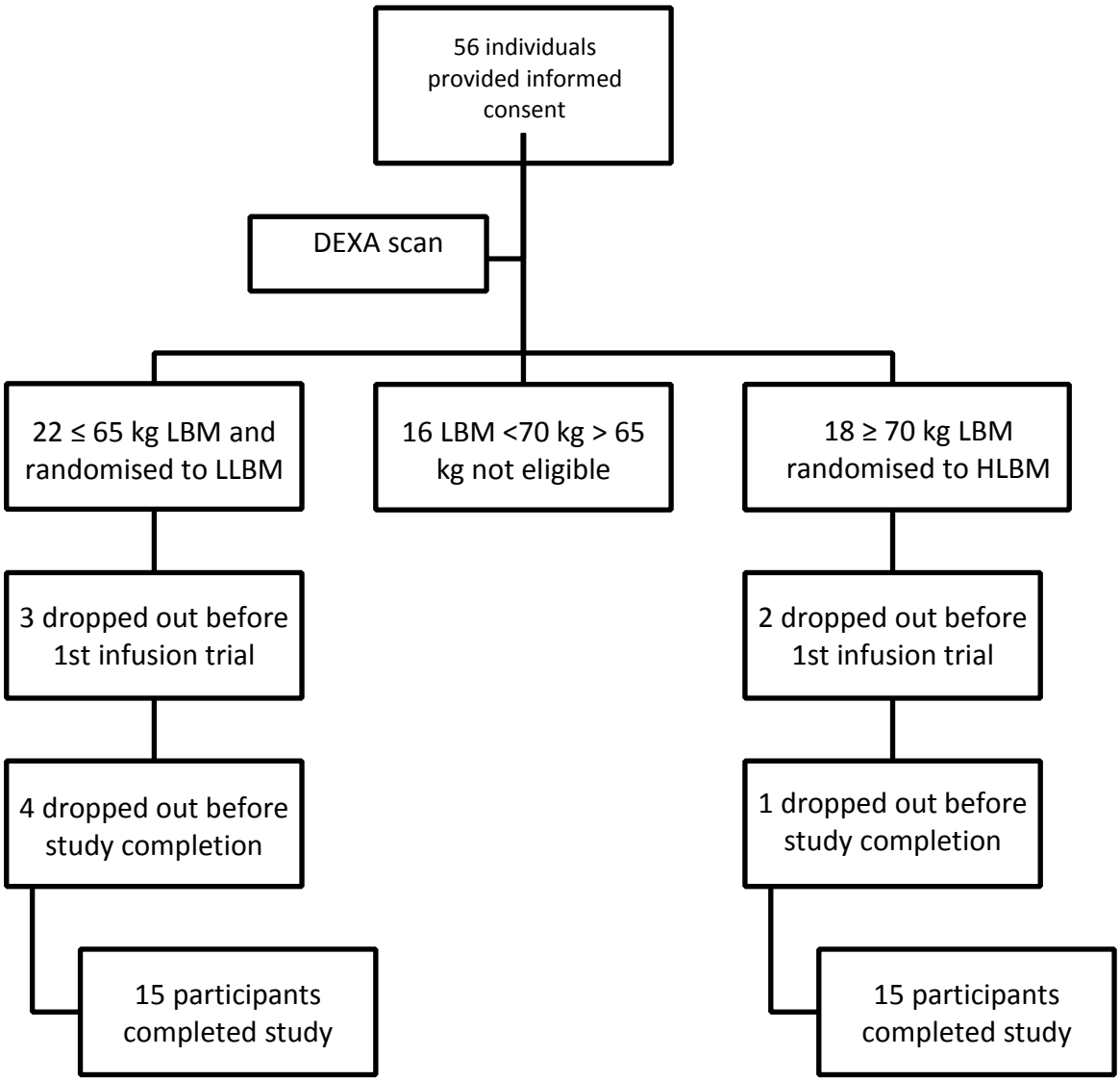


Figure 5.1 - Flow chart of participants that were screened, randomised and completed the study.

5.2.2. Study design

In a two-group, randomised, double-blind, cross-over design, each volunteer participated in two infusion trials designed to measure the response of myofibrillar MPS following whole body resistance exercise and whey protein ingestion. Trials were separated by ~2 wk. Each infusion trial included the ingestion of either 20 (20WP) or 40 (40WP) g of whey protein isolate (GlaxoSmithKline, Middlesex) as a 500 mL drink immediately after exercise. The order of infusion trials, and thus dose

of ingested protein, was random and an independent investigator prepared the drinks.

5.2.3. Preliminary testing

Prior to study inclusion, participant LBM was assessed using a DEXA scanner (GE Healthcare Systems, Hertfordshire). Participants with either ≤ 65 kg lean mass or ≥70 kg lean mass were included in the study. Each participant's 1RM was assessed using a previously validated protocol (Baechle et al., 2008) on selected resistance exercise machines (Cybex International, MA); chest press, latissimus pull-down, leg curl, leg press and leg extension in this order. All leg exercises were carried out on one leg at a time. Participants returned ~1 wk later to confirm their 1 RM.

5.2.4. Dietary and activity control

Each participant completed a 3 d weighed food diary that was analysed using the dietary analysis software Wisp Version 4.0 (Tinuviel Software Systems, Anglesey). Each participant's control diet was based on their self-recorded intakes and matched the energy intake and composition of their habitual diet (Table 5.2). The diets were individually tailored to food preferences and were provided in food packages for a 48 h period prior to both infusion trials. Participants completed a 7 d activity diary and were asked to keep their activity consistent during the study period. Participants were instructed to refrain from strenuous exercise for 48 h before the infusion trials.

Table 5.2 - Habitual diet and diet consumed for 48 h prior to infusion trials.

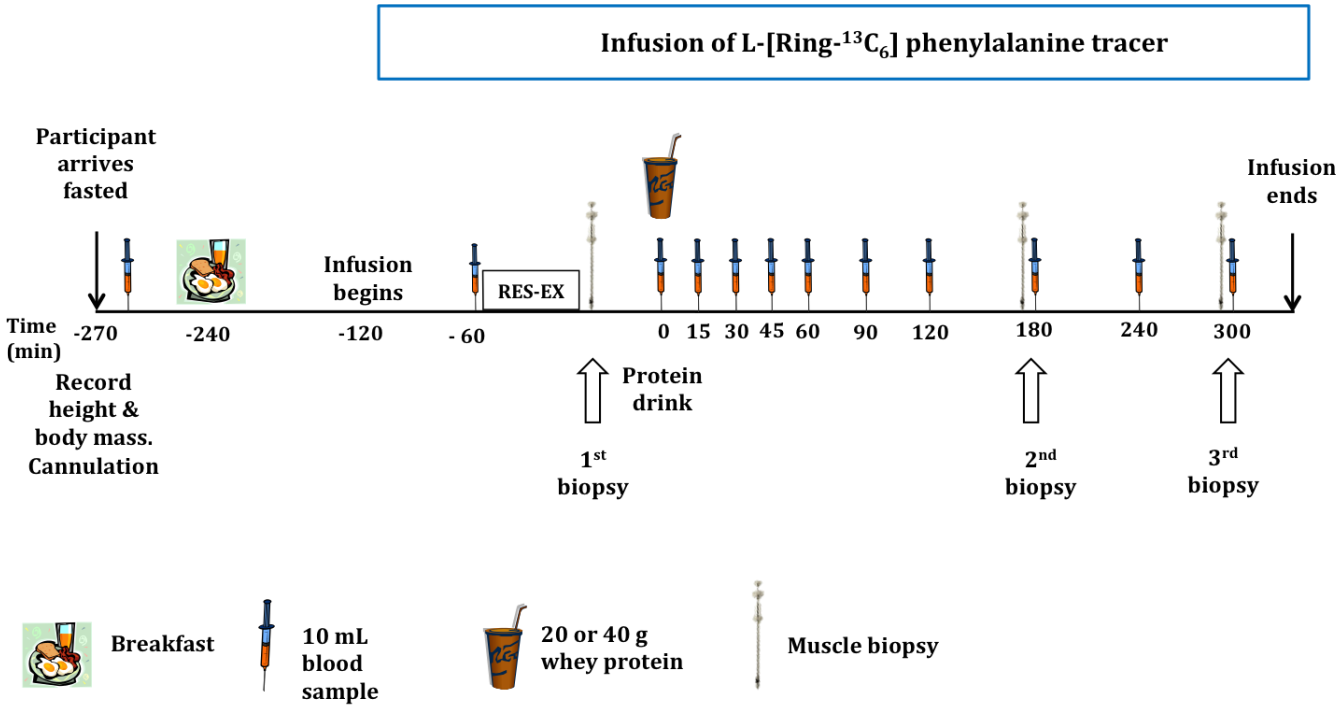
	LLBM (≤ 65 kg lean mass)	HLBM (≥ 70 kg lean mass)
Energy intake (kcal·d^{-1})	2498 ± 676	2851 ± 619*
Carbohydrate intake (g·d^{-1})	269.8 ± 115.4	309.1 ± 108.1
% EI	42 ± 14	37 ± 11
Protein intake (g·d^{-1})	154.5 ± 47.4	185.7 ± 51.3*
% EI	23 ± 9	25 ± 6
Fat intake (g·d^{-1})	77.6 ± 23.3	84.8 ± 19
% EI	31 ± 12	30 ± 8

Values are means ± SD. Habitual diet calculated from 3 day diet records. LLBM – lower lean body mass, HLBM – higher lean body mass, EI – energy intake. * Significantly different from LLBM ($p < 0.05$).

5.2.5. Experimental protocol

A schematic diagram of the experimental protocol is presented in Figure 5.2. Participants arrived at the research laboratories of the Health and Exercise Sciences Research Group at the University of Stirling at ~0600 after an overnight fast. Upon arrival body mass was measured before a 20-gauge cannula was inserted into a forearm vein and a fasted blood sample was collected. Participants were then provided with a standardised breakfast (7 kcal·kg^{-1} body mass) consisting of 50% of energy as carbohydrate, 30% of energy as protein and 20% of energy as fat. After breakfast participants rested in a semi-supine position for 2 h before a primed constant infusion (0.05 μmol·min^{-1}·kg^{-1}; 2.0 μmol·kg^{-1} prime) of L- [ring-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, MA.) was initiated through a 0.2 μm filter. Another 20-gauge cannula was inserted into the contralateral arm for frequent blood sampling. The cannula was periodically flushed with 0.9% saline solution and the arm was wrapped in a heated blanket to allow arterialised blood sampling. Approximately 45 min after starting the infusion a ~1 cm incision was made in the

vastus lateralis under sterile conditions and local anaesthesia (2% lidocaine). Biopsy leg was randomised by left or right. The incision was bandaged and the participants performed an acute bout of resistance exercise on the following machines in the following order; chest press, latissimus pull-down, leg curl, leg press and leg extension. Participants worked at 75% of their 1 RM at a cadence of 1 s concentric, 2 s eccentric. Each participant was instructed to complete 3 sets of 10 repetitions with a 4th final set to volitional failure, to ensure that each participant was working at the same relative intensity, *i.e.,* working to failure. The exercise bout for the second trial was matched to the first. There were no differences in exercise volume between the



two trials for any of the exercises.

Figure 5.2 - Schematic diagram of infusion trial protocol.

Immediately after exercise, a skeletal muscle biopsy was obtained from the incision made before exercise, using a 5 mm Bergström needle modified for manual suction. Participants then consumed a drink that contained either 20 or 40 g of a whey protein isolate made up in 500 mL of water (t = 0 min). Drinks were enriched to 6% with L-

[ring-$^{13}C_6$] phenylalanine. The amino acid composition of the drinks was as follows (percentage of total content) alanine 5.0%, arginine 2.1%, aspartic acid 11.0%, cysteine 2.2%, glutamic acid 1.4%, glycine 1.4%, histidine 1.7%, isoleucine 6.4%, leucine 10.6%, lysine 9.6%, methionine 2.2%, phenylalanine 3.0%, proline 5.5%, serine 4.6%, threonine 6.7%, tryptophan 1.4%, tyrosine 2.6%, valine 5.9%. Subsequent muscle biopsies were obtained from the same leg at 180 and 300 min from new incisions. During the second trial, participants consumed the alternate dose of protein from the first trial and biopsies were obtained from the contralateral leg. Arterialised blood samples were obtained at t =-60, 0, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min. The infusion was stopped following collection of the final blood sample at 300 min. Muscle samples were cleaned with ice cold 0.9% saline solution and were blotted, removing any blood, fat or connective tissue before being frozen in liquid nitrogen and stored at – 80° C for until analysis. Blood samples were dispensed into EDTA and sodium heparin containing vacutainers and were centrifuged at 3500 rpm for 15 min at 4° C. Plasma was extracted into 0.5 mL aliquots and stored at – 80° C until analysis.

5.2.6. Plasma analysis

Plasma insulin concentrations were measured at 0, 15, 30, 45, 60, 90 and 120 min using ELISA kits (Demeditec Diagnostics, Kiel). Plasma samples were analysed for leucine, phenylalanine and threonine concentrations, as well as phenylalanine and tyrosine enrichments as previously described (Witard et al., 2014). Briefly, plasma samples were thawed and acetic acid (1:1 dilution) and internal standard were added (U-[$^{13}C_6$] leucine 0.52 mmol·L^{-1}; U-[$^{13}C_9$ ^{15}N] phenylalanine 0.50 mmol·L^{-1}; U-[$^{13}C_4$ ^{15}N] threonine 0.58 mmol·L^{-1}). Next, amino acids were extracted and purified on

cation-exchange columns (Dowex 50WX8 hydrogen form 100-200 mesh resin, Sigma Aldrich, Dorset). Samples were dried under N_2 gas before being converted to their tert-butyl dimethylsilyl derivative (MTBSTFA). Finally, 2 µL of sample was injected into the gas chromatography mass spectrometer (GC-MS) (Agilent, Santa Clara, CA)(flow rate 2.5 mL·min^{-1}, inlet temperature 250° C; oven gradient 100° C hold for 0.5 min, increased to 170° C at the rate of 10° C·min^{-1}, hold 1.5 min and then increase to 235° C at the rate of 30° C·min^{-1}, hold 1 min and then increase to 250° C at the rate of 50° C·min^{-1}, hold 6 min, total run time 18.47min). Ions were monitored at m/z 302/308 for leucine, 336/346 & 234/240 for phenylalanine, 404/409 for threonine and 466/472 for tyrosine in split mode (1:50 split ratio). Plasma leucine, phenylalanine and threonine concentrations were calculated using the internal standard method (Biolo et al., 1995a). Plasma amino acid concentrations were determined based on the tracer to tracee ratio, known volume of blood and internal standard added to the sample. Plasma urea concentrations were measured at each time point using an automated laboratory analyser (Instrumentation Laboratory, Milano) as a surrogate marker of urea production (Witard et al., 2014).

5.2.7. Muscle analysis

Muscle samples (30-35 mg) were homogenised in 500 µL 0.6 M perchloric acid (PCA) prior to centrifugation at 4,500 rpm for 5 min at 4° C. The supernatant was collected and a further 500 µL 0.6 M PCA was added and spun as before. This step was repeated. The resulting accumulation of supernatant had internal standard (U-[$^{13}C_6$] leucine 0.01 mmol·L^{-1}; U-[$^{13}C_9$ ^{15}N] phenylalanine 0.01mmol·L^{-1}) added to it. The supernatant and internal standard were added to the cation-exchange columns and analysis continued as described above for plasma. Finally, 4 µL of intracellular (IC)

sample was injected into the GC (same conditions as plasma analysis detailed above) and were run in splitless mode. IC leucine was detected at m/z 302/308 and phenylalanine (concentration and enrichment) at m/z 336/342/346.

Following IC extraction from the muscle sample the protein pellet was rinsed with doubly distilled H_2O before being further homogenised in homogenisation buffer (7.5 $\mu L \cdot mg^{-1}$ muscle; 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA 10 mM β-glycerophosphate, 50 mM NaF). Samples were spun at 6000 rpm for 10 min at 4° C, the supernatant discarded and 500 µL doubly distilled H_2O was added before vortexing and spinning at 4500 rpm for 10 min at 4° C. The supernatant was discarded before 1 mL of 0.3 M NaOH was added and the sample heated at 50° C for 30 min with periodic vortexing to separate the pellet from any collagen. The sample was spun at 10,000 rpm for 5 min at 4° C and the supernatant was collected into a glass tube. A further 1 mL 0.3 M NaOH was added to the pellet and spun in the same manner before the supernatant was added to the previous collection. Then 1 mL of 1 M PCA was added to the supernatant and spun at 2000 rpm for 10 min at 4° C. The resulting supernatant was removed and discarded; the pellet that remained was rinsed twice in 1 mL of 70% ethanol by spinning at 1500 rpm for 10 min at 4° C. The pellet was hydrolysed overnight at 110° C in 2 mL of 0.5 M HCl and 1 mL of activated resin. The hydrolysed samples were purified on the cation-exchange columns as previously described and dried under N_2. Samples were converted to their n acetyl, n-propyl ester (NAP) derivative. Finally, 1 µL of derivatised sample was injected into a gas chromatography combustion isotope ratio mass spectrometer (GC-C-IRMS) and run in splitless mode monitoring m/z 44/45 carbon ratio.

P70S6K1 activity assays were run as in Chapter 4.

5.2.8. Calculations

Myofibrillar FSR was calculated using the standard precursor product equation below:

$FSR = [(E_{B2}-E_{B1})/(E_{IC} \times t)] \times 100$

Where E_B (B2 is the biopsy at the later time point, B1 is the biopsy from the earlier time point) is the enrichment of bound phenylalanine, E_{IC} is the IC phenylalanine enrichment of the biopsies and t is time of tracer incorporation (h). IC phenylalanine enrichment was used as the precursor in all FSR calculations.

Plasma and IC amino acid concentrations were calculated by the internal standard method:

$C=Q_{is}/V \times E_{is}$

Where Q_{is} is the amount of internal standard added to the sample, V is the volume of plasma or intracellular water (663 $mL \cdot kg^{-1}$ muscle; (Biolo et al., 1995a) and E_{is} is the internal standard tracer to tracee ratio in the plasma.

Whole body phenylalanine oxidation rates were estimated using the phenylalanine balance model (Munro and Fleck, 1969) based on the hydroxylation of L- [ring-$^{13}C_6$] phenylalanine to L- [ring-$^{13}C_6$] tyrosine, without measuring $^{13}CO_2$ enrichment in the breath (Thompson et al., 1989):

$P_t/P_p \times (Q_p^2/(E_p/E_t)-1) \times (F+Q_p)$

Where P_t/P_p is the molar ration of fluxes of tyrosine and phenylalanine, Q_p equals the rate of disappearance of phenylalanine under steady state conditions, E_p is the enrichment of phenylalanine, E_t is the enrichment of tyrosine and F equals the infusion rate.

Area under the curve (AUC) for plasma insulin, amino acid and urea concentrations and rate of phenylalanine oxidation were calculated using GraphPad Prism Version 6 (Graphpad Software Incorporation, CA). AUC of insulin, urea concentrations and rate of phenylalanine oxidation were calculated from a baseline concentration of 0 and for amino acid concentrations the baseline was taken as the concentration at the 0 min time point.

5.2.9. Statistical analysis

Data were graphed to assess normal distribution using Minitab Version 17.0 (Minitab Software Systems, State College, PA). Box cox transformations were performed on data that were not normally distributed. Significance was accepted at the 95% confidence level ($p<0.05$). Anthropometric, strength and dietary data (HLBM vs. LLBM) were analysed using one factor (group) ANOVA using SPSS Version 21 (IBM UK Ltd, Hampshire). Plasma insulin, amino acid and urea concentrations and rate of phenylalanine oxidation were analysed using repeated measures ANOVA with dose (2 levels) and time (7 levels for insulin, 10 for phenylalanine oxidation and 12 for the other measures) as within-factors and group as a between-factor. AUC for plasma insulin, amino acid and urea concentrations were calculated and analysed using a two-way ANOVA with dose as a within-factor and LBM as a between-factor. FSR and intracellular amino acid concentrations (leucine and phenylalanine) were analysed using repeated measures ANOVA, with dose (2 levels) and time (3 levels) as within-factors and group as a between-factor. AUC was calculated for intracellular leucine and phenylalanine concentrations and analysed using a two-way ANOVA with dose as a within-factor and group as a between-factor. If any interaction was detected, Tukey's post-hoc test was performed using Minitab statistical software. Cohen's effect

size (d) CI were calculated for group and dose. Effect sizes of 0.2 are considered small, 0.5 considered medium and >0.8 are considered large (Cohen, 1969). If 0 is not contained within the CI for the effect size the effect is deemed significant.

5.3. Results

5.3.1. Plasma insulin and amino acid concentrations

Plasma insulin concentrations increased following protein ingestion and peaked at 15 min in LLBM with both doses and HLBM with 20WP. In HLBM with 40WP, plasma insulin concentrations peaked at 30 min. 40WP elicited higher plasma insulin concentrations than 20WP at 45 (d=0.45; CI=-0.06 to 0.97), 60 (d=0.56; CI=0.04 to 1.07) and 90 min (d=0.36; CI=-0.15 to 0.87) (time × dose interaction; $p<0.001$) with no effect of group (Figure 5.3A). Plasma insulin AUC for 40WP was greater than 20WP ($p<0.001$; d=0.37; CI=-0.14 to 0.88) regardless of group (Figure 5.3B).

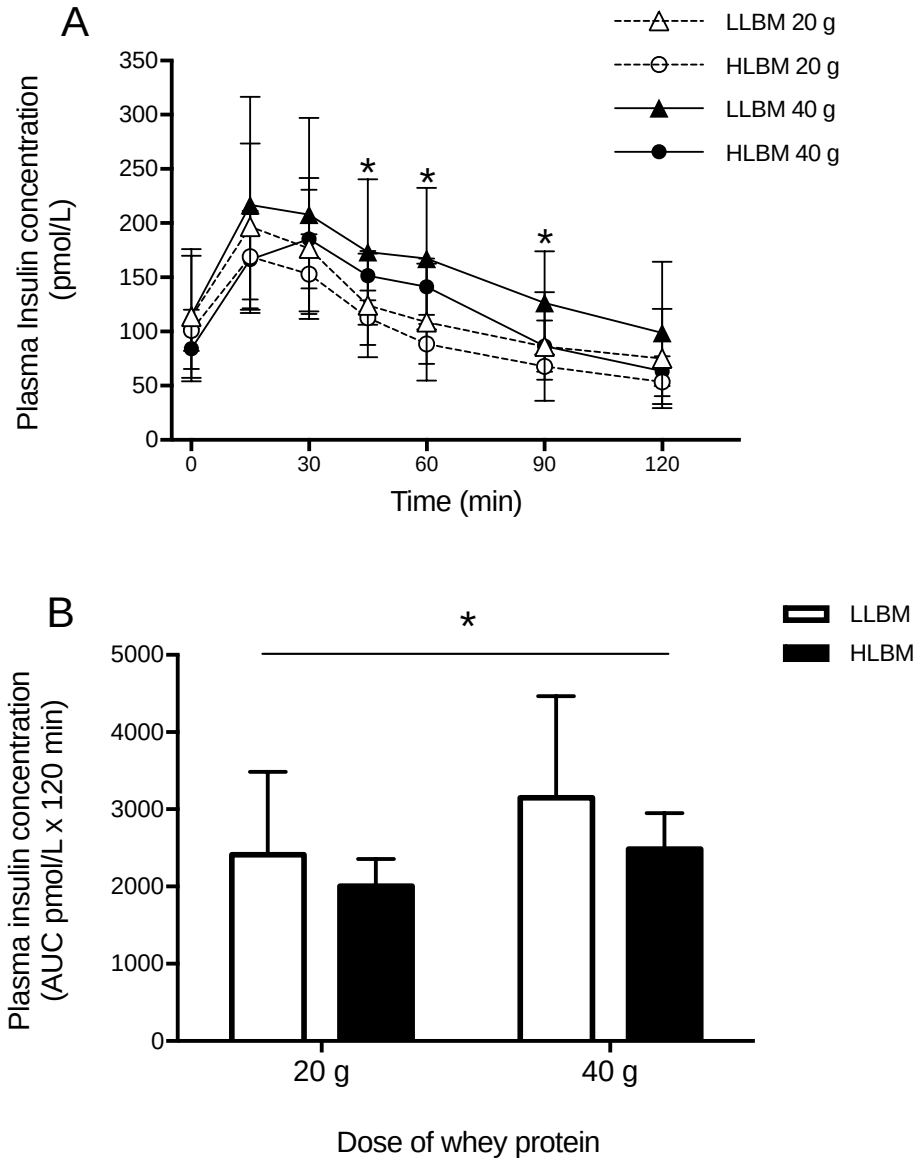
Plasma leucine concentrations peaked at 45 min with 20WP and at 60 min with 40WP in both groups. Plasma leucine concentrations were higher at 45 (d=1.81; CI=1.21 to 2.42), 60 (d=3.13; CI=2.38 to 3.89), 90 (d=2.64; CI=1.94 to 3.33) and 120 min (d=2.00; CI=1.38 to 2.63) in both groups with 40WP compared with 20WP whilst also being elevated at 30 min (d=1.13; CI= 0.36 to 1.90) in LLBM (Figure 5.3C). Plasma leucine concentrations for 40WP were higher in LLBM than HLBM at 90 min (d=1.26; CI=0.48 to 2.04) (group × time × dose interaction; $p=0.048$). The effect sizes calculated between doses at 30 (d=0.98; CI= 0.45 to 1.52), 180 (d=0.97; CI=0.43 to 1.50) and 240 min (d=0.97; CI=0.43 to 1.50) also were large but did not reach statistical significance. The same pattern was apparent between groups at 120 min (d=0.55; CI=0.03 to 1.07). Plasma leucine AUC (Figure 5.3D), was 2.8 fold greater with

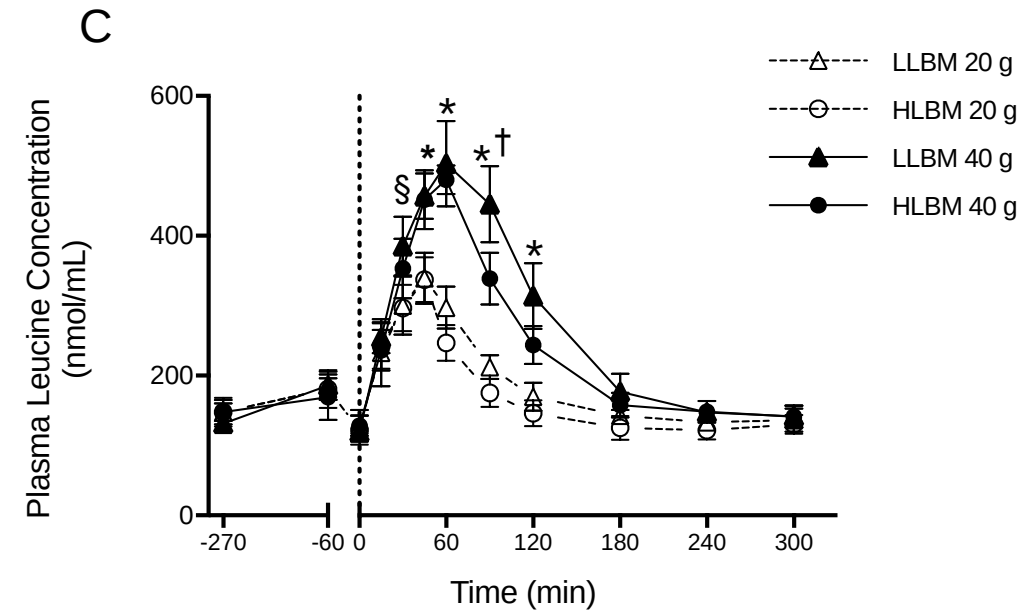
40WP compared with 20WP ($p<0.001$; d=2.68; CI= 1.98 to 3.38). With 40WP plasma leucine AUC was 1.3 fold greater in the LLBM than HLBM (d=1.42; CI=0.62 to 2.22) group (dose × group interaction; p=0.039).

Plasma phenylalanine concentrations were greater with 40WP compared with 20WP in both groups at 30 (d=0.94, CI= 0.40 to 1.47), 45 (d=1.33, CI=0.77 to 1.89), 60 (d=2.08; CI= 1.46 to 2.71) and 90 min (d=1.32; CI=0.76-1.36) (dose × time interaction; $p<0.001$) (Figure 5.3E). Although not statistically significant the effect size between doses at 120 min (d=0.83; CI=0.29 to 1.36) was large. At 30 (d=0.58; CI=0.06 to 1.09) and 90 min (d=0.83; CI=0.30 to 1.36), regardless of dose, LLBM was greater than HLBM for plasma phenylalanine concentration (time × group interaction; p=0.021). The effect size between groups at 120 min (d=0.67; CI=0.15 to 1.19) was considered medium to large. Plasma phenylalanine AUC was 6.2 fold greater in LLBM with 40WP compared with 20WP (dose × group interaction; p=0.022; d=1.30; CI=0.51 to 2.09) (Figure 5.3F).

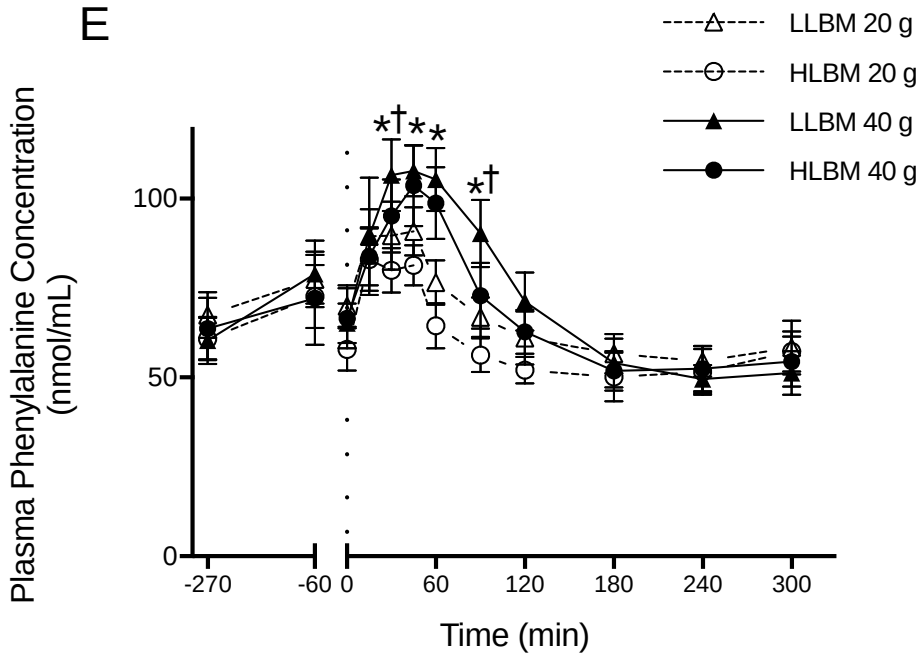
Plasma threonine concentrations peaked at 45 min and were elevated with 40WP compared with 20WP in both groups at 30 (d=1.04; CI=0.5 to 1.58), 45 (d=1.09; CI=0.54 to 1.63), 60 (d=1.54; CI= 0.96 to 1.93), 90 (d=1.37; CI= 0.81 to 1.93) and 120 min (d=1.22; CI=0.66 to 1.77) (time × dose interaction $p<0.01$) (Figure 5.3G). Plasma threonine concentrations were higher in LLBM compared with HLBM (main effect of group; p=0.022) but no interactions were observed. Effect sizes at 30 (d=0.71; CI=0.18 to 1.23) and 90 min (d=0.65; CI=0.13 to 1.17) were considered medium to large. Plasma threonine AUC was 2.6 fold greater with 40WP compared with 20WP (d=1.68; CI=1.09 to 2.27) and with 40WP plasma threonine AUC was 1.5 fold greater

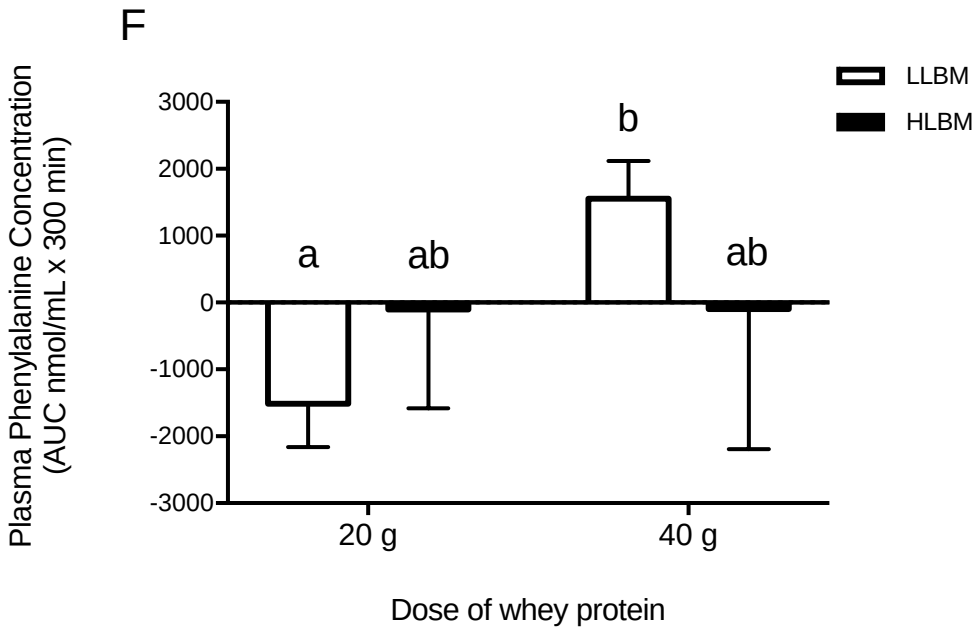
in LLBM than HLBM (d=0.96; CI=0.20 to 1.71) (dose × group interaction; p=0.005) (Figure 5.3H).




C
LLBM 20 g
HLBM 20 g
LLBM 40 g
HLBM 40 g
Plasma Leucine Concentration (nmol/mL)
600
400
200
0
-270
-60
0
60
120
180
240
300
Time (min)
§
*
*†
*


D
LLBM
HLBM
Plasma Leucine Concentration (AUC nmol/mL x 300 min)
50000
40000
30000
20000
10000
0
a
a
b
c
20 g
40 g
Dose of whey protein


E
LLBM 20 g
HLBM 20 g
LLBM 40 g
HLBM 40 g
Plasma Phenylalanine Concentration (nmol/mL)
100
50
0
*†
*†
*
-270
-60
0
60
120
180
240
300
Time (min)


F
LLBM
HLBM
Plasma Phenylalanine Concentration (AUC nmol/mL x 300 min)
3000
2000
1000
0
-1000
-2000
-3000
a
ab
b
ab
20 g
40 g
Dose of whey protein

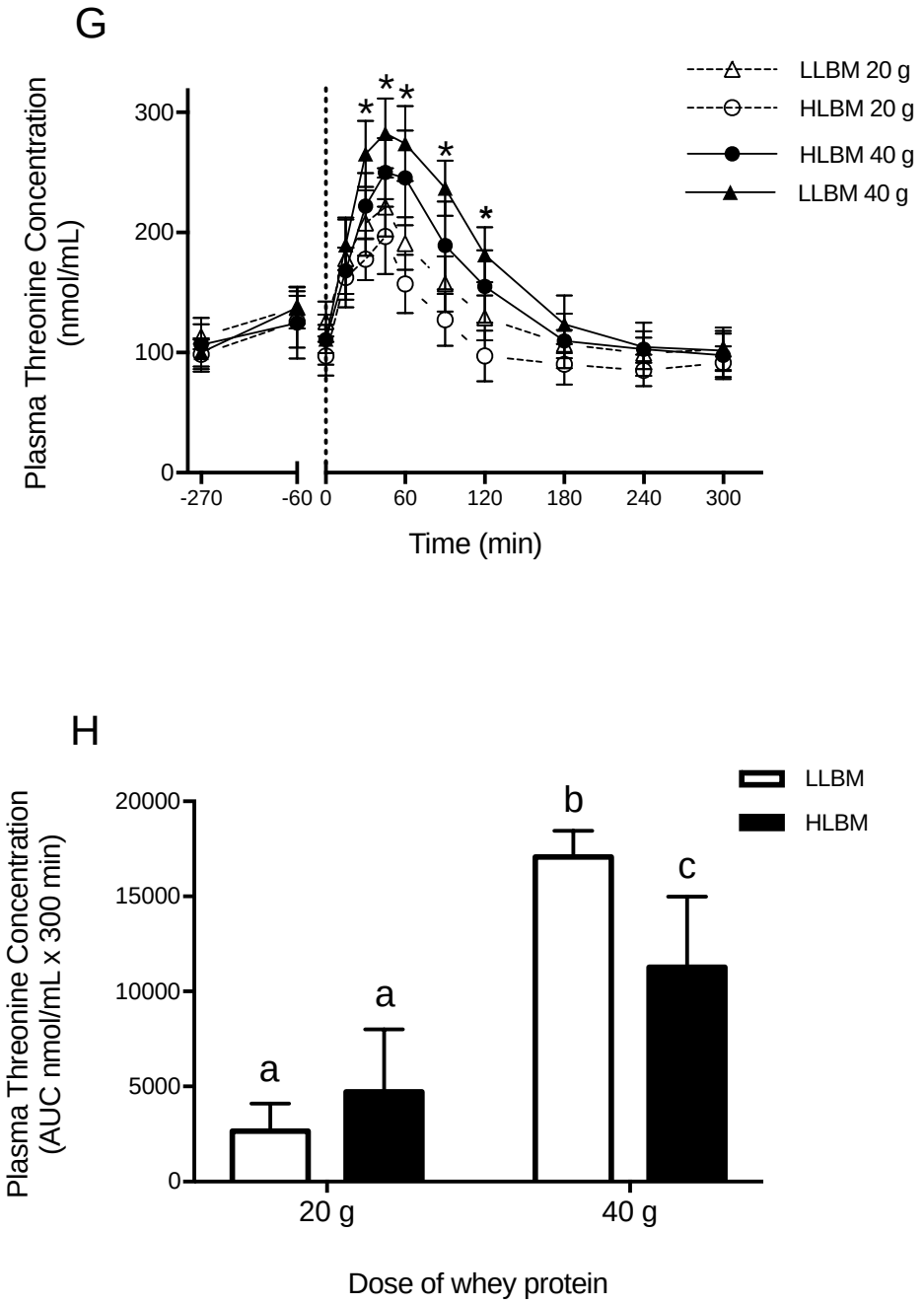
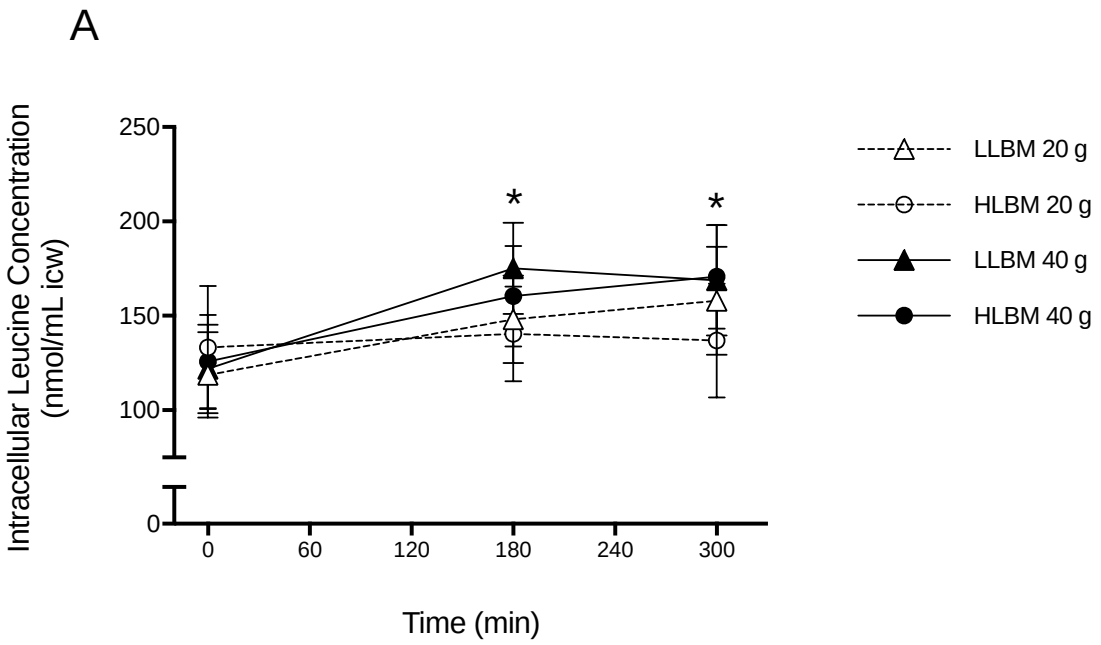


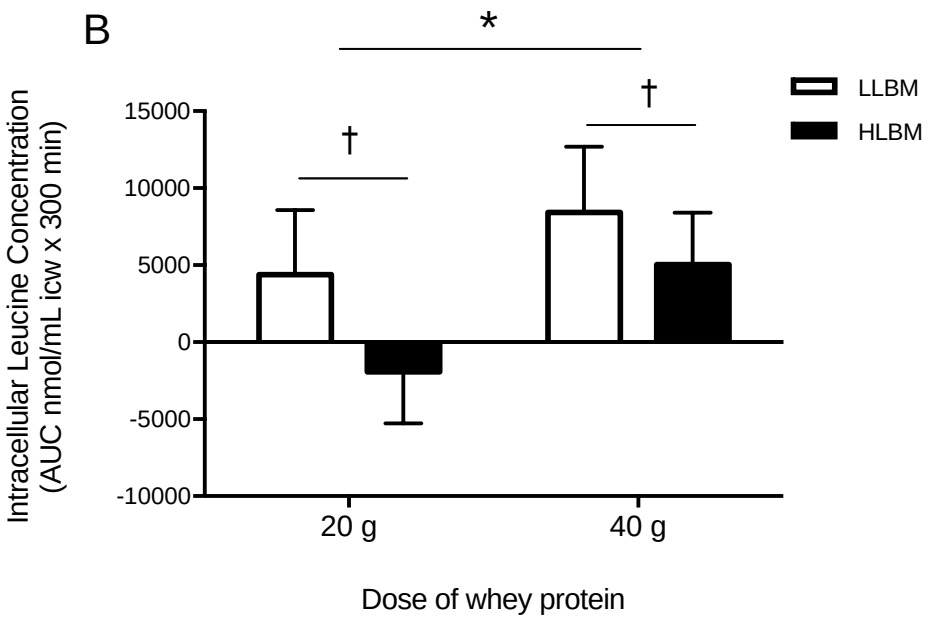
Figure 5.3 - Plasma insulin (A-B), leucine (C-D), phenylalanine (E-F) and threonine (G-H) concentrations following ingestion of either 20 or 40 g of whey protein isolate in both the lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. Data presented as means with 95% confidence intervals. Data expressed over time (A, C, E and G) and as area under the curve (B, D, F, and H). * denotes a significant difference between doses, † denotes a significant difference between groups, means with a different letter are significantly different from each other. A - dose × time interaction $p<0.001$, * $p<0.05$; B - main effect of dose * $p<0.001$ C - group × dose × time interaction $p=0.048$, * all $p<0.020$, § significant difference between doses in LLBM group only, † $p=0.012$; D - dose × group interaction $p=0.039$; E - dose × time $p<0.001$ and time × group interactions $p<0.021$, * $p<0.050$, † $p<0.050$; F - significant dose × group interaction $p=0.022$; G - dose × time interaction $p<0.001$ and main effect for group $p=0.022$, * $p<0.050$; H - dose × group interaction $p=0.005$.

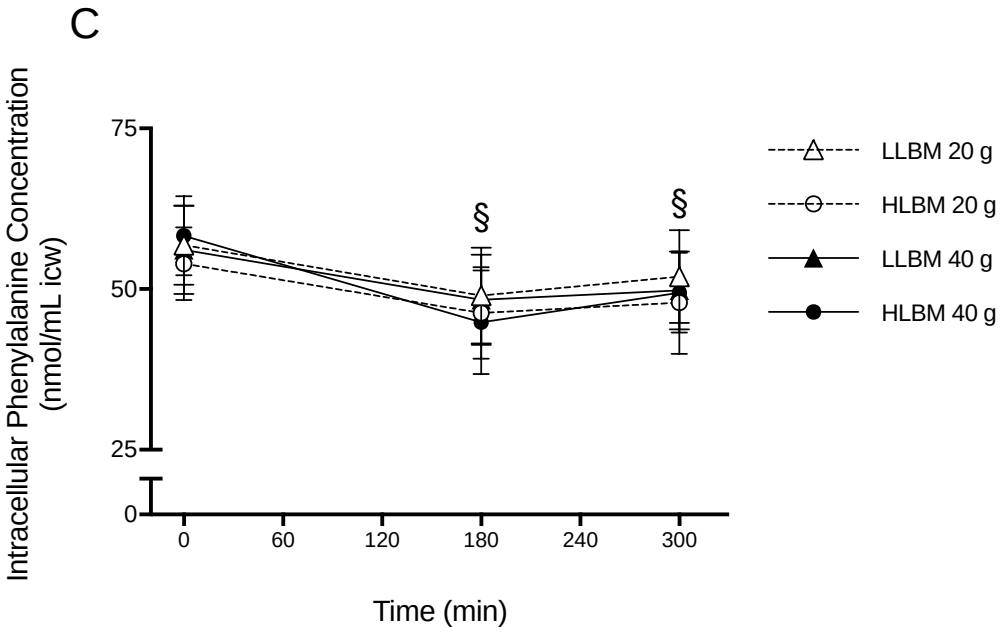
5.3.2. Intracellular amino acid concentrations

IC leucine concentrations were greater with 40WP compared with 20WP at 180 (d=0.57; CI=0.05 to 1.09) and 300 min (d=0.65; CI=0.13 to 1.17) (dose × time interaction; p=0.005) (Figure 5.4A). IC leucine AUC was 3.1 fold greater with 40WP than 20WP (main effect of dose; p=0.001; d=0.82; CI=0.30 to 1.35) and greater in LLBM than HLBM group (main effect of group; p=0.012; d=0.57; CI=0.05 to 1.08) (Figure 5.4B).

There were no differences between groups or doses in IC phenylalanine concentrations (Figure 5.4C). IC phenylalanine concentrations were lower at 180 and 300 min compared with 0 min (main effect of time; p<0.001) and AUC was negative for all groups (Figure 5.4D).




B
*
†
†
LLBM
HLBM
Intracellular Leucine Concentration
(AUC nmol/mL icw x 300 min)
15000
10000
5000
0
-5000
-10000
20 g
40 g
Dose of whey protein


C
Intracellular Phenylalanine Concentration
(nmol/mL icw)
75
50
25
0
§
§
0
60
120
180
240
300
Time (min)
LLBM 20 g
HLBM 20 g
LLBM 40 g
HLBM 40 g

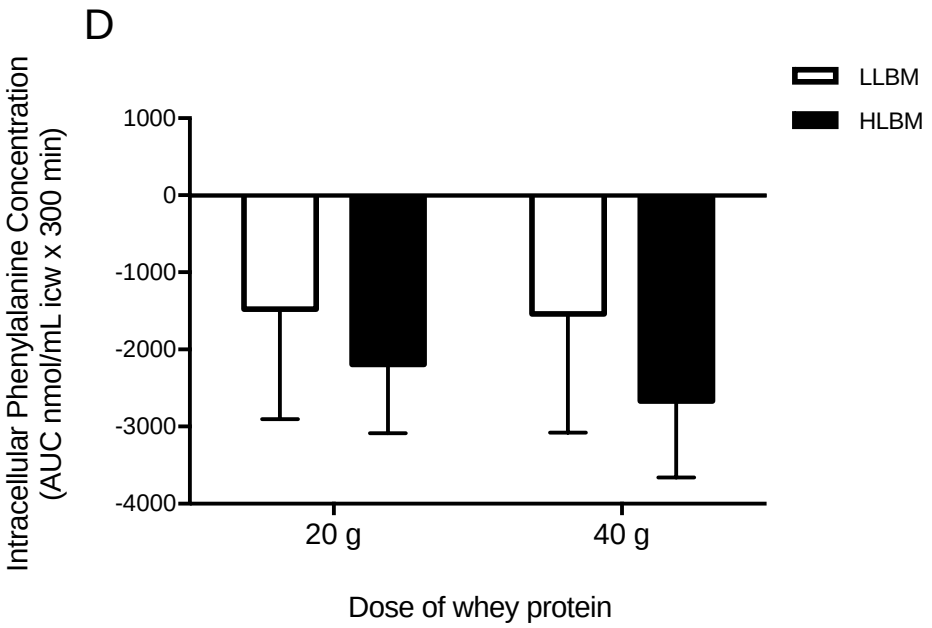


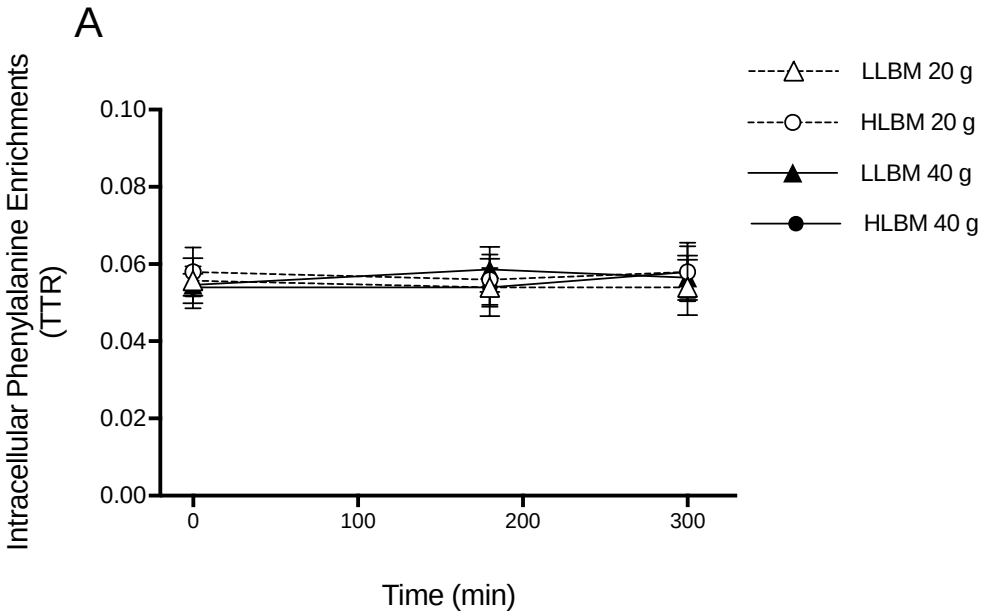
Figure 5.4 - Intracellular leucine (A-B) and phenylalanine (C-D) concentrations following ingestion of either 20 or 40 g of whey protein isolate in both the lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. Data presented as means with 95% confidence intervals. Data expressed over time (A and C) and as area under the curve (B and D). A - dose × time interaction p=0.005 * significant difference between doses; B - main effect of dose *p=0.001 and main effect of group † p=0.012 C - main effect of time p<0.001 § significantly different from 0 min.

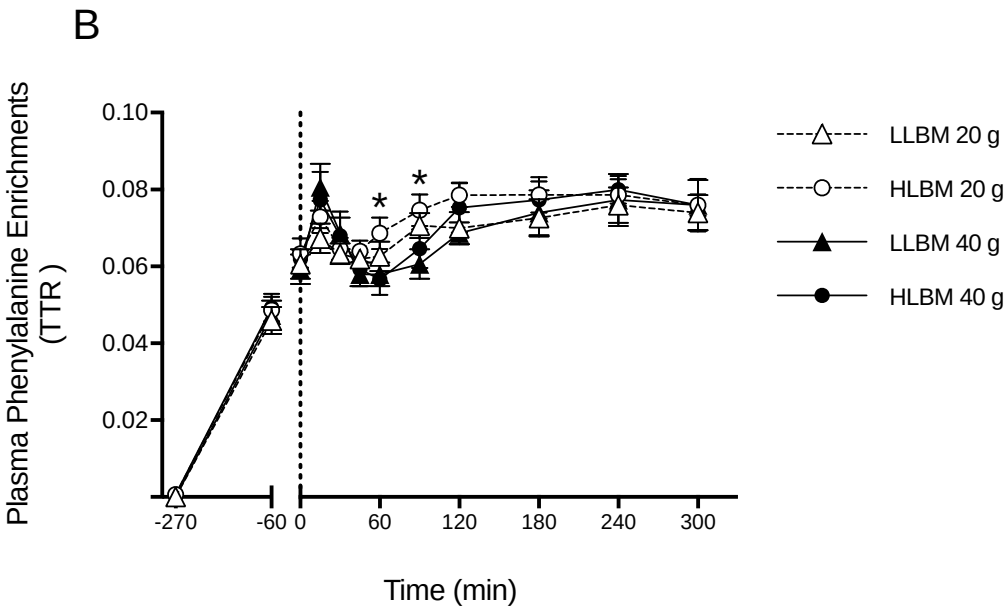
5.3.3. Tracer enrichments

No differences were observed in IC phenylalanine enrichments between groups or doses or across time. Moreover, IC phenylalanine enrichments did not deviate from 0 (p=0.808); therefore the participants were deemed to be in an isotopic steady-state of phenylalanine enrichment (Figure 5.5A). Plasma phenylalanine enrichment fluctuated slightly following protein ingestion (Figure 5.5B). Nevertheless, FSR was calculated using plasma phenylalanine enrichment AUC and the responses were not different from FSR calculated with IC phenylalanine.

Plasma tyrosine enrichments were elevated to a greater extent with 40WP compared with 20WP at 15 min post ingestion (d=0.66; CI=0.14 to 1.18) (dose × time interaction; p=0.011) (Figure 5.5C). Although not statistically significant, the effect

size between doses at 30 min (d=0.63; CI=0.11 to 1.15) was similar to the 15 min time point.





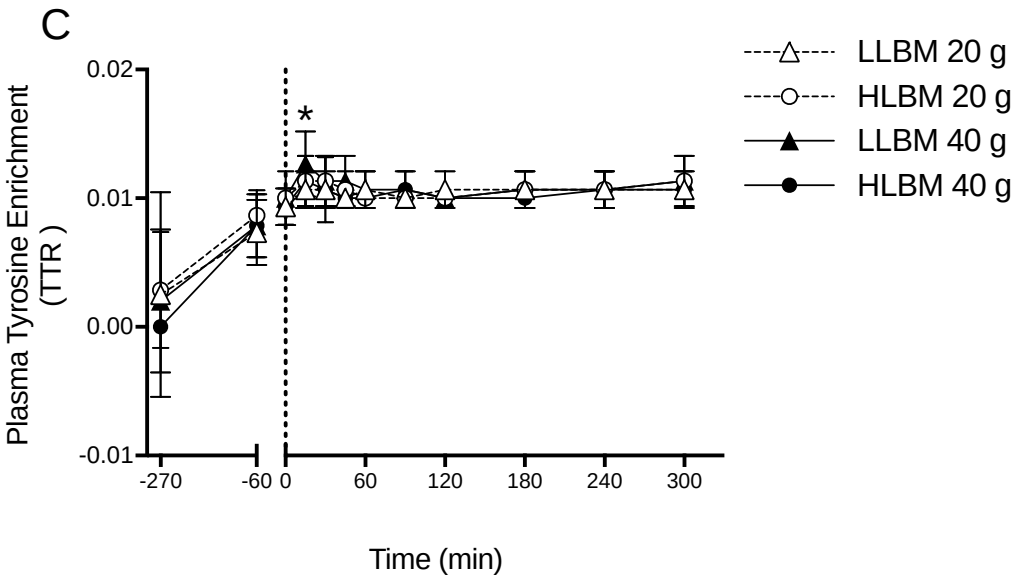


Figure 5.5 - Muscle intracellular (A) and plasma (B) phenylalanine enrichments and plasma tyrosine enrichments (C) expressed over time during L- [ring-13C6] phenylalanine infusion in both the lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. Data presented as means with 95% confidence intervals. Data expressed as tracer to tracee ratio (TTR). Ingestion of either 20 or 40 g whey protein isolate occurred at 0 min. B - dose × time interaction $p<0.001$; C - dose × time interaction $p=0.011$, * significant difference between doses $p<0.050$.

5.3.4. Plasma urea concentrations

Plasma urea concentrations were greater with 40WP compared with 20WP at 120 (d=0.66; CI=0.14 to 1.18), 180 (d=0.55; CI=0.03 to 1.06), 240 (d=0.66; CI=0.14 to 1.18) and 300 min (d=0.58; CI=0.07 to 1.10) (dose × time interaction; $p<0.001$) (Figure 5.6A). Plasma urea AUC was greater with 40WP than with 20WP for plasma urea expressed as AUC (main effect of dose; p=0.002, d=0.48; CI= -0.03 to 0.99) and LLBM was greater than HLBM (main effect of group; p=0.047; d=0.68; CI=0.16 to 1.20) (Figure 5.6B). Since all effect sizes were positive, LLBM was greater than HLBM with medium to large effect sizes, although not statistically significant, at 90 (d=0.63; CI=0.11 to 1.15), 120 (d=0.65; CI=0.13 to 1.18), 180 (d=0.77; CI=0.24 to 1.29), 240 (d=0.76; CI=0.24 to 1.28) and 300 min (d=0.71; CI=0.18 to 1.23). The effect sizes at 180, 240 and 300 min were greater than the effect size observed for plasma urea AUC between groups.

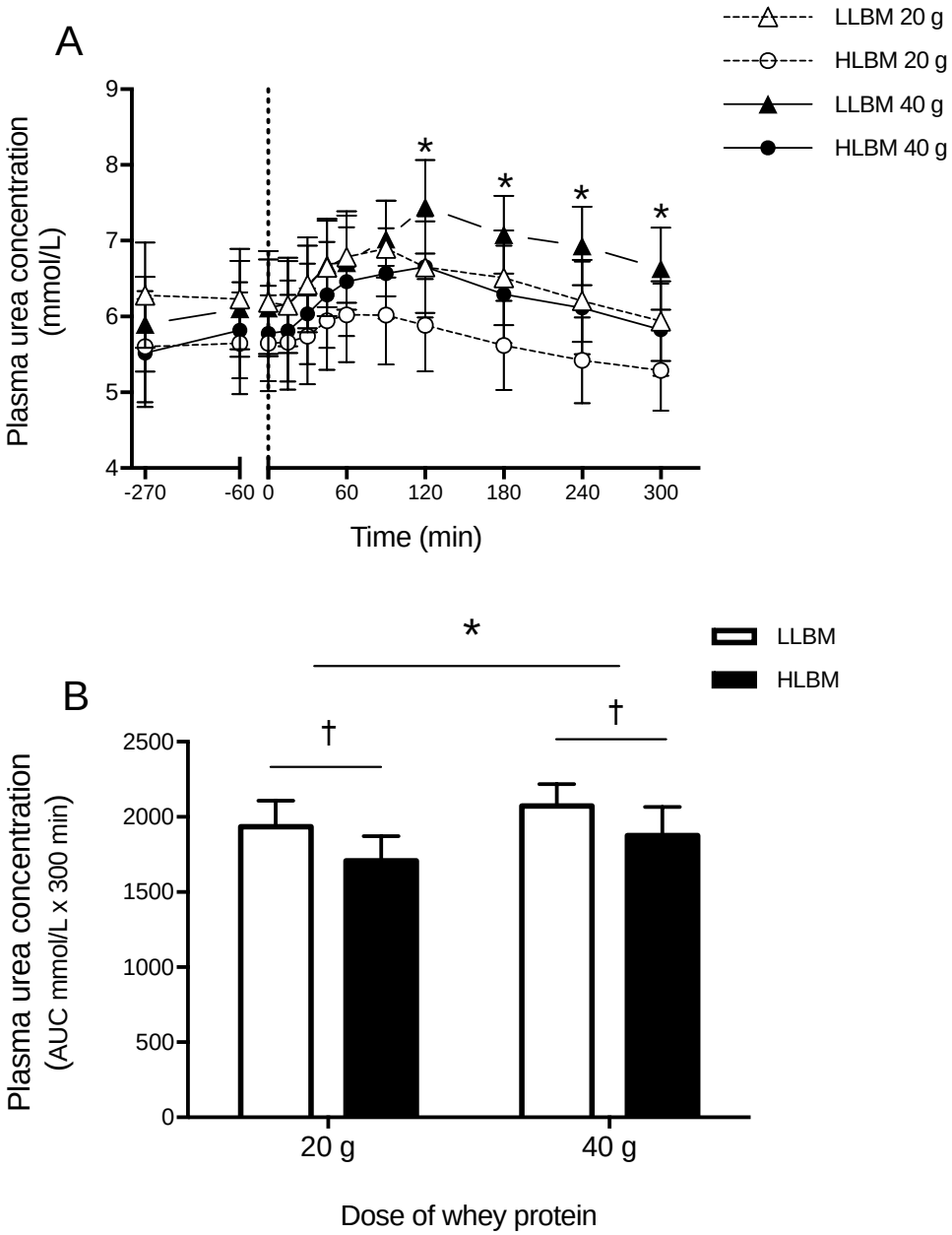


Figure 5.6 - Plasma urea concentrations following ingestion of either 20 or 40 g of whey protein isolate in both the lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. Data presented as means with 95% confidence intervals. A – data expressed over time - dose × time interaction ($p<0.001$), * significant difference between doses all $p<0.05$; B - area under the curve - main effect of dose * $p=0.002$ and group † $p=0.047$.

5.3.5. Phenylalanine oxidation

The rates of phenylalanine oxidation were greater with 40WP compared with 20WP at 60 (d=1.35; CI=0.78 to 1.91) and 90 min (d=1.51; CI=0.93 to 2.08) (dose × time interaction; $p>0.001$) (Figure 5.7A). There was a moderate effect between doses at 45 min (d=0.62; CI=0.11 to 1.14) but this effect was not statistically significant.

Phenylalanine oxidation AUC was greater in 40WP compared with 20WP (main effect of dose; p<0.001; d=0.56; CI=0.05 to 1.08) (Figure 5.7B) but there were no differences between groups (p=0.068; d=0.54; CI=0.03 to 1.06). However, the effect size for the groups for phenylalanine oxidation were between medium and large at 0 (d=0.61; CI=0.09 to 1.13), 90 (d=0.53; CI=0.01 to 1.04), 120 (d=0.79; CI=0.26 to 1.31) and 180 min (d=0.62; CI=0.10 to 1.14).

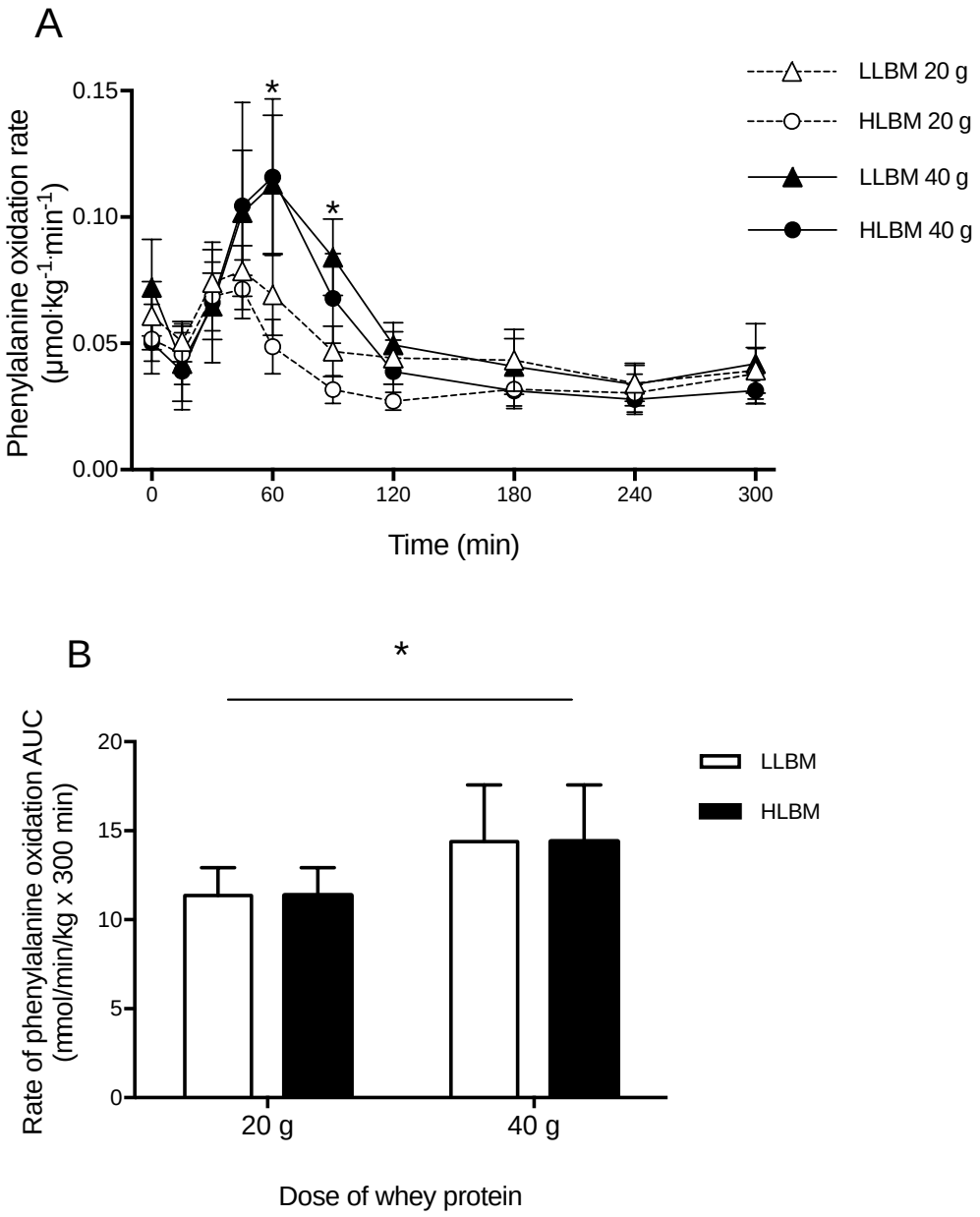
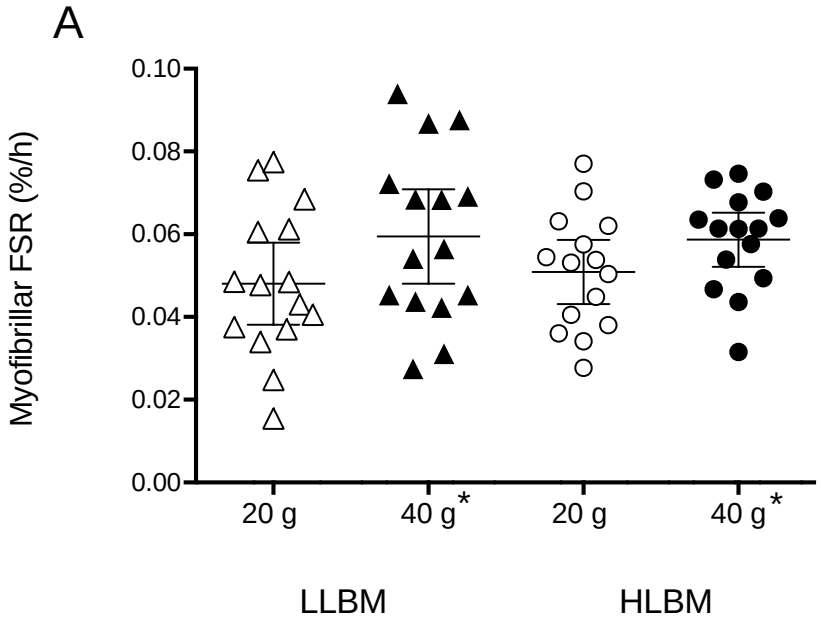


Figure 5.7 - Rate of phenylalanine oxidation following ingestion of either 20 or 40 g whey protein isolate in lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. Data presented as means with 95% confidence intervals. A - data expressed over time – dose

× time interaction p<0.001, * significant difference between doses all p<0.002; B area under the curve – main effect of dose * p=0.001

5.3.6. Myofibrillar muscle protein synthesis

Myofibrillar FSR was higher with 40WP (0.059 ± 0.017%·h^{-1}) compared with 20WP (0.049 ± 0.016%·h^{-1}) following whole-body resistance exercise, irrespective of group or time (Figure 5.8A-C) (main effect of dose p=0.005). Individual data and means for the 0-300 min recovery period are presented in Figure 5.8A (d=0.59; CI=0.08 to 1.11). The early post-exercise recovery period (Figure 5.8B) (0-180 min; d=0.53; CI=0.02 to 1.05) and overall recovery period had medium to large effect sizes while the later recovery period (Figure 5.8C) (180-300 min; d=0.50; CI=-0.02 to 1.01) had a lower effect size. There were no differences between groups at any point in the 300 min recovery period. (20WP - LLBM: 0.048 ± 0.018%·h^{-1}; HLBM: 0.051 ± 0.014%·h^{-1}; 40WP - LLBM: 0.059 ± 0.021%·h^{-1}; HLBM: 0.059 ± 0.012%·h^{-1}) Percentage differences in myofibrillar FSR between 20WP and 40WP are displayed in Table 5.3. There was no significant difference between groups for % difference in myofibrillar FSR.



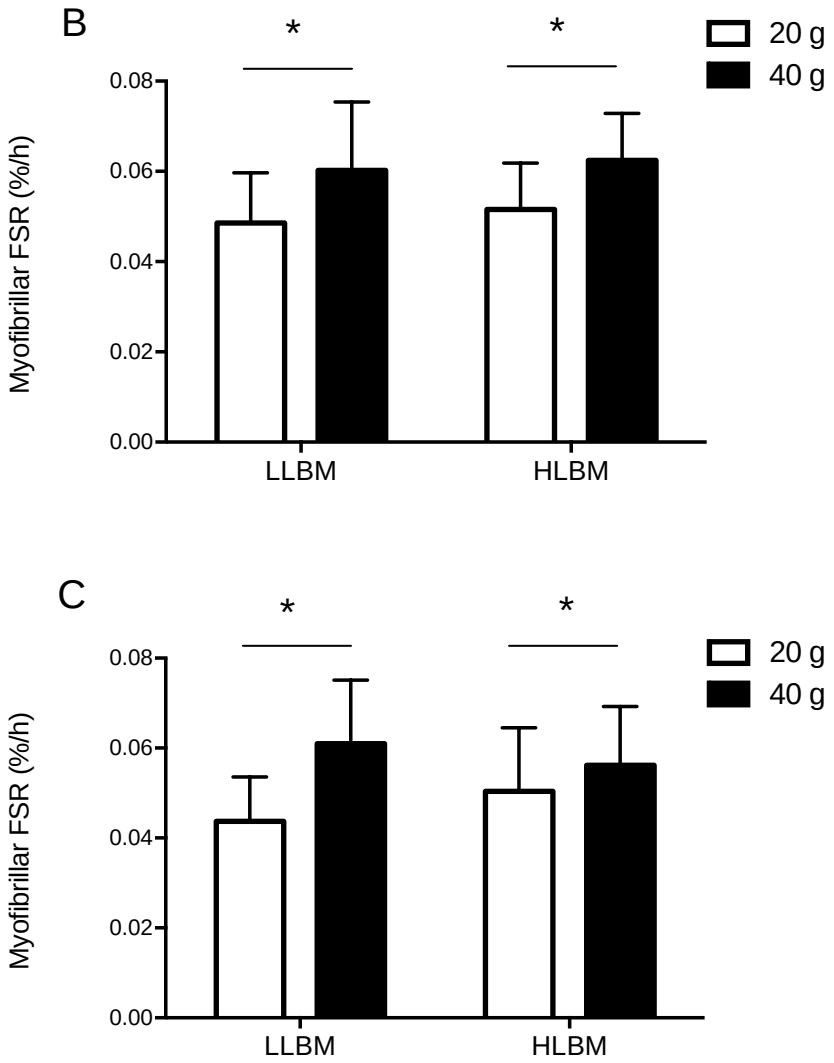


Figure 5.8 - Myofibrillar fractional synthesis rate (FSR) following ingestion of either 20 or 40 g whey protein isolate in lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. A - data presented as individual values with means and 95% confidence intervals from 0-5 h post protein ingestion, main effect of dose * p=0.005. Data presented as means with 95% confidence intervals. B - 0-3 h and C - 3-5 h main effect of dose observed * p=0.005.

Table 5.3 - Percentage difference in myofibrillar FSR between ingestion of 20 and 40 g whey protein isolate.

	0-180 min	180-300 min	0-300 min
LLBM	16.2 ± 60.1%	30.5 ± 52.2%	21.5 ± 45.6%
HLBM	20.2 ± 51.4%	13.1 ± 54.4%	15.5 ± 28.3%
Both groups	18.2 ± 55.0%	21.7 ± 53.1%	18.5 ± 37.4%

Values are means ± SD. Percentage change calculated from means of individual change.

5.3.7. P70S6K1 activity

The activity of p70S6K1 was higher in LLBM than HLBM regardless of time or dose (main effect of group; p 0.002) although this difference appeared to be primarily driven by the 180 min time point (Figure 5.9). The effect sizes for each time point were as follows; 0 min d=0.02; CI=-0.49 to 0.53; 180 min d=0.48; CI=-0.03 to 0.99; 300 min d=-0.08; CI -0.58 to 0.43. The activity of P70S6K1 was greater at 180 min compared with 0 min (main effect of time; p=0.008) but there were no differences in p70S6K1 activity between 0 and 300 min or 180 and 300 min.

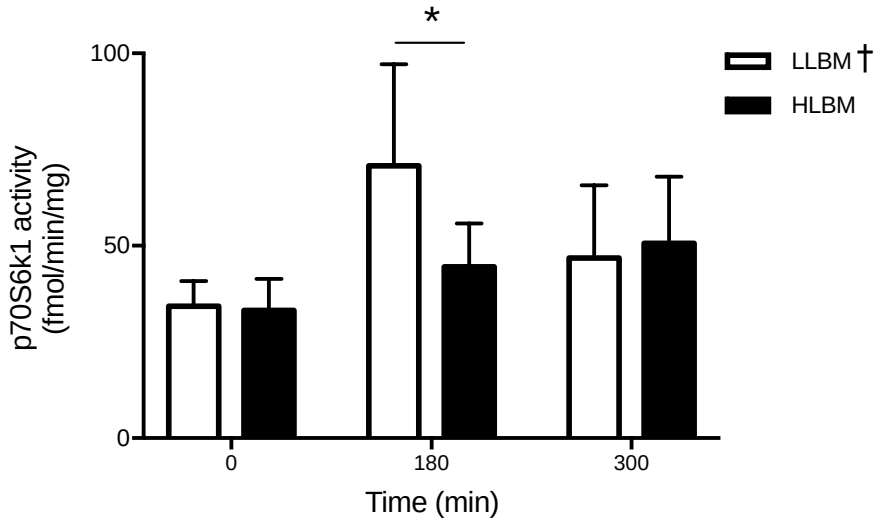


Figure 5.9 - P70S6K1 activity following whey protein isolate ingestion (doses are combined as no effect of dose) in both the lower lean body mass (LLBM) and higher lean body mass (HLBM) groups. Data presented as means with 95% confidence intervals. Main effect of group observed † p=0.002 and main effect of time p=0.008, * significantly different from 0 min.

5.4. Discussion

The primary aim of the present study was to investigate the influence of LBM on the dose of protein required for maximal stimulation of MPS following resistance exercise in trained young males. We hypothesised that those with greater LBM would require more than 20 g of protein to achieve greater stimulation of MPS following resistance exercise. Our novel findings demonstrated that, overall, ingestion of a 40 g dose of whey protein isolate stimulated myofibrillar MPS to a greater extent than a 20 g dose

during acute exercise recovery (0-5 h). However, contrary to our hypothesis, the response of MPS following whole body resistance exercise was similar in both groups of resistance trained males despite a mean difference of 17 kg in LBM between groups.

The general consensus within the scientific and popular literature is that 20-25 g of protein consumed post resistance exercise is sufficient to maximally stimulate MPS (Churchward-Venne et al., 2012a; Morton et al., 2015). Previous protein dose studies reported no difference in MPS between 20 and 40 g of protein in young men following resistance exercise (Witard et al., 2014; Moore et al., 2009). However, in the present study we demonstrated that ingestion of 40 g of whey protein significantly increased myofibrillar MPS compared with ingestion of 20 g of whey protein. The reason our results and previous results do not agree cannot be definitively determined, but there are methodological differences between the three studies. We believe the most likely explanation for the difference in response of MPS to resistance exercise and protein ingestion is the amount of muscle that was activated during the exercise bout. In the current study participants performed a bout of whole body resistance exercise but in previous studies leg only resistance exercise was performed (Moore et al., 2009; Witard et al., 2014). Why might the amount of muscle exercised have an effect on MPS? We suggest that the overall demand for amino acids following a bout of whole body resistance exercise is greater than the demand following a bout of unilateral or bilateral resistance exercise. Resistance exercise sensitises skeletal muscle to amino acids (Biolo et al., 1997). Consequently, MPS is higher in response to resistance exercise followed by protein feeding compared with feeding alone (Witard et al., 2014; Pennings et al., 2011). Also, resistance exercise increases amino acid transport

and uptake into the muscle (Biolo et al., 1995b). Therefore, the greater the amount of muscle activated, the greater the amino acid uptake after feeding. Pennings, et al., (2011) used intrinsically labelled protein to demonstrate that *de novo* MPS from exogenous amino acids was greater in exercised than rested muscle. The authors demonstrated that the amino acids from ingested protein are being used directly to create new muscle proteins. Furthermore, nutritive blood flow increases following exercise (Biolo et al., 1995b) and facilitates the delivery of amino acids to the working muscle. Increased blood flow following exercise facilitates the subsequent increased requirement of amino acid delivery. Additionally, blood flow is reduced to any given muscle when other muscles are activated compared to when one muscle group alone is exercised (Volianitis et al., 2003), thus reducing amino acid delivery to any particular muscle. The muscles' demand for amino acids must be met for maximal MPS stimulation to occur. In the 20 g condition, following whole body resistance exercise, we propose there was insufficient amino acid provision to meet the demands of the exercised muscle so MPS in the measured muscle is lower. Conversely, in the 40 g condition there were more amino acids available for all the exercised muscles and MPS was higher.

Although we believe the amount of muscle exercised is the most likely explanation for the differences in results observed between this study and previous studies (Moore et al., 2009; Witard et al., 2014), alternative non-physiological explanations must be considered. One alterative explanation may be the sample sizes. There was a ~10% mean difference in MPS following ingestion of 20 and 40 g of whey protein in both previous studies. Moore et al., (2009) included six participants in a cross-over design, whereas Witard et al., (2014) recruited twelve participants per group in a parallel

design. The current study included fifteen participants in a cross-over design in each of two groups, resulting in n=30 for each dose. Due to the difference in sample size it is not unreasonable to suggest that Moore et al., (2009) and Witard et al., (2014) may have been slightly underpowered. However, as Moore et al., (2009) and Witard et al., (2014) both observed a difference of ~10% in MPS between the 20 and 40 g conditions and we detected a difference of ~20% there appears a genuine difference in the MPS response to whole body resistance exercise plus protein ingestion and lower limb resistance exercise plus protein ingestion. A second explanation may relate to differences in the type of protein ingested following exercise. The participants in Moore et al., (2009) ingested egg protein, whereas, in the current study and Witard et al., (2014) whey protein was consumed. Ingestion of proteins with different amino acid profiles results in differential MPS stimulation (Tang et al., 2009). The amino acid profiles of whey and egg protein do differ (Moore et al., 2009; Witard et al., 2014) but the influence of these proteins on MPS has not been compared directly. Therefore, it is unclear whether the type of protein ingested altered the MPS response to resistance exercise and feeding. Finally, the protein fraction measured differed between the studies. Myofibrillar MPS was measured in this study and Witard et al., (2014) whereas Moore et al., (2009) measured mixed MPS. Protein synthesis of myofibrillar and other protein fractions have been known to respond differently to the same resistance exercise protocol (Burd et al., 2012). However, the results from Witard et al., (2014) and Moore et al., (2009) agree despite the methodological dissimilarities, *i.e.,* type of protein and protein fraction, between the studies. Accordingly, these differences are unlikely to explain the discord between the results from the previous studies and the present one. Therefore, the disparity in results between our study and the previous research mostly likely are attributed to

the whole body exercise performed.

The whole body exercise bout also may offer some explanation for the lower MPS values observed in the current study compared to the previous dose-response studies (Moore et al., 2009; Witard et al., 2014). It is difficult to directly compare MPS values between studies with different participants and analysis performed in different laboratories at different times. Nonetheless, the MPS values that we recorded following resistance exercise and protein feeding were ~25-40% lower than those reported previously. Due to the greater demand for amino acids following whole body exercise, we propose that the increase in MPS in the *vastus lateralis* is lower. This lower rate of MPS in the *vastus lateralis* could suggest that a 40 g dose of protein is not sufficient to maximally stimulate myofibrillar MPS following whole body exercise. Hence, unless there was sufficient amino acid provision to all the muscles following whole body exercise, we contend that rates of MPS similar to those of the previous studies would not be observed. From the observations made in the present study we can conclude that 40 g of whey protein stimulates myofibrillar MPS to a greater extent than 20 g following whole body exercise. However, it is not possible to conclude that 40 g of protein maximally stimulates MPS during acute recovery from whole body resistance exercise and further study is warranted to determine that dose. Therefore, it seems that whole body exercise influences the amount of protein required to maximally stimulate MPS.

The dose of protein necessary for maximal stimulation of MPS following resistance exercise often has been thought to be greater for those with a greater amount of LBM. Our study is the first to directly address whether the amount of LBM influences the MPS response to resistance exercise combined with protein feeding. Whereas, we did

not observe any influence of the amount of LBM on the MPS response, we did observe that 40 g of protein stimulated MPS to a greater extent than 20 g following whole body resistance exercise. We believe the doses in the current study are not sufficient to highlight differences between groups because the demand for amino acid provision is not being met in all skeletal muscle. Exercise has been shown to elicit a rightward shift in the dose-response relationship of MPS to ingested protein at increasing (0-40 g) doses of whey protein (Witard et al., 2014). We believe that the demand for amino acids in the current study is greater than in previous studies (Moore et al., 2009; Witard et al., 2014) because a larger amount of muscle is being exercised. The additional protein in the 40 g condition is simply meeting these demands in both groups. Differences between groups may only become apparent when the amino acid demands of all the skeletal muscle in the LLBM group have been met. At this dose the amino acid demands in the HLBM will not yet have been fulfilled because participants in this group possess more LBM. A higher dose of protein would be required to meet the amino acid demand in a larger amount of muscle when more of that muscle has been activated, *e.g.,* following whole body exercise. Consequently, we maintain that those with higher LBM may require a higher protein dose to achieve maximal stimulation of MPS following whole body exercise.

The fate of the ingested amino acids also could provide some explanation as to why we did not observe differences between groups. The proportion of dietary amino acids released into the circulation and available for MPS at rest is thought to be ~50-70% while the remainder is retained in the splanchnic bed (Groen et al., 2015; Volpi et al., 1999). Pennings et al., (2011) used intrinsically labelled protein to demonstrate that the amount of amino acids from ingested protein that appeared in the circulation

did not differ between rest and exercise conditions. In previous work only lower limb exercise was performed, therefore it is not known how whole body resistance exercise would affect the digestion and absorption kinetics of amino acids. Consequently, there could be a greater demand for blood flow to the skeletal muscle, directing blood away from the splanchnic bed and therefore reducing the efficiency of amino acid extraction. Furthermore, it is unclear whether the amount of protein ingested would change amino acid absorption kinetics. Plasma amino acid concentrations were higher in LLBM than HLBM when 40 g of whey protein is ingested suggesting there could be an upper limit or capacity of the splanchnic bed for amino acid uptake. It is possible this limit has been reached with 40 g in the LLBM but not the HLBM group, resulting in the release of excess amino acids into the circulation and increasing amino acid availability in the LLBM but not the HLBM group. Amino acid kinetics may vary between LLBM and HLBM groups masking differences that may be present in the MPS response at different doses. Alternatively, higher doses of protein may be required following whole body resistance exercise before differences are observed between those with lower and higher LBM.

Our study is the first to examine directly the influence of the amount of LBM on the MPS response to resistance exercise and protein feeding. Moore et al., (2014) reported the amount of protein necessary to stimulate maximally MPS relative to the amount of LBM at rest, but no determination of the influence of LBM can be made from those data. The study was done retrospectively and by making the dose relative to LBM, the authors actually removed the influence of LBM. Moreover, no MPS data following exercise were reported. Consequently, direct comparisons cannot be made with our results. Witard et al., (2014) observed a significant correlation between LBM

and MPS during their study and that underpinned the basis of the current study. Further research investigating the influence of LBM on the protein synthetic response is required both with whole body exercise and higher protein doses and with uni/bilateral exercise and similar protein doses.

Extracellular (Bohé et al., 2003) and/or intracellular (Biolo et al., 1995b) amino acid concentrations previously have been shown to regulate MPS. However, these regulatory mechanisms did not appear to be entirely responsible for modulating MPS in our study. The differences in plasma amino acid concentrations observed between groups do not correspond to differences in MPS. Considering dose alone, the differences in plasma and intracellular concentrations are reflected in the differences observed in MPS. However, the differences in amino acid concentrations between groups at 40 g are not reflected in the MPS response. A possible explanation for this difference is that the maximum capacity of the muscle cell to dispose of free amino acids *via* incorporation into new muscle protein is being approached in the LLBM group, *i.e.,* the 'muscle full effect' (Atherton et al., 2010). However, there is an insufficient supply of amino acids in the HLBM group to further stimulate MPS. The plasma urea concentration data seem to support this idea to some extent. In the current study, plasma urea AUC was higher in the 40 g trial compared with 20 g and higher in LLBM compared to HLBM for both doses. These data suggest that in the 40 g trial and the LLBM group, some muscle reached maximum capacity for MPS, leading to disposal of excess amino acids. The phenylalanine oxidation data further support this notion with regard to dose only. Rates of oxidation were higher in the 40 than the 20 g trial, suggesting an excess of amino acids. Alternatively, the higher amino acid concentrations at 40 g in the LLBM compared with the HLBM group, could simply be

a consequence of lower body fluid volumes. Individuals with lower LBM have lower body fluid volumes (Boer, 1984). Consequently, plasma and IC amino acid concentrations would be higher in the LLBM, as the amount of amino acids (g) provided was equivalent in both groups. Larger differences in plasma and intracellular amino acid concentrations may be required before they translate to MPS changes and may not predict or influence MPS as much as previously thought.

The translation initiation step of MPS is regulated at the molecular level by the activity of intracellular signalling proteins. The mTORC1 signalling cascade is one of the main regulators of protein synthesis (Kimball and Jefferson, 2010). The signalling molecule p70S6K1 is a downstream target of mTORC1 and the enzyme's specific activity is increased in response to anabolic stimuli (McGlory et al., 2016; Apró et al., 2015a&b). We measured the activity of p70S6K1 in an attempt to gain information, at the molecular level, about the regulation of MPS in response to ingesting different doses of protein. There were no effects of dose on p70S6K1 activity, an effect not reflected in the MPS response. Furthermore, at 180 min p70S6K1 activity was 1.6 fold greater in the LLBM compared with the HLBM group. Our results are similar to Moore et al., (2009) who observed no increase in p70S6K1 phosphorylation at 4 h in response to resistance exercise and increasing doses of egg protein, despite an increase in MPS. Furthermore, the results support our findings in Chapter 4. Consequently, there appears to be a disconnect between the signalling pathways and MPS. The reason behind this disconnect in the current study is unclear but it could be due to less enzyme activity for the same MPS output, *i.e.,* an improved signalling efficiency. The HLBM group achieved the same stimulation of MPS as LLBM but with lower p70S6K1 activity. Increased efficiency of p70S6K1 has been observed

previously in our laboratory (McGlory et al., 2016). However, the disconnect between signalling and MPS is not a new concept (Atherton et al., 2010; Dreyer et al., 2006; Witard et al., 2009). Atherton et al., (2010) observed that p70S6K1 phosphorylation did not match the temporal pattern of MPS. Many studies that measure MPS are not primarily designed to measure anabolic signalling and it is possible we failed to detect the peak signalling response that occurred prior to our measurements at 180 and 300 min. Apró et al., (2015b) showed elevated p70S6K1 activity at 60 min following ingestion of a beverage containing leucine only or leucine+EAA. It is likely that by measuring p70S6K1 activity at 180 min we missed some of the response. However, Moore et al., (2011) observed that although the largest increase in p70S6K1 phosphorylation was at 1 h post resistance exercise and protein feeding, it was still elevated at 3 and 5 h. The MPS data were reported elsewhere (Moore et al., 2009b) but the authors state that the p70S6K1 response displayed a similar time course to MPS following resistance exercise and ingestion of a protein bolus. Leucine appears to be the main amino acid driving mTORC1 stimulation (Apró et al., 2015b). In the LLBM group plasma leucine concentration was higher than HLBM with 40 g of protein. It is possible that this higher plasma leucine concentration was driving the increase in p70S6K1 activity in the LLBM group, however this did not translate to elevated MPS. It also is possible that a threshold exists and only a certain amount of p70S6K1 activity is required to increase MPS. Indeed work by Crozier et al., (2005) suggest further p70S6K1 activation, after a certain threshold, does not potentiate the MPS response. Consequently, once this level has been reached any further increase in activity will not result in the increased stimulation of MPS. Therefore, it is not surprising that differences in p70S6K1 activity do not directly correspond with differences in MPS in the present study.

In summary, our data show that ingestion of 40 g whey protein results in greater stimulation of MPS than 20 g whey protein following whole body resistance exercise. We infer that whole body resistance exercise alters the dynamics of protein feeding compared with exercising a smaller amount of muscle; as is the case with lower limb exercise. LBM does not influence the MPS response to whole body resistance exercise and protein feeding, at least at doses below 40 g of whey protein. We can conclude that more protein is necessary for greater stimulation of MPS following whole body compared with unilateral or bilateral resistance exercise. Further study is required to identify a maximal stimulatory protein dose for MPS following whole body resistance exercise and to further explore if this maximal dose is influenced LBM.

CHAPTER 6 General discussion and concluding remarks

The aim of this thesis was to explore and better understand the relationship between LBM and protein feeding. The aim was addressed from an applied perspective with trained athletes, at whole body level and at both the metabolic and molecular level of muscle. Successful completion of the following objectives resulted in achievement of this thesis' aim:

i. To assess and compare current nutritional practices and body composition of young rugby union players at two different playing standards (Chapter 2).
ii. To investigate the change in dietary habits of young elite rugby union players in different environments (Chapter 3).
iii. To determine whether body composition is maintained over a period of international rugby union competition (Chapter 3).
iv. To examine the signalling response that underpins the MPS response to protein feeding and resistance exercise (Chapter 4 and 5).
v. To evaluate the influence of LBM on the MPS response to protein dose following whole body resistance exercise (Chapter 5).

The main findings of this thesis have been discussed within the experimental chapters as follows:

i. Young elite young rugby union players have greater total body mass, LBM and consume more protein compared with young amateur players.
ii. Over a period of international rugby union competition body composition was unchanged. Dietary habits changed when players were in camp - in particular protein intake increased.
iii. P70S6K1 activity is greater following resistance exercise and feeding compared with feeding alone.

iv. P70S6K1 activity does not alter in response to ingestion of higher protein doses. The degree with which p70S6K1 activity regulates MPS is minimal.

v. Following whole body resistance exercise ingestion of 40 g of whey protein stimulates MPS to a greater extent than 20 g but there is no influence of total LBM at these doses.

6.1. Protein recommendations

Daily protein recommendations for athletes currently range from 1.4-1.8 g·kg^{-1} BM. Other guidelines recommend consumption of 20 g of high quality protein at each eating occasion spread throughout the day and following resistance exercise. At rest and following resistance exercise ingestion of 20 g of protein has been shown to maximally stimulate MPS (Moore et al., 2009; Witard et al., 2014) and these studies are the basis for some protein recommendations. For the rugby players in Chapter 2 the g·kg^{-1} BM recommendations would result in a protein intake of ~ 140-190 g per day. MacKenzie et al., (2015) reported that young Australian elite rugby union players had on average 5.6 eating occasions per day. Consuming 20 g of protein at each of these eating occasions would result in a total protein intake for the day of ~110 g. The data contained within this thesis challenge this recommendation under a certain set of conditions and indicate that not all athletes follow this recommendation. The elite and amateur rugby union players in Chapter 2 consumed on average 202 and 163 g respectively. Consequently, regardless of which recommendations we use, these athletes consumed more protein than is currently recommended. In Chapter 5 we demonstrated that more protein was required for greater MPS stimulation following whole body resistance exercise compared with previous studies using lower limb exercise. MPS was stimulated to a greater extent

following ingestion of 40 g of protein compared with 20 g of protein. Many athletes will perform training that involves their whole body. For example, compound and Olympic lifts engage many of the major muscle groups and would be considered whole body resistance exercise. Rugby players engage in whole body resistance exercise (Jones et al., 2016) and consume more protein than current recommendations as discussed above and in Chapter 2. Therefore, if a 40 g dose of protein replaces a 20 g dose in an individual's daily protein intake the total amount becomes 132 g based on an average of 5.6 eating occasions. This recommended amount of protein is closer to, but still below, the protein consumption of rugby union players presented in this thesis. The highest dose investigated in Chapter 5 was 40 g and it is possible that higher doses could stimulate MPS further following whole body exercise. Consumption of 20 g of protein may be sufficient to maximally stimulate MPS at rest but following whole body exercise 40 g of protein or more may be beneficial when trying to build or maintain LBM.

Protein intake recommendations are often made relative to body mass or LBM. Often it is proposed that those with higher body mass or LBM require more protein to achieve greater stimulation of MPS following resistance exercise, as discussed in Chapter 5. Based on data from previous studies, Moore et al., (2014) concluded that consumption of 0.24 $g \cdot kg^{-1}$ BM or 0.25 $g \cdot kg^{-1}$ LBM of protein is sufficient for maximal MPS stimulation in young individuals. However, the data used in the analysis were obtained from resting muscle only and not from exercised muscle. Witard et al., (2014) demonstrated that there is a difference in the MPS response between rested and exercised muscle. In Chapter 5 in the LLBM group the participants consumed, on average, ~0.26 $g \cdot kgBM^{-1}$ and 0.37 $g \cdot kgLBM^{-1}$ of whey protein for the 20 g dose and ~

0.52 g·kgBM^{-1} and 0.67 g·kgLBM^{-1} of whey protein for the 40 g dose. In the HLBM group the participants consumed, on average, ~0.2 g·kgBM^{-1} and 0.26 g·kgLBM^{-1} of whey protein for the 20 g dose and ~ 0.40 g·kgBM^{-1} and 0.52 g·kgLBM^{-1} of whey protein for the 40 g dose. These data demonstrate the response of MPS to protein feeding following exercise is different than protein ingestion alone. Furthermore, it shows that more protein is required following resistance exercise and that the guidelines are likely underestimating requirements as MPS in Chapter 5 was stimulated to a greater extent with the higher protein doses. There are currently no data that support the notion that athletes with more LBM require more protein to achieve greater MPS stimulation. Therefore, it is interesting that protein recommendations are often provided relative to body mass or LBM. There were differences in absolute protein intake between the elite and amateur rugby union players in Chapter 2. However, when protein intake was calculated relative to body mass and LBM no differences were observed. This finding suggests that larger players eat more protein than smaller players since elite players had more total body mass and LBM than amateur players. However, in Chapter 5 we observed no influence of total LBM on the MPS response to two protein doses consumed following whole body resistance exercise. From the results of this thesis larger athletes consume more protein than smaller ones, yet the MPS response did not differ between athletes with higher *vs*. lower LBM. Nevertheless, despite the fact we observed no differences between the LLBM and HLBM groups in Chapter 5, we still maintain that total LBM could influence the MPS response to protein dose under certain circumstances. The reason we believe we did not observe a difference in the MPS response between groups following ingestion of 20 and 40 g of protein is because of the additional demand for amino acids induced by whole body resistance exercise. There are

currently no scientific data to support the idea that larger athletes require more protein to get similar MPS stimulation in any given muscle, compared with smaller athletes, following resistance exercise. Although rugby union players from Chapter 1 consumed more protein (2.4-2.6 g·kg^{-1} BM daily) than the recommendations for athletes (1.2-1.7 g·kg^{-1} BM daily (Rodriguez et al., 2009)) the protein dose required following whole body resistance exercise is thus far unknown. Therefore, players should continue to consume their current protein intake as long as it causes them no health issues or decrements to their performance.

International rugby union players increased their protein intake from the home environment to the camp environment (Chapter 3). Players consumed ~190 g of protein at home and ~245 g in camp. This amount of protein equates, on average, to 34 g per serving at home and 43 g per serving in camp (based on 5.6 eating occasions per day) (MacKenzie et al., 2015). However, from Chapters 2 and 3 we know that protein distribution is skewed towards the noon and evening portions of the day. Unfortunately, we did not have enough information on the timing of protein intake in these studies so the amounts above are estimates. As long as a sufficient amount of protein is consumed at each sitting (>20 g) then it is unlikely to make a huge difference if more protein is consumed at one sitting compared to another. A skewed distribution may have an impact on LBM accretion if one of the eating occasions contains less than the required amount to stimulation MPS maximally. It is likely that players were already consuming sufficient protein at home and camp to stimulate MPS. However, both elite and amateur players were consuming more than 4 servings of 20 g (Areta et al., 2013) and 1 serving of 40 g (132 g; based on one whole body exercise session per day) yet there is a significant difference in LBM between the

groups. Elite players could have stimulated MPS with their training to a greater extent than amateur players and the protein consumed supported and optimised this training. Similarly, during the 6N (Chapter 3), the training is likely to have changed so optimal conditions for increasing LBM may not have been met but were sufficient for maintenance of LBM.

The activity of p70S6K1 plays an important, though minimal role in MPS regulation. If p70S6K1 is blocked there is a reduction in body mass of mice (Shima et al., 1998) and blocking the mTORC1 pathway prevents an increase in MPS in humans (Dickinson et al., 2011; Drummond et al., 2009). Furthermore, Terzis et al., (2008) demonstrated a strong correlation between p70S6K1 phosphorylation and increases in muscle mass. However, protein dose had no effect on p70S6K1 activity following both whole body and lower limb unilateral resistance exercise (Chapter 4 and 5). Conversely, MPS increased following ingestion of 20 g of protein compared with 10 g following bilateral lower limb resistance exercise (Moore et al., 2009). Additionally, MPS was greater following ingestion of 40 g of protein compared with 20 g following whole body exercise (Chapter 5). However, in both cases there was no increase in p70S6K1 phosphorylation or activity with increasing amounts of protein. Furthermore, p70S6K1 activity only explains ~20% of the variation in MPS following resistance exercise and protein feeding. Activity of p70S6K1 is integral for the stimulation of MPS, as discussed in Chapter 4, but it only contributes to some of the MPS response. It has been suggested previously that p70S6K1 must be stimulated to a certain threshold of activity in order to stimulate MPS and further p70S6K1 activation would not result in further stimulation of MPS (Crozier et al., 2005). We propose that this threshold of p70S6K1 activation to stimulate MPS may have been met by resistance

exercise alone. Accordingly, ingestion of protein does not further increase p70S6K1 activation as MPS has already been stimulated.

6.2 Limitations and future research

The findings of this thesis will contribute to the body of scientific knowledge and inform practice in applied settings. However, there are some limitations that should be considered. Working with elite athletes places constraints on the methodologies that can be used to collect data. Due to time constraints and concerns about compliance we opted to use 3 d food diaries in this body of work. Food diaries kept longer than 4 d result in respondent fatigue which reduces validity (Thompson and Subar, 2013). However, behaviour change is a problem with food diaries as is misreporting (Magkos and Yannakoulia, 2003). Furthermore, athletes may change their behaviour to make the recording process easier for themselves (Magkos and Yannakoulia, 2003).

Food diaries were chosen instead of 24 h recall because several 24 h recalls would have been necessary to capture the participants' habitual diet and a trained interviewer would have been required (Thompson and Subar, 2013). A 3 d period was chosen for the diet recording since this amount of time is suitable for reasonable accuracy while maintaining compliance (Magkos and Yannakoulia, 2003). The lack of detail obtained from the food diaries in Chapters 2 and 3 limited the analysis that could be performed and conclusions drawn. Exact timings of each eating occasion would have allowed comparison between the current studies and work by MacKenzie et al., 2015) examining protein pattern. Protein intake within each segment of the day was calculated, providing more information than total protein intake alone. All

groups of players detailed in this study had a skewed protein distribution, *i.e.,* less protein consumed in the morning and more protein consumed in the afternoon and evening. However, it is unclear what the effect of a skewed protein distribution is on long term lean mass accretion. Although, MacKenzie-Shalders et al., (2016) demonstrated that increasing the number of protein feeds from 4 to 6 did not result in greater lean mass accretion during a rugby pre-season. The effect of skewed or even distribution of protein intake on lean mass accretion would be an interesting area for future research.

The lack of usable training data from the rugby players in this thesis should be considered a limitation. It is difficult to assess the appropriateness of the diet when training information is not available. The timing of protein intake, in relation to training sessions, could not be assessed accurately in the current body of work as a result of the lack of detail within the food diaries and lack of training data. Future work analysing the diet of rugby union players should take into account training type, load and volume as well as timing of nutrient intake in relation to exercise.

Chapter 3 discusses the use of in camp nutrition provision as a tool to educate athletes about nutrition. This tool could be expected to be beneficial, as it would reach a large group of athletes at once. However, the effectiveness of this tool could not be assessed in this thesis because a further at home food diary was not recorded following the players' time in camp. Future research could assess the effectiveness of in camp nutrition provision on the dietary habits of athletes.

Skinfolds and DEXA were used to measure body composition of the elite and amateur rugby union players in Chapter 2 but only DEXA was used to measure the body composition of the U20 players in Chapter 3. The recent use of DEXA scanners to measure body composition as well as BMD has become routine and the method is becoming widely accepted (Buehring et al., 2014; Toombs et al., 2012; Van der Ploeg et al., 2003). However, the only way to accurately measure fat mass or %BF is using a cadaver and, perhaps surprisingly, no one has ever DEXA scanned a cadaver to validate body composition measures from DEXA (Toombs et al., 2012). The four-compartment (4C) model, considered the 'gold standard' criterion method for measuring body composition, is used for validation of DEXA (Toombs et al., 2012; Van der Ploeg et al., 2003). Toombs et al., (2012) summarise a number of studies validating %BF from DEXA to 4C model in non-athletic and athletic populations. Half of the studies in the athletic populations demonstrated an underestimation of %BF with DEXA and half an overestimation of %BF (Toombs et al., 2012). Santos et al., (2010) demonstrated that DEXA overestimated %BF and fat mass but underestimated fat free mass (LBM) compared with the 4C model in judo athletes. The difference in %BF was statistically significant but the same was not true for fat mass and fat free mass. A validation study using a whole body phantom was carried out in a group of Australian Football players. The study showed that DEXA accurately estimated LBM and BMD but tended to underestimate fat mass (Bilsborough et al., 2014). There is some disagreement in the literature regarding the measurement of %BF using DEXA but this tool seems to give accurate results for BMD and fat free mass/LBM. The Lunar iDEXA model used in the current study is the most up-to-date narrow fan beam scanner. It has improved resolution compared with older scanners resulting in better image quality. The Lunar iDEXA has not yet been compared to the

4C model but the precision of the scanner has been measured. The CV(%) for LBM ranges from 0.4-0.5%, fat mass from 0.7-1% and %BF from 0.6-0.9% (Toombs et al., 2012) which is more precise than the Lunar Prodigy (CVs). The validity of DEXA for measuring body composition is greater for fat free mass/LBM and BMC than for fat mass but the accuracy of the Lunar iDEXA specifically is yet to be fully established. The precision of body composition measurements using Lunar iDEXA is relatively high. Body composition measured by DEXA is relatively precise and valid for LBM but less valid for fat mass.

DEXA and skinfold data correlated fairly well for elite and amateur rugby union players – particularly LBM and LMI in Chapter 2. However, there was a difference in %BF, with results from the skinfold measurements significantly different between groups while results from DEXA were not. It is unclear, however, how changes over time in body composition between DEXA and skinfolds would agree. It has been shown that the DEXA can detect changes in body composition in athletes even when the stringent scanning controls are not in place (Colyer et al., 2016). This finding makes DEXA a slightly more practical measure in elite populations than previously thought. However, DEXA scans tend to be less accessible for practitioners out in the field than skinfold measurements, which are typically used by practitioners in rugby union (Jones et al., 2016). Therefore, fully understanding how these measures related to each other in rugby union players would provide practitioners with valuable information that might inform how they monitor body composition. For example, quarterly or bi-annual DEXA scans coupled with skinfolds and followed with skinfolds every 8 wk may be more convenient and cost effective for practitioners

working in elite rugby union. Future research should establish the relationship between the two methods of body composition measurement.

P70S6K1 activity was measured in Chapter 4 and 5 of this thesis. Unfortunately, a full dose response analysis could not be performed in Chapter 4 due to a lack of tissue. It is likely that the timing of the biopsies, as discussed in Chapter 4 and 5, was more suitable for the measurement of MPS and not for analysis of signalling. Therefore, any early response of p70S6K1, as seen in previous studies at 1 h (Moore et al., 2011) may have been missed. Consequently, it is entirely possible that there was a difference between doses before the first measures at 3 and 4 h in Chapters 5 and 4 respectively. An ambitious but extremely useful area of research would be to identify the time-course of the response between p70S6K1 activation and MPS in various exercise and nutrition situations. Furthermore, it would be beneficial to identify the time course of p70S6K1 activation in response to resistance exercise and protein feeding. The identification of the time course of these responses would help to identify when it would and would not be worthwhile measuring p70S6K1 activity in MPS studies. It is entirely possible, as mentioned in Chapter 4 that the timing of the biopsies for MPS calculation are not ideal for measuring p70S6K1 activity.

The study design in Chapter 5 meant that no direct comparison could be made between whole body and lower limb resistance exercise. Comparisons can only be made between separate studies. However, it does seem clear that the dynamics of protein nutrition are altered by the amount of muscle exercised. It is for this reason that the influence of LBM should not yet be dismissed. If the amount of muscle mass exercised influences the MPS response to protein feeding then if there is more muscle

present (higher LBM) it would follow that it results in a greater amount of muscle being exercised. However, this difference may become apparent only at doses that maximally stimulate MPS. For this reason, future studies should use higher protein doses than those in Chapter 5 to identify the dose of protein required to maximally stimulate MPS following whole body resistance exercise in athletes with varying amounts of LBM. Repeating the study of Chapter 5 but with lower limb exercise, which we know coupled with ingestion of 20 g protein maximally stimulates MPS, would be informative. A comparison of whole body and lower limb resistance exercise would provide valuable information that could be used to inform study design and protein recommendations.

6.*3. Practical Applications*

There are a number of practical applications that arise from this thesis. Monitoring changes in body composition of team sport athletes should be carried out on an individual as well as group basis. The effectiveness of training coupled with nutrition strategies can be assessed. Building a database of players' body composition changes across, and between, seasons allows practitioners to put any alterations observed into context for an individual. The importance of monitoring the diet of individual athletes also has been highlighted in this body of work. While as a group there may be no change in dietary habits, individuals could vary greatly which could have implications for their performance.

Chapter 2 identifies that the difference in body mass between young elite and amateur rugby union players is due to a difference in LBM. It is unclear whether players reached a higher playing standard because they have a higher LBM or

whether they achieved a higher LBM when they reached a higher playing standard. However, players aiming to compete at higher levels should place some degree of focus on increasing their LBM. Although this concept is not new in the field, we provide scientific evidence to support it. An increase in LBM can be achieved through an appropriate resistance training programme but the athletes' training must be supported by an appropriate nutrition strategy. Players must ensure that they are consuming sufficient protein to aid recovery and facilitate remodelling of their muscle proteins.

Previously, 20 g was the amount of protein recommended to be consumed following resistance exercise. However, in Chapter 5 we demonstrated that 40 g of whey protein, or more, should be consumed following whole body resistance exercise to increase MPS stimulation. This higher dose of protein is relevant for any athlete performing whole body resistance exercise, not just rugby players. These findings have implications for anyone wishing to optimise their nutrition to support the development or maintenance of LBM. Although not the focus of this programme of work, the results could have implications for elderly individuals. ACSM recommend whole body resistance exercise for the elderly population (Garber et al., 2011). It is well documented that elderly individuals require more protein to stimulate MPS to the same extent as young people (Yang et al., 2012). We have demonstrated that following whole body resistance exercise 40 g protein stimulates MPS to a greater extent than 20 g in young people. It is likely that for elderly individuals, ingestion of a higher dose of protein following whole body exercise should be recommended.

This thesis addresses the relationship between lean body mass and protein feeding at a number of levels. We have investigated the dietary habits of athletes and compared how their habits relate to the recommendations that are available. The impact that the protein ingested has at a whole body, muscular and molecular level has been presented. The broad range of levels that we have looked at in this thesis provides a greater understanding of the relationship of lean body mass and protein feeding. Furthermore, it demonstrates some of the processes or science behind recommendations and practice within the field of sports nutrition.

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APPENDICES

Volactive UltraWhey 90 instant
Supplementary Technical Data Sheet

Product

Whey protein isolate 90%; agglomerated and instantised

Description

Volactive UltraWhey 90 instant is manufactured from sweet cheese whey using cross-flow membrane filtration. The resulting protein concentrate is spray-dried with concurrent agglomeration and instantised with soya lecithin (E322). This product provides an excellent source of natural protein for use in a variety of food and beverage applications in an easy mixing form.

Trace minerals and heavy metals, ppm	
Aluminium	<5.00
Arsenic	<0.25
Cadmium	<0.10
Chromium	<1.00
Cobalt	<1.00
Copper	<5.00
Iron	<10.0
Lead	<0.02
Manganese	<1.00
Mercury	<0.05
Molybdenum	<1.00
Selenium	<1.00
Tin	<5.00
Titanium	<10.0
Zinc	<5.00

Minerals, mg per 100g powder	
Calcium	450
Chloride	50
Magnesium	50
Phosphorus	220
Potassium	400
Sodium	200

Vitamins, mg per 100g powder		
Choline chlorine		10.0
Niacin	(Vit B3)	0.00
Thiamin	(Vit B1)	0.10
Riboflavin	(Vit B2)	0.15
Pantothenic acid	(Vit B5)	0.01
Folic acid	(Vit B9)	0.10

Amino acid profile, % of total amino acid	
Alanine	5.00
Arginine	2.10
Aspartic acid	11.0
Cystine	2.20
Glutamic Acid	18.1
Glycine	1.40
Histidine	1.70
Isoleucine	6.40
Leucine	10.6
Lysine	9.60
Methionine	2.20
Phenylalanine	3.00
Proline	5.50
Serine	4.60
Threonine	6.70
Tryptophan	1.40
Tyrosine	2.60
Valine	5.90

Protein fractions, % of protein	
Beta-Lactoglobulin	50 - 60
Glycomacropeptide	15 - 20
Alpha-Lactalbumin	15 - 20
Bovine Serum Albumin	1.0 - 2.0
Immunoglobulin G	1.0 - 2.0
Immunoglobulin A	0.1 - 1.0
Lactoferrin	0.1 - 0.5

Nutritional Information, g per 100 g powder

Energy value, KJ	1,563
Energy value, Kcal	368.0
Protein (Nx6.38; dry matter basis)	93.0
Carbohydrates	2.50
of which sugars	2.50
Fat	0.30
of which unsaturated	0.25
of which monounsaturated fatty acids	0.15
polyunsaturated fatty acids	0.05
trans fatty acids	<0.05
saturated	0.05
Cholesterol	<0.01
Dietary fibre	0.00

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Strength and Conditioning and Concurrent Training Practices in Elite Rugby Union

AU1 Thomas W. Jones,[1] Andrew Smith,[2,3] Lindsay S. Macnaughton,[4] and Duncan N. French[5,6]

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Abstract

Jones, TW, Smith, A, Macnaughton, LS, and French, DN. Strength and Conditioning and Concurrent Training Practices in Elite Rugby Union. *J Strength Cond Res* XX(X): 000–000, 2016–There is limited published research on strength and conditioning (S&C) practices in elite rugby union (RU). Information regarding testing batteries and programme design would provide valuable information to both applied practitioners and researchers investigating the influence of training interventions or preperformance strategies. The aim of this study was to detail the current practices of S&C coaches and sport scientists working in RU. A questionnaire was developed that comprised 7 sections: personal details, physical testing, strength and power development, concurrent training, flexibility development, unique aspects of the programme, and any further relevant information regarding prescribed training programmes. Forty-three (41 men, 2 women; age: 33.1 ± 5.3 years) of 52 (83%) coaches responded to the questionnaire. The majority of practitioners worked with AU3 international level and/or professional RU athletes. All respondents believed strength training benefits RU performance and reported that their athletes regularly performed strength training. The clean and back squat were rated the most important prescribed exercises. Forty-one (95%) respondents reported prescribing plyometric exercises and 38 (88%) indicated that periodization strategies were used. Forty-two (98%) practitioners reported conducting physical testing, with body composition being the most commonly tested phenotype. Thirty-three (77%) practitioners indicated that the potential muted strength development associated with concurrent training was considered when programming and 27 (63%) believed that strength before aerobic training was more favorable for strength development than vice versa. This research represents the only published survey to date of S&C practices in northern and southern hemisphere RU.

Key Words combined exercise, interference, physical preparation, programme design, questionnaire

Introduction

Rugby union (RU) is a contact team sport that is popular worldwide. Match analysis has indicated that RU is a multidirectional, intermittent, invasion game incorporating multiple high-intensity efforts. These vary in nature and consist of sprinting, accelerations, and sport-specific activities including tackling, rucking, mauling, and scrummaging (12,13,30,33). The physical demands of RU are specific to the individual positions (24). A 15-player side consists of forwards ($n = 8$) and backs ($n = 7$), the forwards are further subcategorised in to; "front row," "second row," and "back row" positions. Backs also are subcategorized into "half backs," "centers," and "outside backs." In many cases, players are allocated to certain positions based on their anthropometric and physical performance characteristics, with forwards tending to be heavier and stronger and backs tending to be leaner and faster (11).

A growing body of research has examined the physical demands of competitive RU matches through performance, time motion, and global position system analyses (8,9,33). More recent research has examined the influence of standardized and controlled conditioning interventions on physical performance phenotypes associated with successful RU performance (1,3,42). In addition, studies have investigated the influence of preperformance strategies including postactivation potentiation and hormonal priming on physical performance factors necessary for effective RU performance (2,18,26).

The availability of the literature quantifying both the physical demands of elite RU and the influence of conditioning interventions has allowed practitioners to gain a greater understanding of the physiology of RU and potentially programme more effectively for their athletes. Despite this increased understanding, RU remains a challenging sport to support. In contrast to many (particularly

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Table 1. Level of athlete respondents support.

National	Proclub	Senior academy	University
24	30	11	6

*Many respondents detailed more than 1 level of athlete.

Olympic) sports, RU requires differing and in some cases contrasting physical qualities for successful performance. Research has indicated that strength and power (both absolute and relative to body mass) are important physical qualities in elite RU union (1,11), in contrast as players can cover an average of ~7 km during a competitive match (8) athletes also require aerobic and fatigue resistance capabilities (33). This required contrast may present practitioners with problems when programming as responses to strength and power training can be muted as a result of endurance type stimulus (21,22,25,28). This inhibited strength development or "interference effect" (22) associated with concurrent strength and aerobic training also warrants consideration during training phases such as preseason, in which practitioners often have limited time to promote gains in strength and power phenotypes.

Despite the growing global profile of RU and increasing attention in the scientific literature, there is little published information available pertaining to practices and strategies used by strength and conditioning (S&C) and sports science practitioners in elite RU. Although S&C practices have been examined in various North American and Olympic sports (10,14–16,19,38), there are no available data detailing how specific conditioning is prescribed and monitored in elite RU. In addition, is it is presently unknown if the "interference effect" associated with concurrent strength and aerobic type training is (a) considered and (b) managed by practitioners working with RU athletes.

Information relating to common trends in training prescription and management could act as a useful reference source for applied practitioners. This information also may inform training programme design for future studies seeking to examine the influence of conditioning interventions in elite RU athletes. As such, the aim of this study was to survey and examine training and monitoring strategies of practitioners responsible for the S&C of RU athletes.

Methods

Experimental Approach to the Problem

The survey titled "Strength and Conditioning Questionnaire" was adapted from that used by Ebben and Blackard (14). The questionnaire was made specific to RU and pilot tested on a group of 7 S&C coaches. The survey contained 7 sections: personal details, physical testing, strength and power development, concurrent training, flexibility development, unique aspects of the programme, and any further relevant information regarding prescribed training programmes. The survey was distributed to S&C coaches and sport scientists working with either professional rugby clubs/franchises/provinces or national teams in both the northern and southern hemispheres. It was hypothesized that this study would provide a comprehensive view of S&C and concurrent training practices in elite RU.

Subjects

Before all experimental procedures, the Northumbria University research ethics committee approved the study. All subjects were informed of the risks and benefits of the investigation before signing an approved informed consent document to participate in the study. Surveys were sent out electronically through e-mail and a survey collating Web site. Data were collected between September 2014 and February 2015. The study conforms to the Code of Ethics of the World Medical Association (approved by the ethics advisory board of Swansea University) and required players to provide informed consent before participation.

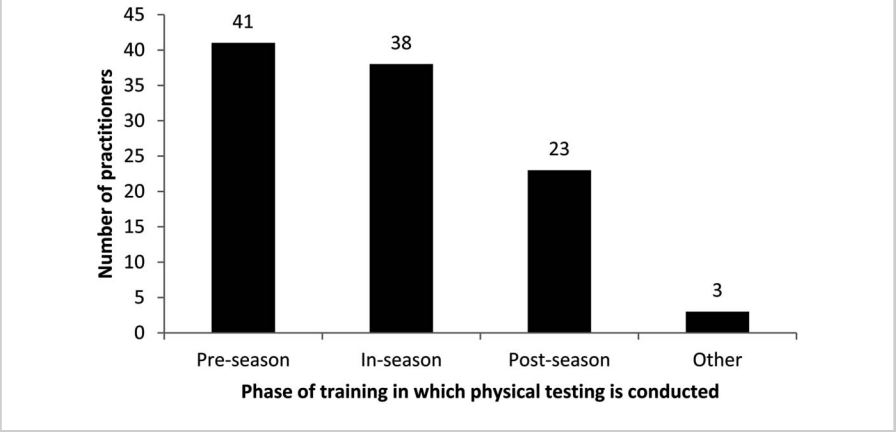

Figure 1. Times when physical performance phenotypes are assessed.

Statistical Analyses

The survey contained fixed-response and open-ended questions. Answers to open-ended questions were content analyzed

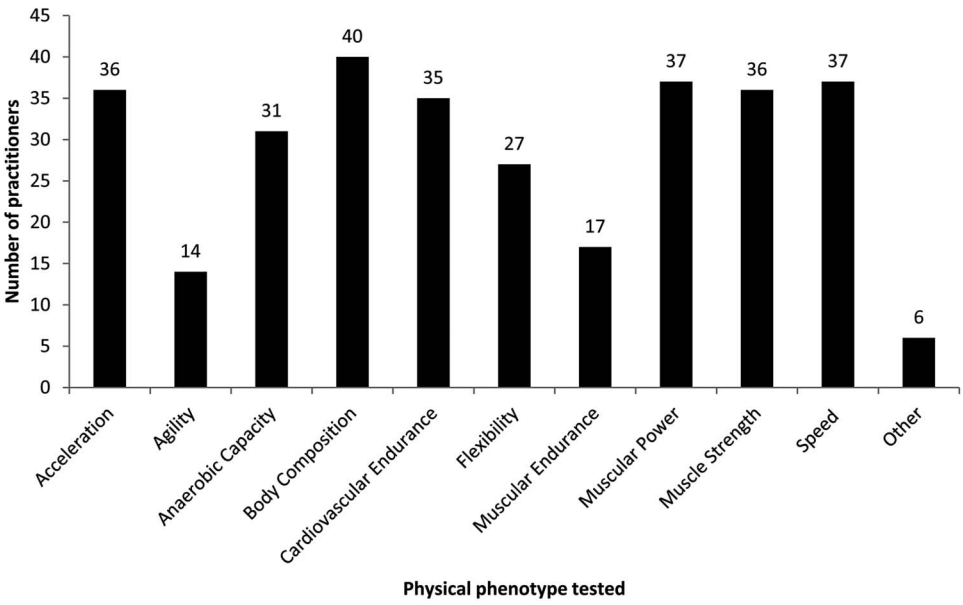

Figure 2. Physical phenotypes tested.

according to methods described by Patton (31), which have previously been used in other surveys of S&C practices in elite and professional sports (10,15,16,38). Researchers had experience with qualitative methods of sports science and S&C research. When analyzing data, investigators generated raw result data and higher-order themes through inductive content analysis and compared individually generated themes until agreement was reached at all levels of analysis. When higher-order themes were developed, deductive analysis was used to confirm that all raw data themes were represented.

Results

Personal Details

Forty-three (41 men, 2 women; age: 33.1 ± 5.3 years) of 52 (83%) coaches responded to the questionnaire. The respondents consisted of 21 S&C coaches, 12 head S&C coaches, 3 senior S&C coaches, 3 academy S&C coaches, 2 performance managers, and 2 sport scientists. Forty-two practitioners reported having fellow coaching and support staff. Examples of fellow coaching staff given by respondents were "Assistants," "Interns," and other S&C staff such as performance mangers and "Travelling S&C Coach" (text in double quotes are direct quotations taken from questionnaires). Four practitioners were based in Australia, 3 in France, 4 in New Zealand, 2 in South Africa, 1 in Hong Kong, 1 in Japan, 1 in Samoa, and 27 in the United Kingdom. Information

Table 2. Sets and repetitions used during in-season programmes.

Higher-order themes	No. responses	Select raw data representing responses to this question
Set range of 3–5	24	
Set range including >5 sets	9	
Repetition range of 3–5	12	
Repetition range including >5 repetitions	18	
Miscellaneous*	6	"Huge variance depending on the outcome" "Neural–less than 3, cellular–to failure" "Dependent of team and athlete experience" "Depends upon the individual player" "Repetition ranges vary hugely based on the individual aims of the programme, training history, loading scheme, etc"

*Answers that could not be associated with any of the broad identified themes.

Table 3. Sets and repetitions used during off-season programmes.

Higher-order themes	No. responses	Select raw data representing responses to this question
Set range of 3–6	22	
Set range including >6 sets	6	
Repetition range of 3–8	12	
Repetition range including >8 repetitions	20	
Miscellaneous*	2	"During the off-season, we typically use higher volumes"
		Dependent of team and athlete "experience and aim of programme"

*Answers that could not be associated with any of the broad identified themes.

on the types of athlete coached by the respondents is presented in Table 1. T1

Formal Education

Seventy-nine percent of respondents had an undergraduate degree in Sport and Exercise Science or a related subject and 61% held a master's degree in a Sport Science–related field.

Table 4. Recovery time prescribed between strength training and rugby training and competitive matches.

Question	Same day	24 h	36 h	48 h	>48 h
Time prescribed between Olympic style lifting session and high-quality rugby session	34	9	2	2	1
Time prescribed between general strength session and high-quality rugby session	31	8	4	5	0
Time prescribed between Olympic style lifting session and competitive rugby match	4	6	11	22	9
Time prescribed between general strength session and a competitive rugby match	1	4	11	20	14

In addition, 2 coaches held Post Graduate Certificates in Education and 2 stated they were completing PhDs in Exercise Physiology and S&C.

Certification

The most commonly held professional certification was United Kingdom Strength and Conditioning Association Accreditation ($n = 10$). Nine respondents were certified S&C specialists with the National Strength and Conditioning Association (USA), 5 were accredited at various levels by the Australian Strength and Conditioning Association, and 6 were British Amateur Weightlifting Association certified. Other qualifications held included "British Association of Sport and Exercise Sciences High Performance Sport Accreditation," "International Society for the Advancement of Kinanthropometry Accreditation," and "United Kingdom Athletics Coaching Qualification."

Physical Testing

Forty-two of 43 respondents indicated that physical testing was conducted on their athletes. Participants were asked when testing was performed (Figure 1) and what aspects of physical performance were tested (Figure 2). The most commonly used test of acceleration was 10-m sprint time ($n = 30$). Tests of agility included proagility test, "reactive agility," Illinois agility run, T-test, 5-0-5 test, change of direction and acceleration test, and "in depth lateral jumps." Measures of anaerobic capacity included Rugby Football Union anaerobic test, Welsh Rugby Union WAT test, "repeat sprint ability," Yo-Yo test, "Watt-Bike repeat sprints (10 × 6 seconds in at 30-second intervals)", "Watt-Bike 30-second sprint," "Watt-Bike 6-minutes test," 500-m rowing, phosphate decrement test, "3 × 60-second running test," "intermittent shuttle test," anaerobic shuttle, "lactate test on treadmill," "Bronco shuttle test," "GPS work capacity," "Australian 30 seconds × 6 test," Wingate test, "rugby anaerobic fitness test," "150-m Shuttle Test," "club-specific conditioning test," "rugby-specific testing," "anaerobic training threshold zone (ATTZ) runs," and "6 × 30-m sprints." F1 F2

The most commonly used measure of body composition was sum of 8 site skinfolds ($n = 22$) with 7 ($n = 5$) and 3 ($n = 1$) site skinfolds also utilized. Other measures of body composition included body mass, height, dual-energy x-ray absorptiometry, body fat%, and one respondent designed their own method of assessing body composition, although no other details were given. Twenty-three respondents stated that the Yo-Yo incremental test was utilized as a measure of cardiovascular (CV) endurance, other used tests of CV

Table 5. Practitioners rank order of the 5 most important weightlifting exercises within their training programme.

Order of importance	Exercises (no. coaches reporting)
1	Squat (30)
	Clean (9)
	Any single leg strength variation (1)
2	Clean (19)
	Deadlift (6)
	Squat (3)
	Pull-up and bench press (2)
	Nordics, unilateral lower body, high pull, push press, Romanian deadlift, snatch, hamstring variations, and split squat (1)
3	Bench press (7)
	Olympic lift variation (5)
	Romanian deadlift (4)
	Push press, split squat, and split jerk (3)
	Bench pull, squat, overhead press, and horizontal row (2)
	Chin, single arm row, deadlift, lunge, and dumbbell press (1)
4	Chin (8)
	Push press, deadlift, snatch, and clean (2)
	Bench press, bent over row, landmine, power jerk, jump squat, split jerk, squat, high pull, single leg squat, military press, Romanian deadlift, bench pull, weighted step ups, and single leg deadlift (1)
5	Bench press (7)
	Chin (5)
	Overhead press, clean, bent over row, snatch, and Romanian deadlift (2)
	Floor hip thruster, push press, deadlift, high pull, dumbbell incline press, bench throw, split squat, shoulder rotation, bench pull, and single arm row (1)

endurance included 1,500-m run, "30-15 aerobic test," "a 4-minute shuttle test," 1-km run, "MAS test TUB 2," "1-km repeat," "3-min Watt-Bike test," 2.4-km time trial, "7-minute test," "modified bleep test," "Watt-Bike 20-minute test," "GPS work capacity," "incremental treadmill test," "ATTZ test," and "1.6-km time trial."

Functional movement screening was the most commonly utilized measure of flexibility ($n = 8$), other measures of flexibility included "physio screening" "subjective assessments," sit-and-reach test, "physical competency assessment," Thomas test, hamstring capacity, thoracic rotation, knee to wall test, "internally developed movement competency screen," "range of motion tests," and overhead squat. Seventeen respondents tested indices of muscular endurance (Figure 2): these included glute bridge, calf raise, max push ups, max sit ups, "modified test involving body weight exercises, and timed run devised around facility layout," max chins, max dips, max pull-ups, "capacity tests on calves, glutes and hamstrings," plank, side plank, back extension, and single leg glute bridge.

The most commonly used test of muscular power was maximum countermovement jump height ($n = 19$), 11 (26%) practitioners assessed 1–3 repetition maximum (RM) in Olympic lifts (clean or snatch), or their variations (i.e., from hang position), additionally 17 (40%) assessed reactive strength index or other jump variations including broad jumps, drop jumps, squat jumps, "triple response jumps," etc. A variety of other measures of muscular power were utilized by respondents including "velocity test," velocities of movements through "GymAware" and "Attacker" systems, 10- and 30-m sprints, tendon stiffness, 1RM in bench press, back squat and half squat, "bench throw and pull," and peak power output in 6 seconds on Watt-Bike and medicine ball throw. Twenty-eight practitioners utilized 1RM testing to assess muscular strength with bench press ($n = 22$) and back squat ($n = 20$) the most common lifts. Other methods of assessing muscular strength included mid-thigh isometric pulls on a force plate and "predicted RMs taken from strength training performance." All 37 respondents who stated that testing speed phenotypes examined sprint times with distances ranging from 10 to 80 m, additional speed tests used included "speed bounce" and GPS maximum velocity.

Strength and Power Development

The initial question in the section asked if practitioners believed that strength training benefits RU performance, all 43 respondents answered yes. Eight practitioners left additional comments such as "stronger players are more resilient," "it helps the players develop the appropriate physical qualities that are required to play the game," "But a focus on quality of lifting through a full range if safe for the athlete is critical as well as the combination of movement skills, awareness and integration with the rest of the rugby programme is critical to maximum carryover into performance" and "it is a very important part of preparation but in my experience it's importance is overstated by the rugby community." All 43 respondents also stated that strength training was regularly performed by their athletes.

In-Season Training

The current section was divided into 2 subsections, the first of which focused on in-season strength and power training practices. The first question in this subsection asked how many days of the week that in-season strength and power training was performed; 1 practitioner reported 1 $d \cdot wk^{-1}$,

Table 6. Determination of training loads.*

Higher-order themes	No. responses	Select raw data representing responses to this question
RM and max strength testing	31	Percentage of 1RM. All % based from preseason testing. % of a 1–3RM test.
Athlete lead	3	Athlete lead, occasional last set max repetitions of weight used to see if appropriate weight. Players determine their own weights based on how they physically feel.
Coaches subjective assessment	3	Coaching eye, then prescribed in vital training blocks. Assessment of the required effort vs. technical breakdown/quality.
Periodization and phase of training	3	Current needs and stage of season. Consideration given to current aims of programme and training history.

*RM = repetition maximum.

14 reported 2 $d \cdot wk^{-1}$, 35 reported 3 $d \cdot wk^{-1}$, 4 reported 4 $d \cdot wk^{-1}$, and 1 reported 5 $d \cdot wk^{-1}$.

The second question within this subsection asked coaches to detail the days of the week in which strength and power training is performed in relation to next scheduled match day (MD); 6 practitioners reported MD-6, 31 reported MD-5, 36 reported MD-4, 14 reported MD-3, 35 reported MD-2, 6 reported MD-1, and 3 reported strength and power training was conducted on MD. The third question in this section asked practitioners the typical duration of an in-season strength and power session; 2 practitioners reported 15–30 minutes, 12 reported 30–45 minutes, 26 reported 45–60 minutes, and 7 reported 60–75 minutes. The final question in the subsection asked practitioners to indicate the number of sets and repetitions typically used for strength training exercises in-season. Responses were content analyzed and resulted in the creation of 5 higher-order themes including (a) set range of 3–5, (b) set range including >5 sets, (c) repetition range of 3–5, (4) repetition range including >5 repetitions, and (5) miscellaneous. Further information on higher-order themes, practitioner responses, and representative raw data is presented in Table 2. T2

Off-Season Training

The first question in the off-season subsection asked practitioners the number of days per week their players engage in strength training. Three practitioners reported 2 $d \cdot wk^{-1}$, 11 reported 3 $d \cdot wk^{-1}$, 25 reported 4 $d \cdot wk^{-1}$, 10 reported 5 $d \cdot wk^{-1}$, and 4 reported 6 $d \cdot wk^{-1}$. The following question addressed the average length of an off-season strength/power session; 2 respondents reported 15–30 minutes, 4 reported 30–45 minutes, 22 reported 45–60 minutes, 12 reported 60–75 minutes, and 1 reported >75 minutes.

The final question in the off-season training subsection asked practitioners to indicate the number of sets and repetitions typically used for strength training exercises during the off-season. Content analysis resulted in the creation of 5 higher-order themes including (a) set range of 3–6, (b) set range including >6 sets, (c) repetition range of 3–8, (d) repetition range including >8 repetitions, and (e) miscellaneous. Further information on higher-order themes, practitioner responses, and representative raw data is presented in Table 3. T3

Table 7. Training methods used by coaches for speed development.

Higher-order themes	No. responses	Select raw data representing responses to this question
Unresisted (free) sprinting	25	Actual max speed running. Sprinting on a track. Free sprinting.
Plyometrics	13	Plyos. Plyometrics. Plyometric movements.
Sprint mechanics and technique	13	Running mechanics. Technical delivery. Technique development.
Resisted sprinting	13	Weighted sleds. Resisted accelerations. Sled and Bungee cord work.
Improving max strength	9	Max strength development. Increasing strength and power through weight training. Creating a high strength base.
Olympic lifts	4	Olympic lifts. Hang cleans.

*Many respondents detailed more than 1 training method.

Programme Design

The initial question in this subsection asked whether practitioners included Olympic style weightlifting exercises in their prescribed training programme. Thirty-eight respondents indicated that Olympic style weightlifting exercises were included in conditioning programmes.

The next questions within this subsection were related to

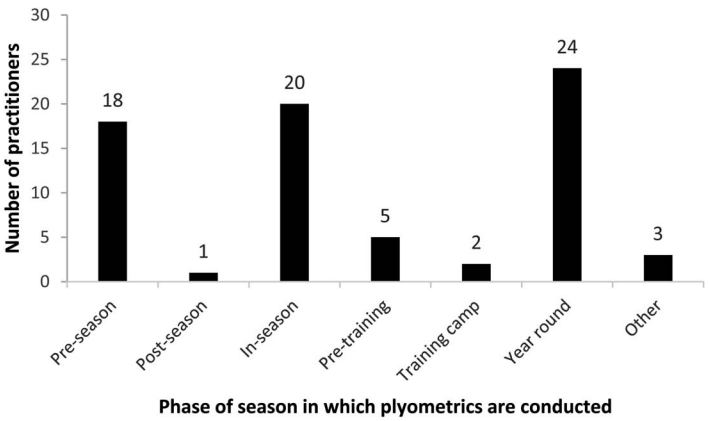

Figure 3. Times in which plyometrics are conducted.

recovery time prescribed between (a) an Olympic weightlifting style strength session and a high-quality rugby training session, (b) a general strength training session and a high-quality rugby training session, (c) an Olympic weightlifting style strength session and a competitive rugby match, and (d) a general strength training session and a competitive rugby match. Responses to these 4 questions are detailed in Table 4. Practitioners were then asked the extent to which they agreed that strength and power training influenced rugby performance; 26 coaches indicated that they strongly agreed, 14 strongly agreed, and 1 indicated they were unsure. The next question asked coaches to identify and rank the top 5 weightlifting exercises that are most important in their programmes, responses to this question are detailed in Table 5.

Question 7 in this subsection asked practitioners if they used periodization strategies to structure training plans. Thirty-eight (88%) respondents indicated that periodization strategies were used. Practitioners comments in response to this question included "To target specific outcomes in a specific period," "Better long-term results, prevents stagnation," and "Monitoring and assessing load and volume with intensity is vital, so you need to know when to delay and load at appropriate times of the year."

The final question in this section asked practitioners how load (weight) was determined during typical strength training sessions. Responses were content analyzed into 4 categories including (a) RM and max strength testing, (b) athlete led, (c) coaches subjective assessment, and (d) periodization and phase of training. Data pertaining to higher-order themes, total number of practitioners whose responses made up the theme and selected raw data within higher-order themes, are presented in Table 6.

Speed Development

Forty of 43 (93%) respondents who completed the survey reported incorporating aspects of speed development in their programming. Responses were content analyzed and resulted in the creation of 6 higher-order themes: (a) unresisted (free) sprinting, (b) plyometrics, (c) sprint mechanics and technique, (d) resisted sprinting, (e) improving max strength, and (f) Olympic lifting. Table 7 details the aforementioned higher-order themes, the total number of coaches whose responses made up the theme, and select raw data within each higher-order theme.

Table 8. Methods of integration of plyometrics into prescribed training programme.

Higher-order themes	No. responses	Select raw data representing responses to this question
Within strength and/or power session	25	Within strength programme. Mainly in strength/power sessions. With strength or Olympic lifts.
Dependant on individual athlete	4	Individually based around the needs of the athlete. Depends on individual.
Within warm-up	2	Part of warm-up. Part of field warm-ups in-season.
Part of movement skills	1	Part of movement skills.

Plyometrics

Forty-one (95%) respondents reported using plyometrics. The subsequent question in this section asked why coaches prescribed plyometrics, 16 (37%) coaches reported prescribing plyometrics for improving rate of force development, 7 (16%) for training the stretch shortening cycle, 4 (9%) for improving stiffness, and 2 (5%) for injury prevention. The third question in this subsection focused on the phases of the year plyometrics

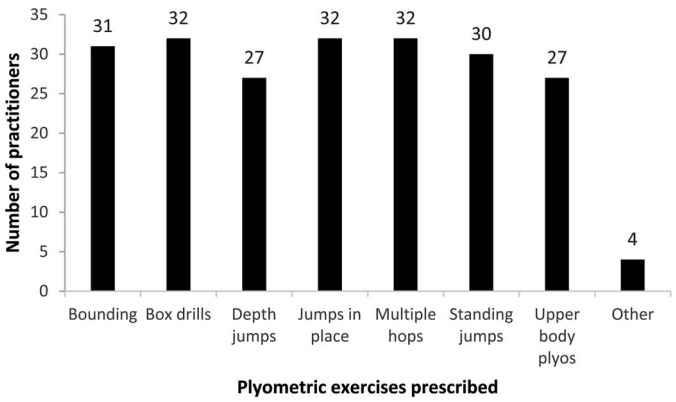

Figure 4. Specific plyometric exercises prescribed.

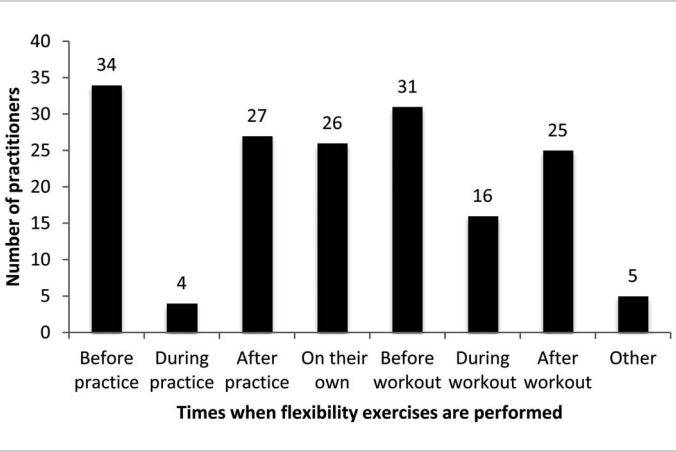

Figure 5. Times when athletes were encouraged or required to perform flexibility exercises.

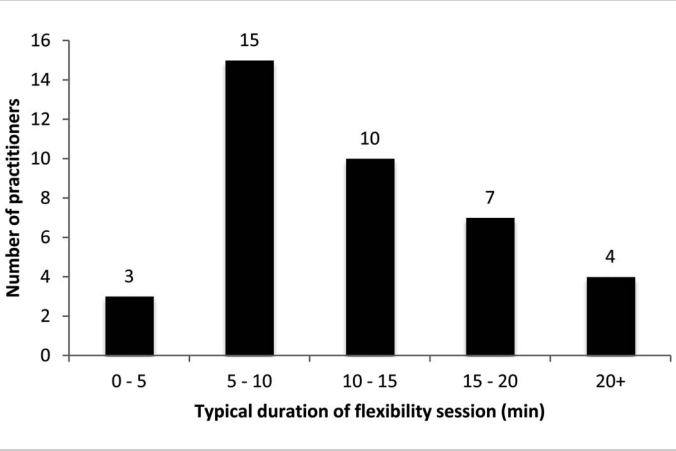

Figure 6. Duration of a typical flexibility session prescribed by coaches.

are used; Figure 3 illustrates the responses to this question. F3

The forth question in this subsection examined integrated plyometrics. Responses were content analyzed and resulted in the creation of 4 higher-order themes; (a) within strength and/or power session, (b) dependant on Individual athlete, (c) within warm-up and (d) part of movement skills. Table 8 lists the higher-order themes, number of practitioners whose responses make up the theme and representative raw data within each theme. The final question within this subsection asked practitioners to identify types of plyometric exercises regularly used in their programme. Reponses to this question are detailed in Figure 4. T8 F4

Flexibility Development

Forty-one (95%) practitioners indicated that some form of flexibility training was included in players' physical programmes. Thirty (70%) respondents indicated that static stretching was performed, 26 (60%) reported using proprioceptive neuromuscular facilitation and 37 (86%) indicated that dynamic stretching was performed. Six (14%) respondents reported using other methods of flexibility development including yoga, body balance, band distraction, and stretch bands. The following question asked practitioners when their athletes performed flexibility training, the typical duration of flexibility sessions and the duration athletes were encouraged to hold a static stretch. Results from these questions are presented in Figures 5–7.

Concurrent Strength and Endurance Training

The first question in the subsection asked practitioners if

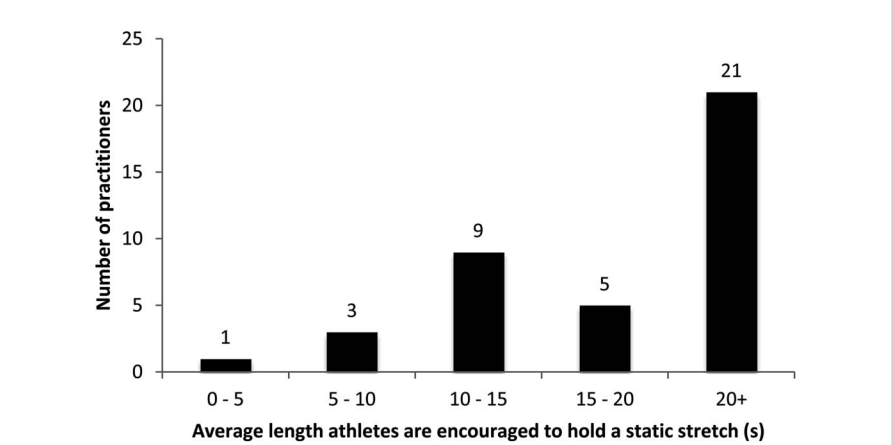

Figure 7. Amount of time athletes are encouraged to hold a static stretch.

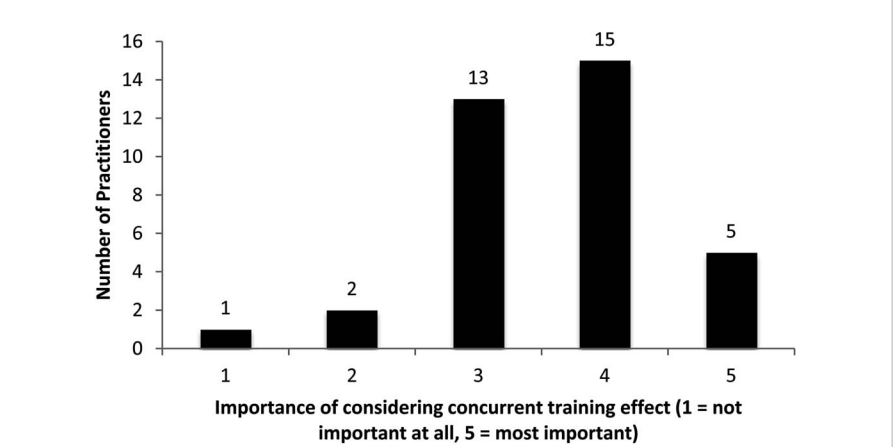

Figure 8. Importance of considering of concurrent training effect when programming for strength/hypertrophic development (1 = not important at all, 5 = most important).

they considered any potential muting effect of endurance training on strength/hypertrophic development, 33 (77%) practitioners indicated that they did and 8 (19%) indicated that they did not. Reasons for not considering any potential interference effect consisted of "Rugby is concurrent," "Players must develop both motor qualities," and "If programmed correctly can balance both into programmes."

The following question in this subsection asked practitioners how important they felt it was to consider any concurrent training effect when programming for strength/hypertrophic development (1 = not important at all and 5 = most important), the responses to this question are detailed in Figure 8. The penultimate question asked participants to rank the following programme variables in order of importance when attempting to avoid any muting effect of endurance type stimulus on strength/hypertrophic development; periodization, order of strength and endurance training, volume of endurance training, volume of strength training, and time between strength and endurance training. Responses to this

Table 9. Order of importance of programme variables when attempting to avoid any muting effect of endurance stimulus on strength/hypertrophic development (1 = most important, 5 = least important).

	Order of importance, 1 = most important, 5 = least important (no. responses)				
Programme variable	1	2	3	4	5
Periodization	18	4	3	3	11
Order of strength and endurance training	11	11	9	7	1
Volume of endurance training	6	12	11	6	4
Volume of strength training	2	2	8	16	11
Time between strength and endurance training	2	10	8	7	12

Table 10. Unique aspects of practitioners prescribed conditioning programmes.

Higher-order themes	No. responses	Select raw data representing responses to this question
Individualization	7	Individualism for every player. We provide position and player specific programmes. Degree of individuality of programming.
Nothing unique	7	Nothing is unique. Nothing unique, do the basics well. None.
Miscellaneous	7	Game day primers and hypoxic sessions. Include functional movement screening corrective exercise.
Integration	4	Combination of speed and agility work into lifting sessions. Integrating rugby drills in reactive agility sessions.
Periodization	3	The periodization of our season. Periodization of the anaerobic training threshold zone.

Table 11. What practitioners would like to do differently in their conditioning programmes.

Higher-order themes	No. responses	Select raw data representing responses to this question
Have more time	11	Have more time to train. Allow more 1 on 1 time. Have more training time.
Miscellaneous	10	Create more self-motivated and reliable athletes. More metabolic conditioning sessions. Better detail in the basics.
Improved facilities/ equipment	7	Greater resourcing. More equipment. Our facilities restrict everything.
Greater individualization	6	Individualize conditioning. Individualize with more detail per player. Individualize much more depending on assessment.
Improved monitoring	6	Heart rate variability would be the next step. More precision in monitoring. Increased monitoring to be able to prescribe more accurately and monitor change.
More staff	5	More staff. More staff to supervise sessions.

*Many respondents detailed more than 1 thing they would like to change.

T9 question are detailed in Table 9. The final question in this section asked practitioners which order of strength and endurance training they felt was more conducive to strength and/or hypertrophic development, 27 (63%) practitioners believed that strength then endurance training was more favorable and 12 (28%) believed endurance then strength.

Unique Aspects of the Programme

The unique aspects (if any) of practitioners physical conditioning were content analyzed and divided into 5 higher-order themes: (a) individualization, (b) nothing unique, (c) miscellaneous, (d) integration, and (e) periodization. Table 10 details T10 these themes and the number of practitioners' responses that make up each theme. The second question within this section asked practitioners what they would like to do differently in their conditioning programmes. Responses were content analyzed and resulted in the creation of 6 higher-order themes; (a) have more time, (b) miscellaneous, (c) improved facilities/equipment, (d) greater individualization, (e) improved monitoring, and (f) more staff. Table 11 details T11 these themes and the number of practitioners' responses that make up each theme.

Discussion

The present study sought to conduct a comprehensive survey of S&C and concurrent training practice in elite RU. To the authors' knowledge, this is the first qualitative assessment of practitioners S&C practices in RU. A total of 43 practitioners responded to the questionnaire, this is the highest number of responses obtained in a study examining S&C provision in a single sport. Previous studies examining S&C practices in North American sports have received between 20 and 26 responses (14–16,38) and a more recent study in British Rowing received 32 responses (19). The response rate to our survey was high (83%), previous comparable studies have reported return rates of between 69 and 87%. As such, 43 responses at a return rate of 83% were deemed sufficient for analysis. Many respondents stated that they worked with more than 1 level of RU athlete. The most commonly supported level of athlete played for either a professional club, province or franchise, and/or a national team (30 and 24 responses). Therefore, the data presented in this article are reflective of elite RU.

Practitioners reported testing 11 aspects of physical fitness (additional are details presented in Figure 2). This number is notably more than previously reported in other sports including Major League Baseball (MLB) (3–4 aspects) (16), National Hockey League (NHL) and National Basketball Association (NBA) (7–8 aspects) (15,38), and Rowing (4–5 aspects) (19). The 11 aspects of physical fitness tested in the present study are, however, similar to that previously reported in National Football League (NFL) (9–10 aspects) (14). It is possible that this is reflective of the similarities between RU and NFL as they are both contact, intermittent, invasion based team sports. However, comparisons should perhaps be interpreted with caution as Ebben and Blackard (14) reported S&C practices in NFL in 2001 and it is very likely that assessment batteries in NFL have progressed and been adapted over the past ~14 years.

The most commonly tested aspect of physical fitness was body composition, which was assessed by 40 of 42 (95%) of practitioners. Similarly, body composition was commonly assessed by practitioners working with North American sports with 83–100% of respondents indicating body composition was assessed (14–16,38). To the authors' knowledge, there are no empirical data demonstrating that "favourable" changes in body composition (increased lean mass and lower levels of subcutaneous fat) result in improved RU performance. However, when % body fat from separate studies are combined, a linear relationship between playing standard and % body fat is evident and it seems that as playing standard increases, % body fat of RU athletes decreases (full summary provided by Duthie et al. (11)). It is also reasonable to suggest that increases in lean mass and reduction in % body fat may result in improvements power to body mass ratio, acceleration, and other performance phenotypes associated with RU performance. Monitoring body composition may also be useful for assessing (any) gains in lean mass following any prescribed hypertrophy type training. Other commonly assessed aspects of physical fitness were max speed, muscular power (both 37), acceleration, and muscular strength (both 36). It is likely this indicates the practitioners who responded to the survey consider these physical qualities important for RU performance. There was a notable variance in measures of anaerobic capacity employed, with 17 different measures used across the 31 practitioners who indicated that they performed anaerobic capacity testing. This may indicate that there is a need for future work to construct a valid and standardized protocol for assessing anaerobic capacity in RU athletes. Overall physical testing was most commonly conducted pre- and in-seasons with 41 and 38 respondents indicating that physical testing was conducted during these phases.

All 43 respondents indicated that strength training was regularly performed by their athletes; in addition, all practitioners believed that strength training is beneficial for RU performance. This belief is supported by research indicating that RU performance requires high levels of contractile strength (29,35). Thirty-eight of the 43 practitioners (90%) reported implementing Olympic style weightlifting exercises within strength and power training. This practice is similar to those reported in Rowing (87% of practitioners surveyed), NFL (88%), NBA (95%), and NHL (91%) (14,15,19,38). These data indicate that Olympic style weightlifting exercises are widely prescribed in team sports and rowing, this prescription is most likely due to the association with Olympic lifting training and improvement in power output and acceleration (5,41) which have been identified as important physical qualities in RU and other sports (33,37). The squat and clean were considered the most important exercise within players training programmes. The aforementioned lifts were seen also as the 2 most important by practitioners working in Rowing, NBA, NFL, and NHL (14,15,19,38). Gee et al. (19) hypothesized that the clean and squat are valued across a range of sports as they relate to sports-specific performance phenotypes such as sprint and jump ability (23,32).

With regard to strength training frequency, 35 (81%) practitioners reported prescribing strength training 3 $d \cdot wk^{-1}$ in-season, whereas in the off-season 25 (58%), practitioners reported prescribing strength training 4 $d \cdot wk^{-1}$. The most common set/repetition/load scheme prescribed in-season was 3–5 sets of >5 repetitions based on RM and max strength testing, this scheme differed to the most common prescription of 3–6 sets of >8 based on RM and max strength testing. This increased volume of strength training also was reflected in practitioners' comments which included "during the off-season, we typically use higher volumes." These alterations in strength training volume may reflect the shift of conditioners focus from maintenance (in-season) to development (off-season) of physical qualities and that S&C staff tend to have more contact time with athletes outside the competitive season (anecdotal observations and reports from practitioners).

Speed development training was prescribed by 40 respondents (93%), which is similar to that reported in NFL, MLB, NBA (all 100%), and NHL (96%) (14–16,38). Unresisted or "free" sprinting was the most popular method of speed development, training methods included "max speed running" and "track sprinting." The second most popular method of speed development was plyometrics and 41 (95%) respondents reported implementing plyometrics within their conditioning plans (for speed development or otherwise). As with speed development, this method is similar to NBA (100%), MLB (95%), and NHL (91%) (15,16,38). It is somewhat surprising that the prevalence of plyometrics prescribed in NFL was notably lower (73%) (14) than that in RU given that both sports require physical qualities such as power and acceleration for successful performance (4). However, as previously stated, it is likely that S&C practices in NFL have changed since the study of Ebben and Blackard (14) was conducted.

Thirty-eight of 43 respondents (90%) reported implementing periodization strategies in their conditioning programmes, this practice is similar to that of coaches in Rowing (97%), NBA (91%), NHL (90%), and MLB (83%) (15,16,19,38). Periodization strategies have been demonstrated to result in greater improvements in strength, power and body composition than linear training (27,40). Periodization has also been reported to be an effective means of avoiding any potential muting effect of aerobic type stimulus on strength and power development (17). Thirty-three respondents (77%) indicated that the "interference effect" associated with concurrent strength and aerobic training was considered whilst programming for RU athletes. In addition, 20 (47%) practitioners believed that it was very important to consider when constructing conditioning plans. As previously stated, periodization has been reported to be an effective means of concurrently developing strength and aerobic physical qualities (17), as such it is perhaps unsurprising that periodization was ranked as the most important programme variable when attempting to avoid any interference effects (Table 9). Time between strength and endurance training was considered the least important variable to consider. This finding is somewhat surprising as research has indicated allowing sufficient time (≥6 hours) between strength and aerobic stimuli allows strength development to occur uninhibited (17,34). In addition, elite Kayakers have been reported to separate strength and aerobic training sessions by 6–8 hours to allow full glycogen restoration (17). The majority of practitioners scheduled strength and Olympic lifting sessions (72 and 79% respectively) on the same day as high-quality RU sessions; however, the recovery period afforded between sessions was not detailed.

Twenty-seven (63%) practitioners believed that strength before endurance training was more conducive to strength development rather than vice versa. Researchers have reported similar magnitudes of strength development when strength training is conducted prior endurance training and vice versa (6,20,36). However, Collins and Snow (7) reported maximal strength development was greater when strength training was conducted subsequent to endurance training rather than vice versa. In contrast, it has been reported that in well-trained individuals, strength training performance is lessened for up to 8 hours after aerobic type training (39), which over time may result in muted strength development. As such, it presently remains unclear which order of concurrent strength and aerobic training is most favorable for strength development and how it should be programmed in sports such as RU, which require both strength and aerobic physical qualities.

From analysis of survey data, key research findings emerged. Physical testing was commonly conducted amongst practitioners with body composition, max speed, muscular power, and strength and acceleration being the most commonly tested variables. Olympic lifting was widely prescribed within strength training and most practitioners used periodization strategies when programming. Most respondents consider the interference effect associated with concurrent strength and aerobic training and many believed that it was an important factor to consider whilst programming. Periodization was identified as the most common programme variable to consider when attempting to avoid any muting effect of endurance stimulus on strength/hypertrophic development, whereas time between strength and aerobic stimuli was considered the least important. With further regard to concurrent training, most practitioners believed that strength before endurance training was more favorable for strength development than vice versa. Unresisted/free sprinting was the most popular method of speed development, and plyometrics were the second most popular. Plyometrics were also prescribed by almost all practitioners for the development of physical qualities such as speed, power, and acceleration.

Practical Applications

This study describes S&C and concurrent training practices of practitioners supporting RU athletes in the Northern and Southern hemispheres. As most respondents supported international and/or professional level RU athletes, practitioners now have a source of data describing S&C practices at the elite end of RU. Coaches and sports science practitioners who work with RU athletes at all levels of the game may use this summary of S&C practices as a resource to inform and improve their practices. Information presented in this article may also influence the design of experimental protocols in future studies investigating effects of conditioning interventions on physical performance phenotypes associated with RU performance.

Acknowledgments

The authors thank all individuals who volunteered to participate in the study. The results of the present study do not constitute any endorsement from the National Strength and Conditioning Association.

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nutrients

MDPI

Review

Protein Considerations for Optimising Skeletal Muscle Mass in Healthy Young and Older Adults

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Abstract: Skeletal muscle is critical for human health. Protein feeding, alongside resistance exercise, is a potent stimulus for muscle protein synthesis (MPS) and is a key factor that regulates skeletal muscle mass (SMM). The main purpose of this narrative review was to evaluate the latest evidence for optimising the amino acid or protein source, dose, timing, pattern and macronutrient coingestion for increasing or preserving SMM in healthy young and healthy older adults. We used a systematic search strategy of PubMed and Web of Science to retrieve all articles related to this review objective. In summary, our findings support the notion that protein guidelines for increasing or preserving SMM are more complex than simply recommending a total daily amount of protein. Instead, multifactorial interactions between protein source, dose, timing, pattern and macronutrient coingestion, alongside exercise, influence the stimulation of MPS, and thus should be considered in the context of protein recommendations for regulating SMM. To conclude, on the basis of currently available scientific literature, protein recommendations for optimising SMM should be tailored to the population or context of interest, with consideration given to age and resting/post resistance exercise conditions.

Keywords: muscle hypertrophy; muscle protein synthesis; amino acid availability; protein source; protein dose; protein timing; protein pattern; macronutrient coingestion

1. Introduction

Skeletal muscle is crucial for metabolic health and sport performance. Beyond the positive relationship between skeletal muscle mass (SMM), strength and athletic performance, skeletal muscle also plays an important, and often underappreciated, role in reducing risk of diseases such as obesity, cardiovascular disease, insulin resistance, diabetes and osteoporosis [1]. Therefore, strategies to preserve or increase SMM are vitally important for both clinical and athletic populations.

Skeletal muscle tissue displays remarkable plasticity. This plasticity allows for adaptation, including an increase in SMM. Skeletal muscle proteins are continuously being remodelled through the simultaneous processes of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). In turn, skeletal muscle protein remodeling is a prerequisite for increasing SMM [2]. Exercise and nutrition influence SMM through changes in MPS more than MPB [3]. Thus, MPS is accepted to be the dominant process of muscle remodelling responsible for regulating SMM in healthy adult humans. Whilst a high degree of muscle remodelling also is associated with other phenotypic adaptations, including the repair of old and/or damaged muscle proteins and modifications to the type and functionality of muscle proteins, the present review refers to skeletal muscle protein remodelling in the context of optimising muscle mass.

Protein or amino acid feeding stimulates MPS at rest [4] and during exercise recovery [5]. Thus, it follows that protein ingestion is a key stimulus for preserving SMM under resting conditions and increasing SMM under exercise training conditions. The stimulation of MPS is fundamentally regulated by extracellular and intracellular amino acid availability [6]. Figure 1 depicts the role of amino acid availability in regulating MPS in response to amino acid/protein ingestion and exercise. Amino acid availability is modulated by several dietary factors, including the amino acid/protein source, amount ingested (as a single dose), timing, pattern and macronutrient coingestion. These factors independently and synergistically impact rates of protein digestion and amino acid absorption, the splanchnic extraction of amino acids, microvascular perfusion (capillary recruitment and dilation), the delivery of amino acids to skeletal muscle and the uptake of amino acids by skeletal muscle, and thus regulate postprandial rates of MPS. In addition, exercise enhances the ability of skeletal muscle to respond to amino acid provision [7,8]. The most likely contributing mechanism is an exercise-induced increase in blood flow to the muscle [5] that increases the delivery of amino acids to the muscle, thus increasing the provision of substrate for MPS [9]. Crucially, the responsiveness of MPS to amino acid ingestion deteriorates with advancing age [10–12]. This phenomenon is referred to as "anabolic resistance" and is thought to be mediated by impairments in each of the dietary factors introduced above.

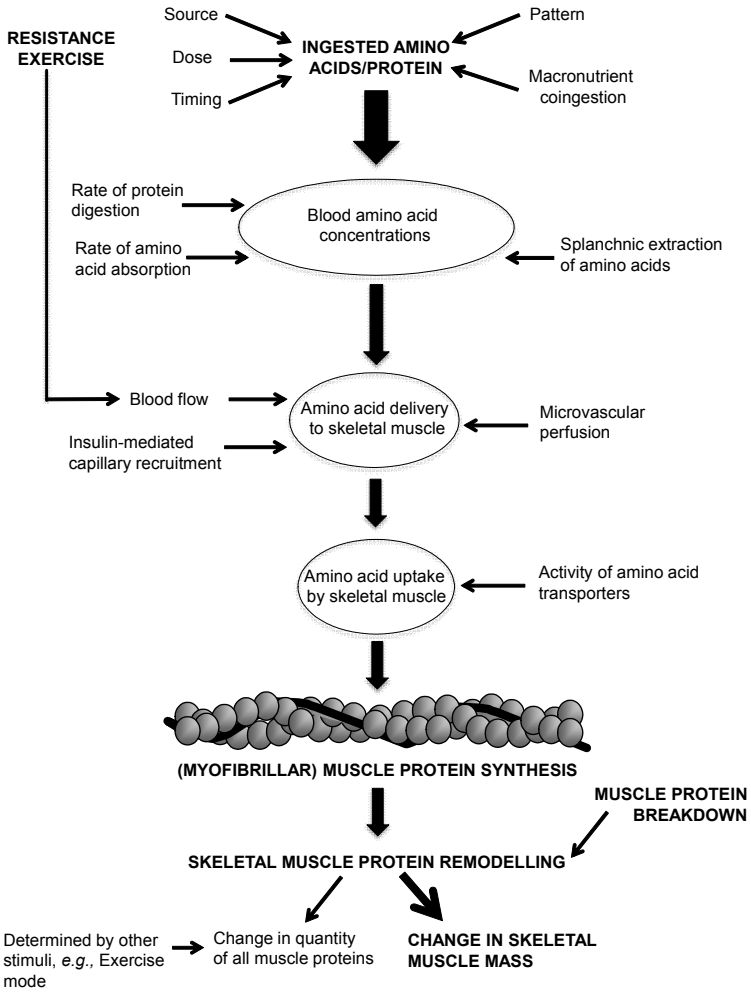

Figure 1. Simplified diagram detailing the role of amino acid availability in regulating muscle protein synthesis with amino acid/protein ingestion and exercise. Whilst resistance exercise preferentially stimulates the synthesis of contractile myofibrillar proteins (e.g., actin, myosin, troponin), resistance exercise also stimulates the synthesis of non-contractile proteins (e.g., mitochondrial and sarcoplasmic) in skeletal muscle.

To our knowledge, no previous authors have conducted a narrative review, using a systematic search strategy, to evaluate scientific evidence used to inform the latest protein recommendations for optimising MPS and SMM in healthy adult humans. Therefore, the primary objective of this review was to examine the impact of five key factors related to protein nutrition that regulate MPS, defined herein as:

i Amino acid/protein *source* refers to the origin source of ingested protein, e.g., isolated intact whey, casein or soy; animal or plant. Amino acid/protein *form* refers to the matrix form of ingested protein, e.g., liquid or solid.

ii Amino acid/protein *dose* refers to the quantity of amino acids/protein contained in a single serving.

iii Amino acid/protein *timing* refers to the timed intake of amino acids/protein in relation to exercise (before and after) or to ingestion of other nutrients.

iv Amino acid/protein *pattern* refers to the distribution pattern of ingested amino acids/protein over a given period of time, accounting for the dose, timing and frequency of Amino acid/protein ingestion.

v *Macronutrient co-ingestion* refers to the concurrent ingestion of carbohydrate (CHO) and/or fat alongside an amino acid/protein source.

For clarity, this review has been structured to address each factor of protein nutrition independently. However, an important point of discussion concerns the interaction of these factors for modulating MPS in healthy young and older adults. An understanding of recommended protein nutrition practice for optimising MPS and SMM could lead to the provision of improved advice to aid the muscle health of young and older adults.

2. Methods

A systematic search strategy was employed to identify citations for this narrative review. We searched the National Library of Medicine database (PubMed) and Web of Science from their inception through to December 2015. The terms "muscle anabolism" OR "muscle protein synthesis" OR "muscle hypertrophy" OR "skeletal muscle protein remodelling" AND "protein feeding" OR "protein ingestion" OR "protein supplementation" OR "AA ingestion" AND "humans" were entered into both databases and filters including "articles" and "humans" were used to refine the search. After initial screening of title and abstracts, selected papers were examined, including the reference lists of the retrieved articles.

Studied participants met the eligibility criteria if classified as healthy with no medical contraindications. Participants were young (mean age of studied cohort $\leqslant$35 years) and older (mean age of studied cohort $\geqslant$65 years) adult men and women, resistance-trained ($\geqslant$2 exercise sessions/week) or untrained volunteers, who were studied under resting or post resistance exercise conditions in the fed or fasted state. Several exclusion criteria were applied. We excluded intervention studies where the control condition was not considered appropriate to answer the question. For example, in the context of macronutrient coingestion, several studies included an iso-energetic CHO only [13] or a non-energetic placebo [14] rather than an amino acid/protein- only control condition. Additionally excluded were case studies and descriptive studies whereby no control group was used. Studies were excluded if they had a specific purpose of weight loss, if the method of protein intake was not oral (e.g., nasogastric/enteral intake of protein or the infusion of amino acids) and the exercise mode was not resistance-based. Finally, we excluded studies where participants were classified as patient groups (*i.e.*, not healthy, including overweight) and any non-human studies. Screening of studies resulted in the assessment of 64 citations for this narrative review. Of these, 24 citations were focused on amino acid/protein source, 8 dose, 11 timing, 6 pattern, and 15 macronutrient coingestion.

3. Synthesis of Findings

3.1. Amino Acid/Protein Source

Amino acid composition and digestive properties can vary between different isolated types of intact proteins, protein blends *vs.* isolated intact proteins and different forms of the same protein source. The Digestible Indispensable Amino Acid Score (DIAAS) is the latest and preferred index for differentiating between protein sources. The DIAAS score reflects the essential amino acid (EAA) content and digestion properties of any given protein source.

3.1.1. Isolated Types of Intact Protein

The most common comparison of intact proteins is between rapidly digested whey protein that is high in leucine content (~12.5% of total protein) and slowly digested casein protein that exhibits a relatively lower (~8.5% of total protein) leucine content. Studies in young [15] and older [16,17] adults have consistently demonstrated a greater resting postprandial stimulation of mixed-MPS with ingestion of whey compared with casein protein. However, studies that compared the response of MPS or net muscle protein balance (NBAL; difference between MPS and MPB and thus indicative of the aggregate muscle protein anabolic response) to the post-exercise ingestion of whey and casein protein report equivocal results in both young [15,18,19] and older [16,20] adults. In young adults, studies report both a greater post-exercise response of mixed-MPS to ingestion of whey protein compared with micellar casein protein [15] and also no differences in the post-exercise response of NBAL (measured over 5 h) [19] and myofibrillar-MPS (measured over a 6 h period) [18] between whey and casein conditions. Additionally, a recent study in young adults reported no difference in the chronic resistance training-induced increase in lean body mass (LBM) between whey and casein protein conditions [21]. Similarly, studies in older adults have reported both a greater post-exercise stimulation of myofibrillar-MPS (measured over a 4 h period) following ingestion of whey protein isolate compared to micellar casein [16] and also no difference in the post-exercise response of mixed-MPS (measured over a 6 h period) [20] between whey and casein protein conditions. No longitudinal endpoint study in older adults has compared intact whey and casein protein sources on any outcome measure of SMM.

The discrepant findings between studies that fed whey and casein protein after exercise, at least in terms of acute measurements of MPS and NBAL, may be reconciled by general differences in study design. These differences include the form of intact protein ingested post-exercise (whey hydrolysate, whey isolate, micellar casein or calcium caseinate), the chosen endpoint measurement of muscle anabolism (e.g., mixed-MPS, myofibrillar-MPS or NBAL) and/or the time period over which MPS or NBAL was measured after protein ingestion. Micellar casein is insoluble and therefore is often treated with alkaline compounds such as calcium hydroxide to produce calcium caseinate. This treatment alters the digestion kinetics of casein, such that the rate of blood amino acid appearance with caseinate ingestion more closely mimics whey protein compared with micellar casein protein. Interestingly, acute studies that reported a differential post-exercise response of MPS between whey and casein protein ingestion administered micellar casein [15,16]. Conversely, those studies that reported a similar post-exercise response of MPS or NBAL between whey and casein protein conditions administered calcium caseinate protein [18–20]. Taken together, these data suggest that ingesting the more rapidly absorbed caseinate elicits a greater anabolic stimulus compared with ingesting micellar casein. This insight expands other reviews [22] and the common perception that whey protein, due to amino acid composition (high EAA, BCAA and leucine content) and rapid digestibility properties, is the highest-quality intact protein source popularised in protein supplements. In summary, these data consistently demonstrate that ingestion of whey protein stimulates a greater resting postprandial response of MPS compared to casein protein in young and older adults. Similarly, a direct comparison between "fast" whey protein and "slow" micellar casein protein reveals a superior post-exercise response of MPS to whey protein ingestion in young and older adults.

Variation in the time periods over which MPS or NBAL was measured also may explain the discrepant findings. An interesting observation is that studies reporting a greater response of MPS to whey compared with casein protein conducted measurements of MPS over a 4 h period or less after protein ingestion [15,16], whereas studies reporting no differences between whey and casein conditions obtained measurements of MPS or NBAL over 5 h or more [18,19]. It is conceivable that "rapidly" digested whey protein stimulates a greater response of MPS in the early postprandial period ($\leqslant$4 h), however this advantageous "muscle protein anabolic response" is cancelled out in the late ($\geqslant$4 h) postprandial period by the more "slowly" digested casein. Whereas this notion is supported by currently available data, more studies are necessary to substantiate this speculation. Moreover, given the disparate digestive properties and subsequent differences in pattern of blood amino acid appearance between whey and micellar casein protein, physiological rationale underpins the notion that casein should be ingested pre-exercise, whereas whey protein should be ingested post-exercise. However, despite promising rationale [23] surprisingly no study has directly compared the post-exercise response of MPS to ingestion of casein protein before exercise *vs.* whey protein after exercise. Future confirmatory work in young and older adults is necessary to strengthen the quality of this evidence.

Three other direct comparisons of isolated types of intact protein have been studied in young adults: whey *vs.* soy protein which is relatively low in leucine (~7.5% of total protein) content, whey *vs.* rice protein which is slowly digested and relatively low in leucine (~8% of total protein) and casein *vs.* soy protein. A similar resting postprandial response of mixed-MPS to ingestion of whey and soy protein has been reported [15]. However, acute metabolic data that demonstrate a greater post-exercise response of mixed-MPS with whey compared with soy protein ingestion [15] are consistent with a tightly controlled longitudinal endpoint study of ~20 participants [24] that measured greater gains in LBM during a nine-month resistance training period with whey compared to soy protein supplementation. A smaller-scale (n = 12 per condition) well-controlled (administration of meal plans) study that compared whey and rice protein isolate supplementation observed similar gains in LBM between conditions during an eight-week training period [25]. Finally, greater rested and post-exercise responses of MPS were reported with soy compared with casein protein ingestion [15]. In summary, given the sparse body of evidence for each comparison (one or two studies), there remains ample scope for future work that compares the response of MPS and SMM to ingestion of various isolated types of intact protein, both from animal (e.g., egg, fish, *etc.*) and plant (e.g., lentil, quinoa, maize, hemp, *etc.*) sources in young and older adults [26].

3.1.2. Protein Blends

A protein blend combines two or more intact proteins. The scientific rationale for ingesting a protein blend is that combining more than one type of protein will capitalise on the unique digestive properties of each type of protein, allowing for an optimal blood availability of amino acids to increase the amplitude and duration of MPS stimulation. The efficacy of a protein blend for the stimulation of MPS was first evaluated by two studies in young adults that compared the ingestion of skimmed milk (casein + whey protein) with isolated soy protein [27,28]. The finding of a greater acute post-exercise response of mixed-MPS and NBAL with milk compared to soy protein ingestion [27] was extended by a longitudinal study that measured a greater increase in LBM after 12 weeks of resistance training in the milk compared to soy protein condition [28]. However, a recent study demonstrated milk ingestion elicits a similar post-exercise response of MPS compared with beef ingestion in young adults [29]. Two other studies compared the post-exercise response of MPS to ingestion of a protein blend (soy + casein + whey protein) with an isolated whey protein control in young adult men [30,31]. The protein blend composition was 25% whey protein, 50% casein and 25% soy protein. Conditions were matched for total EAA (~8.8 g) and leucine (~1.9 g) content, however, the blend condition comprised a greater total protein content compared with the whey protein condition (~19.3 *vs.* ~17.7 g). As anticipated, in both studies [30,31] the amplitude of rise in amino acid concentrations during the

early postprandial period was greater in the whey protein compared with protein blend condition. However, with the exception of valine, and to a lesser extent phenylalanine, ingestion of the protein blend failed to sustain elevated plasma amino acid (leucine, isoleucine, total BCAA) concentrations during the late (2–4 h) postprandial period compared with whey protein ingestion. Since the casein source included in the blend was sodium caseinate, which exhibits similar transient amino acid kinetics to whey protein [17,18], it was not surprising that no difference in the duration of increased amino acid availability was observed between protein blend and whey protein conditions. In both studies, the response of mixed [30] and myofibrillar [31] MPS followed the same pattern. At 0–2 and 0–4 h post protein ingestion, a similar increase in the response of MPS above basal values was observed between conditions. These data suggest that whey protein ingestion is similarly effective compared to a dose-matched (for leucine content) protein blend for the stimulation of MPS. Interestingly, despite a similar amino acid profile during late recovery, over the 2–4 h postprandial period, the response of MPS was increased above basal rates in the protein blend condition only. Although these data imply that the duration of MPS stimulation may be extended with a protein blend compared with an isolated type of intact whey protein, this observation also may be an artifact of the additional total protein content of the blend condition compared with the whey protein control. Moreover, the physiological significance of stimulating a greater response of MPS during the late (2–4 h) acute recovery period, without augmenting the aggregate (0–4 h) acute response of MPS, is not obviously apparent. Future work also is warranted to evaluate the response of MPS and SMM to other protein blend combinations, including egg, rice and hemp protein. The implications of these data are of particular relevance to the protein industry that is interested in producing cheaper and more sustainable protein-based products.

An important line of research worthy of future investigation is comparing the response of MPS to animal and plant-derived protein sources, or blends of plant-derived proteins [26]. In particular, combinations of plant-derived protein sources with divergent amino acid profiles that when combined allow for a "complete" EAA profile (e.g., relative to animal-derived proteins, wheat is low in lysine yet high in methionine, whereas lentil is high in lysine, yet low in methionine). A recent study reported a similar increase in SMM with the post-exercise ingestion of pea protein compared with whey protein [32]. However, the limited information available in humans implies that animal-derived protein sources stimulate a greater response of MPS compared with plant-derived protein sources [15,28]. However, the overall completeness, applicability and quality of evidence are weak. To date, a limited number of controlled laboratory studies in humans has directly compared the acute response of MPS to ingesting an animal-derived compared to a plant-derived protein source. No acute metabolic studies in humans have compared other animal-derived protein-rich foods, such as eggs, yoghurts, meat and fish with other plant-derived protein-rich foods, such as lentil, maize, pea, rice and wheat. The implications of these data are particularly relevant to the protein industry for aiding the production of more economically and environmentally sustainable protein-based products [33].

3.1.3. Manipulating Amino Acid Composition

Several studies have investigated the impact of manipulating the composition of an amino acid/protein source for stimulating an increased response of MPS to amino acid/protein ingestion [34–37]. In terms of amino acid profile, the leucine content of a protein source is of particular importance for stimulating a postprandial response of MPS. Leucine not only provides substrate for the synthesis of new muscle protein, but also serves as a key anabolic signal for skeletal muscle by activating enzymes within the mammalian target of rapamycin (mTOR) signalling pathway [38]. Indeed, the leucine threshold hypothesis [39] has been proposed to explain the observation that young muscle appears relatively sensitive to the anabolic action of small (~1 g) quantities of ingested leucine, whereas older muscle requires ⩾2 g of leucine (typically contained in ~20 g of high-quality protein) to increase MPS above resting rates [40]. Accordingly, studies have manipulated amino acid composition in two ways: by adding leucine to an amino acid source or modifying the leucine profile of an AA source.

In addition, longitudinal studies have investigated the impact of chronic leucine supplementation on long-term changes in SMM.

Based on available evidence, the efficacy of adding leucine to an amino acid source or modifying the leucine profile of an amino acid source for increasing the stimulation of MPS depends on the interaction of two factors. These factors include the leucine content of the original amino acid source and whether the amino acid source was ingested at rest or after exercise. Two studies in older adults demonstrated the addition of leucine (3.5/2.5 g) to a casein protein (30/20 g) source increased the resting postprandial stimulation of mixed-MPS [39,41]. Conversely, studies in young [42] and older [43] adults reported a similar post-exercise response of mixed-MPS to coingesting leucine (3.4 g) with a whey protein (16.6 g) plus CHO mixture compared to whey protein alone. With regards to modifying leucine profile, studies in young [34] and older [44,45] adults matched the dose of ingested EAA (6.7/10/10 g) between conditions, but manipulated the leucine content (2.8/3.5/3.5 g) of the ingested EAA source. Study outcomes were dependent on the dose of ingested EAA. Leucine-enriched EAA ingestion increased the resting postprandial [34] and post-exercise [44] response of MPS to a suboptimal (for maximal stimulation of MPS—see Amino Acid/Protein dose) dose of EAA, but not to an optimal (for maximal stimulation of MPS) dose of EAA in young [34,45] and [44] older adults. In summary, on the basis of available evidence, leucine coingestion and leucine enrichment effectively stimulates an increased resting postprandial response of MPS to an amino acid source, such as casein protein, that contains a relatively low leucine content (*vs.* whey). In contrast, adding leucine to an amino acid source such as whey protein that already contains sufficient leucine to stimulate a pronounced rise in blood leucine concentration, and thus surpass the leucine threshold for stimulation of MPS, is surplus to increasing post-exercise rates of MPS.

Other studies have manipulated the leucine content of a protein source. A recent study in young adults measured the resting postprandial and post-exercise response of myofibrillar-MPS to ingestion of 25 g whey protein (optimal dose) compared to 6.25 g of whey protein (suboptimal dose) in young adults [46]. Whereas the protein dose was not matched between conditions, leucine intake was equated by adding 2.25 g of leucine (to match the leucine content of the 25 g whey protein dose) to the lower protein dose, thus introducing a leucine-enriched suboptimal dose of whey protein. The impact of leucine-enriching a lower dose of whey protein on the stimulation of MPS differed between resting and post-exercise conditions. In rested muscle, ingestion of a leucine-enriched 6.25 g dose of whey protein resulted in rates of MPS similar to those stimulated with ingestion of a 25 g dose of whey protein. Likewise, ingestion of an EAA-enriched (with the exception of leucine) suboptimal dose of whey protein stimulated a similar MPS response compared with the ingestion of 25 g whey protein. However, notwithstanding the equivalent amount of leucine ingested, an inferior post-exercise response of MPS was observed with ingestion of 6.25 g of leucine-enriched whey protein compared to 25 g of whey protein. This differential response between rested and exercised states may be reconciled by the enhanced ability of muscle to utilise ingested amino acids for the stimulation of MPS following exercise [47]. Hence, it may be speculated that in this study [46], EAA availability was rate limiting for potentiating the post-exercise response of MPS to a suboptimal dose of whey protein. These results support the notion that, rather than blood leucine availability *per se*, the availability of a full complement of EAA is the critical factor for stimulating a maximal response of MPS during exercise recovery.

A follow-up study in young adults by the same authors [35] demonstrated a greater post-exercise response of MPS to ingestion of 25 g of whey protein compared with ingestion of a low dose (6.25 g) of whey protein plus additional leucine (a total of 3 g of leucine) when ingested as part of a mixed macronutrient beverage. However, ingestion of a higher dose of leucine added to 6.25 g of whey protein (totalling 5 g of leucine) resulted in a similar post-exercise response of myofibrillar-MPS to ingestion of 25 g of whey protein. Collectively, these data [35,46] suggest that enriching a suboptimal dose of whey protein with leucine may potentiate the post-exercise response of MPS to a suboptimal

protein dose, but only when the suboptimal protein dose is consumed alongside other macronutrients and is leucine-enriched above a certain undetermined threshold.

Based on the rationale that older adults often experience low levels of appetite [48] and routinely consume suboptimal doses of protein, a similar study [49] was recently conducted in older adults. The ingestion of a leucine-enriched (1.2 g) suboptimal dose of EAA (3 g) stimulated a similar resting postprandial and post-exercise response of myofibrillar-MPS compared to a 20 g whey protein bolus containing 9.6 g of EAA and 2 g of leucine. These data suggest that a less satiating (low energy) leucine-enriched suboptimal dose of EAA stimulates a similar resting and post-exercise response of myofibrillar-MPS compared with ingestion of a larger bolus dose of whey protein in older adults. Hence, fortifying a suboptimal quantity of protein with leucine may be a viable strategy for promoting MPS and increasing SMM in older adults. Given that the optimal dose of whey protein to stimulate a maximal post-exercise response of MPS has been shown to exceed 20 g in older adults (see Amino acid/Protein dose), it remains unknown if a leucine-enriched protein source rescues a maximal response of MPS in older adults. Future studies should be designed to provide a similar comparison between a leucine-enriched suboptimal protein dose (*i.e.*, 20 g of whey protein) and an optimal protein dose (~40 g of whey protein) in older adults during exercise recovery.

Finally, two studies in older adults have evaluated the impact of chronic leucine supplementation on outcome measures of SMM and reported equivocal findings [50,51]. Whereas two weeks of leucine supplementation increased the resting postabsorptive and postprandial response of MPS to a suboptimal dose of EAA plus CHO in one study [50], Verhoeven *et al.* [51] reported no change in LBM after 12 weeks of leucine supplementation. Based on these contrasting findings, the efficacy of a prolonged period of leucine supplementation on outcome measures of SMM remains unclear in older adults and warrants investigation in young adults.

3.1.4. Protein Form

Three studies in older adults have manipulated the form of an amino acid/protein source and measured resting postprandial rates of MPS [17,52–54]. Koopman *et al.* [52] compared liquid supplements of intact casein and casein hydrolysate and reported a greater blood amino acid availability, and a trend for a greater response of MPS, to ingestion of casein hydrolysate. The same research group recently reported that ingestion of casein in its naturally occurring milk matrix form resulted in a reduced blood amino acid availability (possibly due to delayed amino acid digestion/absorption kinetics), but did not modulate postprandial rates of MPS compared with ingestion of isolated intact micellar casein [53]. A similar result was reported by Pennings *et al.* [54] whereby the ingestion of minced beef, that is easily masticated and digested, stimulated a more rapid increase in arterialised blood EAA availability compared with an equivalent amount of intact steak, however no difference in the 6 h postprandial response of MPS was observed between conditions. These findings [17,53] suggest that, at least in the early resting postprandial period, the rate of blood amino acid availability does not translate into an increased stimulation of MPS. However, it must be recognised that these findings are in the context of a single feeding period under resting conditions. Whether a more rapid blood amino acid availability stimulates a greater response of MPS in the context of repeated feeding and/or during exercise recovery deserves consideration.

3.2. Amino Acid/Protein Dose

Several acute metabolic dose-response studies have been designed to characterise the optimum dose of amino acids/protein contained in a single serving for the maximal stimulation of MPS [10,47,55–58]. These studies examined a range of protein sources, including free crystalline amino acids, intact proteins and complete foods in young and older adults at rest and during exercise recovery.

3.2.1. Young Adults

The optimal dose of ingested amino acids/protein for stimulating a maximal resting postprandial response of MPS is well established in young adults. In the context of a realistic meal-like setting, ingesting a standard portion of lean beef (containing ~30 g protein) was shown to stimulate a similar response of MPS compared with an over-sized portion of lean beef (containing ~90 g protein) [59]. Although a study design that compares only two conditions does not allow for a true dose-response relationship to be characterised, these data suggest a saturable protein dose exists regarding the feeding-induced stimulation of MPS. Consistent with the notion of a saturable dose of protein, we [47] and others [10] observed a plateau in the resting postprandial response of MPS to ingesting 10 g of EAA (2.5 < 5 < 10 = 20 g) [10] or 20 g of intact whey protein (10 < 20 = 40 g) [47]. The ingestion of 20 g EAA [10] or 40 g intact protein [47] failed to elicit an additional resting postprandial stimulation of MPS. Instead, we [47] reported a pronounced stimulation of irreversible amino acid oxidation and ureagenesis, implicating a shift toward fates of ingested amino acids other than MPS. Taken together, these data [10,47] often are interpreted to suggest that, when expressed as an absolute intake, 10 g of EAA (equivalent to ~20 g of protein) is the optimal dose for stimulating a maximal response of MPS in young adults at rest. Expanding these data, a retrospective analysis of previous studies revealed that, expressed relative to body mass, the optimal protein dose for maximal stimulation of MPS in young adults at rest is 0.24 g/kg body mass/serving [60].

In young adults, the optimum dose of protein to ingest during exercise recovery is less well defined. We [47] and others [61] reported no statistical difference in the post-exercise response of MPS to ingestion of 20 compared to 40 g of protein. However, it was intriguing that both studies [47,61] reported an ~10% increase in mean values for the post-exercise stimulation of MPS when the protein dose was increased from 20 to 40 g. Given that increasing the dose of ingested protein from 10 to 20 g stimulated a ~20% greater post-exercise response of MPS without a marked increase in amino acid oxidation or urea production, a diminishing return in terms of stimulating MPS, at the very least, was achieved with ingestion of >20 g of protein [47,61]. The physiological relevance, in terms of long-term changes in SMM, of a 10% increase in the response of MPS during exercise recovery is unknown and warrants further investigation.

3.2.2. Older Adults

In older adults, the optimal dose of ingested protein at rest and during exercise recovery is not well established. Consistent with young adults, Symons *et al.* [59] reported a similar resting postprandial response of MPS to ingesting 113 g (~30 g protein) compared with 340 g (~90 g protein) of lean beef. Moreover, the seminal EAA dose-MPS response study by Cuthbertson and colleagues [10] reported a similar resting stimulation of myofibrillar-MPS with the ingestion of 20 (≈40 g protein) or 40 g (≈80 g protein) of EAA in older adults. Hence, in the context of stimulating a postprandial response of MPS, a saturable dose of ingested protein also exists in older adults. However, several recent dose-response studies of intact protein sources [55,57,58] and protein-rich foods [56] in middle-aged (~60 y) [56] and older adults [55,57,58] failed to observe a saturated response of MPS to graded protein intakes. These studies reported a dose-dependent, graded increase in the response of MPS to increasing doses (0–40 g) of intact whey protein [55,58], soy protein [57] and minced beef [56]. Since no previous study has observed a plateau in the response of MPS to increasing doses of ingested protein [55–58], the optimal single bolus dose of ingested protein for stimulating a maximal response of MPS in older adults cannot be firmly established.

Despite being inconclusive, two lines of evidence provide an informed estimate of the optimal protein dose for stimulating a maximal response of MPS in older adults. First, previous work has demonstrated that ingesting >36 g of beef protein [56] or 35–40 g of whey protein [55,58] stimulated a pronounced increase in the rate of irreversible amino acid oxidation. These data [55,58] imply the rate of MPS was approaching, or had indeed reached, an upper limit with ingestion of 35–40 g of protein. Second, the maximal effective protein dose at rest is higher in older compared with young

adults. A retrospective analysis of previous studies [60] estimated that, when expressed relative to body mass, the dose of protein required to stimulate a maximal response of MPS at rest was ~68% greater in older (0.40 g/kg body mass) *vs.* young (0.24 g/kg body mass) adults. Moving forward, to refine the optimal protein dose for the maximal stimulation of MPS in middle-aged or older adults, future studies should measure the postprandial response of myofibrillar-MPS to 0, 20–40 and 50–60 g doses of ingested protein.

In addition to age, several other nutritional, physiological and/or methodological factors could impact the optimal dose of protein for the maximal postprandial stimulation of MPS in young and older adults. Protein source has been shown to affect the dose-response relationship in older adults. A greater dose of soy protein (⩾40 g) [57] was required to stimulate a comparable postprandial MPS response to whey (⩾20 g) protein [58]. As such, a rightwards shift in the dose-response relationship was observed with soy protein compared with whey protein. Intuitively, these findings suggest that protein source alters the optimal protein dose for the maximal stimulation of MPS in older adults.

Physiological factors, including body composition and sex-differences, also may impact the dose-response relationship. It is intuitive that individual differences in SMM will affect the optimal protein dose for maximal stimulation of MPS. However, no study has compared the dose-response relationship between individuals with higher *vs.* lower amounts of SMM. Hence, a protein dose exceeding 20 g may be optimal in young adults with high amounts of SMM, particularly during exercise recovery when muscle is sensitised to protein ingestion [8]. Whereas a sex-specific difference in the response of MPS to exercise and nutrition has not been consistently shown in young adults [62–64], sexually dimorphic postprandial responses of MPS have been shown in older adults [65]. Thus, although not directly evaluated, these data suggest that sex-specific differences are more likely to affect the optimal single bolus dose of protein in older compared with young adults. Future studies are warranted to test this thesis.

3.3. Amino Acid/Protein Timing

The majority of studies have focused on the timing of amino acid/protein ingestion after exercise. Whereas resistance exercise stimulates MPS for at least 48 h during recovery, the magnitude of the post-exercise response of MPS diminishes over time (*i.e.*, 3 > 24 > 48 h) [66]. This time resolution could be explained by the notion that, as time elapses, muscle progressively loses anabolic sensitivity to protein ingestion. An extreme interpretation of this concept is the belief that the anabolic responsiveness of skeletal muscle will be impaired—or even abolished—if an amino acid/protein source is not ingested within as little as 45–60 min following exercise [67]. This time period has been coined the "anabolic window of opportunity."

The timing of amino acid/protein ingestion before and during exercise also should be considered in the context of stimulating MPS. In theory, amino acid/protein ingestion before and/or during exercise increases blood amino acid concentrations at a time when blood flow also is increased by exercise. During exercise, a net loss of muscle protein is apparent because MPS is either decreased [68] or unchanged [69], whereas MPB is (generally) increased [66]. Moreover, the stimulation of MPS by protein ingestion is refractory, with a latent period of ~1 h [70]. Intuitively, ingestion of an amino acid/protein source before or during exercise, will increase amino acid delivery to skeletal muscle during and immediately post-exercise and counteract the net loss of muscle protein during exercise and in the initial post-exercise recovery period by providing additional substrate for the stimulation of MPS.

Scientific rationale exists also to support the notion that post-exercise amino acid/protein ingestion should be timed in relation to CHO intake. The post-exercise response of NBAL to CHO ingestion is delayed until ~1 h after CHO ingestion [71]. Given that the post-exercise response of NBAL to ingested amino acids is rapid [72], one may speculate that delaying protein ingestion for 1 h after CHO ingestion may superimpose these muscle protein anabolic responses. Thus, it could be argued that amino acid/protein timing should consider the timing of other ingested nutrients, as well as proximity to exercise.

3.3.1. Time-Focused *vs.* Time-Divided Amino Acid/Protein Timing

Surprisingly few studies have compared the impact of time-focused (amino acid/protein ingestion in close temporal proximity to exercise) and time-divided (amino acid/protein ingestion at times other than close to exercise) amino acid/protein ingestion on MPS or SMM. Acute metabolic studies do not support the notion that timing amino acid/protein ingestion immediately post-exercise is critical for optimising the muscle anabolic response. These data reveal a similar response of MPS and NBAL to EAA ingestion 1, 2 or 3 h following resistance exercise in untrained young men [73–75]. Hence, it has been argued that the purported "anabolic window of opportunity" may extend beyond the first hour or less following exercise [76]. In addition, a recent study demonstrated protein ingestion 24 h following resistance exercise resulted in a greater response of MPS than protein ingested with no exercise [77]. A direct comparison of the response of MPS to ingestion of protein immediately and 24 h following exercise has yet to be made and thus the stimulation of MPS could, in fact, be slightly greater with protein ingestion immediately following, rather than 24 h after exercise. Nonetheless, it is clear, at least in young adults, that skeletal muscle is still responsive to protein ingestion for at least 24 h following exercise [77]. Thus, according to results from acute metabolic studies, the importance of immediate post-exercise amino acid/protein ingestion does not seem as critical as has often been championed [67,78].

Longitudinal endpoint studies that investigated the efficacy of timing amino acid/protein ingestion in close temporal proximity to exercise for increasing SMM, report inconsistent and, in some cases, puzzling results. A study by Cribb and Hayes [79] reported the ingestion of protein immediately before and after each training session (time-focused protein supplementation regimen) over a 10-week training period resulted in greater improvements in LBM, cross-sectional area of type II muscle fibres and strength compared with ingestion of protein before breakfast and prior to bedtime (time-divided protein supplementation regimen). Similarly, Esmarck *et al.* [80] reported SMM gains after 12 weeks of resistance training in a group of older adults that consumed a protein supplement (within a mixed macronutrient beverage) immediately after a training session, whereas no change in SMM and negligible strength gains were achieved in the group that consumed protein 2 h after exercise. However, it is easy to be sceptical about these data [80]. The magnitude of muscle hypertrophy measured with immediate post-exercise ingestion of the protein supplement was similar to that reported in other resistance training studies with older adult volunteers that included no particular feeding intervention [81,82]. Hence, on closer inspection, the results of this study [80] suggest that immediate post-exercise ingestion of protein does not confer any advantage over resistance training with unsupervised nutrition, at least in older adults. Moreover, it should be noted that waiting 2 h to ingest the protein actually inhibited the "normal" anabolic response to resistance training, making these results [80] puzzling and difficult to interpret. In contrast, other longitudinal studies in young adults fail to support the notion that protein ingestion in close temporal proximity to resistance exercise is critical for maximising SMM. Accordingly, studies by Burk *et al.* [83] and Hoffman *et al.* [84] reported time-focused protein supplementation resulted in a similar [84] or inferior [83] change in LBM after training compared to time-divided protein supplementation. Given that resistance training is an established anabolic stimulus for increasing SMM, it may be considered surprising that no improvement in LBM was observed following the training period with the time-focused supplementation regimen.

3.3.2. Pre- *vs.* Post-Exercise Timing of Protein Ingestion

Other timing considerations may hold similar importance as post-exercise protein timing for optimising the response of MPS. Indeed, ingestion of an EAA plus CHO mixture immediately pre-exercise stimulated a greater response of MPS during 2 h of exercise recovery compared with ingesting an identical EAA-CHO mixture immediately post-exercise [74]. However, an acute study of similar design in young adults, but this time ingesting intact whey protein, reported no difference in NBAL during exercise recovery between pre and post-exercise whey protein conditions [85].

Moreover, the exercise-induced stimulation of MPS was similar when a protein-containing meal was ingested 2 h prior to exercise [86] compared with when an amino acid source was provided after exercise [9,87]. Accordingly, a longitudinal endpoint study reported similar increases in LBM after 12 weeks of resistance training between groups of older adults that consumed a protein blend supplement either before or after each exercise session [88]. Taken together, these data [9,86–88] suggest that skeletal muscle is, at the very least, comparatively responsive to amino acid/protein ingested pre or post-exercise.

3.3.3. Timing of Amino Acid/Protein Ingestion in Relation to Other Nutrients

Only one study has tested the hypothesis that separating, rather than combining, the post-exercise ingestion of amino acids and CHO increases the muscle anabolic response during exercise recovery [75]. However, despite the separate ingestion of EAA and CHO stimulating a transient physiological increase in NBAL in the first 2 h of recovery, no difference in NBAL was demonstrated between combined or separate ingestion of EAA and CHO over an extended 6 h recovery period [75]. Thus, from a practical perspective, separating ingestion of EAA and CHO should be considered unlikely to be an important component of protein recommendations for maximising the muscle protein anabolic response during exercise recovery. Instead, a more simple approach of ingesting CHO and EAA together is sufficient to engender increased muscle anabolism.

3.3.4. Bedtime Protein Feeding

The timed ingestion of amino acids/protein in relation to overnight recovery is a topic of recent investigation [89,90]. It has been proposed that ingesting a protein source that releases amino acids slowly into the blood immediately prior to sleep promotes a more positive NBAL during overnight recovery [89,91]. By maintaining increased blood amino acid availability throughout the night, it may be possible to stimulate MPS and/or attenuate MPB, thereby improving NBAL during overnight recovery from exercise—a period often associated with an extended phase of negative NBAL. Indeed, the timed ingestion of protein before bedtime has been shown to increase the nighttime stimulation of MPS in young and older adults [89,91], and thus may be an effective strategy to increase muscle anabolism during overnight recovery. However, in previous studies [89,90], no time control condition was included, e.g., protein ingestion at a time point other than before bedtime. Hence, the impact of protein timing *per se* cannot be distinguished from the increased protein intake over the day.

3.4. Amino Acid/Protein Pattern

Amino acid/protein pattern accounts for the dose, timing and frequency of ingestion. A balanced pattern is characterised by the equal spread of total daily protein intake between servings, whereas, an unbalanced pattern—shown to be the norm for young [92] and older [93] adults—is characterised by consuming a large proportion of total daily protein intake in a single serving, usually in the evening meal. The aggregate daytime response of MPS is a direct function of the cumulative MPS response to each individual protein serving during the course of a day. In theory, the divergent profiles of blood amino acid concentrations associated with manipulating the timing and frequency of amino acid/protein intake during the course of a day will explain differences in the cumulative response of MPS to balanced and unbalanced protein meal patterns. Accordingly, acute metabolic studies have investigated the influence of amino acid/protein feeding pattern on the aggregate daytime stimulation of MPS while longitudinal endpoint studies have investigated the influence of protein meal pattern on chronic changes in SMM and strength.

3.4.1. Young Adults

Four studies in young adults have investigated the influence of protein pattern on the daytime stimulation of MPS or chronic changes in SMM [94–97]. Acute metabolic studies are not comparable given the discrepancies in research design including exercise state (rest *vs.* post-exercise), and protein

feeding regimen (intact protein *vs.* mixed macronutrient meals). Moreover, the unbalanced pattern implemented in these study designs may be considered somewhat extreme and not reflective of real-world practice. These studies provide ~70% of total daily protein intake in the evening meal [96] which is more than typically consumed during dinner under free-living conditions. Areta *et al.* [94] demonstrated a greater 12 h post-exercise response of myofibrillar-MPS to distributing 80 g of whey protein as 4 × 20 g servings compared with 2 × 40 g servings 6 h apart, or 8 × 10 g servings 1.5 h apart. In a more practical study design, Mamerow *et al.* [96] demonstrated a greater 24 h resting postprandial response of MPS to a balanced meal pattern that distributed 90 g of protein evenly between three meals (3 × 30 g), spaced 3.5–4 h apart *vs.* a conventional [92,93] unbalanced protein meal pattern that biased 70% of daily protein intake towards the evening meal. Hence, despite an equal total daily protein intake (90 g) between conditions, the aggregate daytime stimulation of MPS was greater with a balanced compared to unbalanced protein feeding pattern. A theoretical explanation for the improved aggregate daytime stimulation of MPS with a balanced protein meal pattern may be attributed to the muscle full effect [98] and thus repeatedly reaching the leucine threshold for the maximal acute stimulation of MPS. However, these data are not supported by a recent short-term acute metabolic study [97] that demonstrated no difference in the 3 h resting response of MPS to ingestion of 15 g of EAA either as a single bolus or distributed between four small boluses. Moreover, the only published chronic study by Arnal and colleagues [95] reported no changes in LBM following 14 days of either a balanced or unbalanced protein meal pattern. However, a drawback of this study [95] was that 2/4 meals contained 13–15 g of protein, rather than the optimal 20 g dose [47,61]. At this juncture, acute [96,97] and chronic studies [95] in young adults investigating the influence of protein pattern on MPS and SMM provide inconsistent results. Future studies in young adults should be designed to compare a balanced *vs.* unbalanced distribution pattern of daily protein intake on the daytime stimulation of MPS (under resting and post-exercise conditions) and training-induced changes in SMM, whilst taking into consideration the established optimal dose of protein contained in a single serving for young adults.

3.4.2. Older Adults

Two studies have investigated the influence of protein meal pattern on the response of MPS and SMM in older adults [99,100]. In contrast to studies in young adults, no study has reported that protein meal pattern affects the aggregate response of MPS to total daily protein intake. Kim and colleagues [100] reported no difference in the 22 h response of MPS to an unbalanced pattern that biased 65% of daily protein intake towards the evening meal compared with a balanced pattern that spread total daily protein intake evenly between meals. In this study [100], the balanced pattern consisted of three meals that each contained a protein dose (~37 g) that was likely sufficient for stimulating a maximal resting postprandial response of MPS in older adults [55,58,100]. However, the statistical power of this dataset [100] may be considered to be insufficient given that the sample size of the unbalanced group was only four participants. The only published chronic study by Arnal and colleagues [99] reported no changes in LBM following 14 days of either a balanced or unbalanced protein meal pattern. Thus, on the basis of statistical analysis, results are consistent between acute [100] and chronic [99] studies that investigate the influence of protein pattern on MPS and SMM. To date, no study has investigated the influence of protein feeding pattern on the aggregate post-exercise response of MPS to daily protein intake in older adults.

3.5. Macronutrient Coingestion

Irrespective of whether protein is consumed in food (mixed-macronutrient meal) or supplement (liquid beverage or solid bar) form, it is often coingested with CHO and/or fat. Hence, it is important to understand the impact of macronutrient coingestion on MPS and SMM.

3.5.1. Carbohydrate Coingestion

Macronutrient coingestion alters physiological factors known to regulate the stimulation of MPS. CHO coingestion increases plasma insulin concentrations compared to CHO [101] or protein [102] alone and the anabolic action of insulin on muscle protein metabolism is two-fold. First, under conditions of sufficient amino acid availability [103,104], insulin increases amino acid delivery to skeletal muscle (a rate limiting step in the stimulation of MPS) by increasing capillary recruitment and microvascular perfusion [105]. Second, insulin initiates a suppression of MPB via the ubiquitous proteasome pathway [106]. Therefore, CHO coingestion theoretically has the potential to facilitate the stimulation of MPS and suppress the stimulation of MPB.

A systematic series of hypothesis-driven studies has investigated the influence of CHO coingestion on the response of muscle anabolic response to an amino acid/protein source. Based on available evidence, the efficacy of CHO coingestion to increase the muscle anabolic response and SMM in response to amino acid/protein ingestion is dependent, at least in young adults, on the dose of ingested amino acids/protein. Two acute metabolic studies indicate that coingesting CHO with ~6 g of amino acids increased the muscle protein anabolic response in young adults, compared with the independent ingestion of amino acids [107,108]. These findings of a 60% greater utilisation of ingested amino acids [108] and suppression of urinary 3-MH excretion [107]—a crude marker of MPB—in response to exercise with CHO-amino acid coingestion indicate a greater acute stimulation of MPS and inhibition of myofibrillar-MPB, respectively. Accordingly, the findings of Bird *et al.* [107] were extended to a longitudinal training study [109] whereby young adults achieved greater gains in type II muscle fibre cross-sectional area after 12 weeks of resistance training when consuming a CHO plus amino acid-containing supplement during each exercise session compared with an amino acid-only supplement. As detailed previously, in the absence of sufficient blood amino acid availability [9], the anabolic action of a CHO-mediated increase in blood insulin concentration is likely to target a suppression of MPB, rather than stimulation of MPS [3]. Prior work demonstrated the insulin-mediated suppression of MPB to be linearly graded up to an insulin concentration of ~30 uU/mL [106]. Taken together, these data in young adults suggest the increased muscle anabolic response to coingesting CHO with small ($\leqslant$6 g) doses of EAA is mediated by a suppressed response of MPB [106,107,109]. To date, no study has investigated the impact of coingesting CHO with a suboptimal dose of protein (rather than amino acids) on MPS in young or older adults.

A handful of acute metabolic studies in young [3,102,110,111] and older [110,112] adults report that coingesting CHO with a moderate/large dose of amino acid/protein elicits no change in rested [102,110,112] or post-exercise rates of MPS [3,102,111] or MPB [102]. Consistent with these data [3,102,110–112], similar improvements in LBM, fibre-specific muscle hypertrophy and strength were reported when resistance-trained young males consumed either a protein or mixed protein-CHO supplement immediately after each exercise bout of a 10 weeks resistance-training period [79]. This absence of an additive effect of protein and CHO was evident despite CHO coingestion stimulating a robust increase in circulating insulin concentrations [102,111]. Given that basal insulin concentrations are known to be sufficient for stimulating MPS in the presence of amino acids [106], the insulin response to moderate or large protein doses could be considered sufficient to saturate mTORC1 signalling, thus rendering the CHO-mediated increase in insulin concentration permissive for increasing the stimulation of MPS.

3.5.2. Fat Coingestion

Preliminary, albeit inconsistent, evidence also suggests that fat coingestion increases the muscle anabolic response [113–115]. Mechanistic studies have demonstrated that increasing free fatty acid concentrations in blood had no impact on the responsiveness of NBAL to amino acid ingestion [114,115]. Moreover, results from a recent study demonstrated that coingesting milk fat with casein protein failed to increase the postprandial stimulation of MPS in older adults [53]. In contrast, a study of greater physiological relevance by Elliot *et al.* [113] demonstrated that ingestion of whole-fat milk stimulated

a superior post-exercise utilisation of ingested amino acid compared with ingestion of skimmed-fat milk matched for volume (239 g) and similar in protein content (8.0 *vs.* 8.8 g, respectively). To date, no study has directly assessed the response of MPS to coingesting fat with an amino acid/protein source under rested or exercised conditions in young or older adults.

A topic of recent interest is the role of fish oil derived long chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) in increasing MPS and SMM [116–119]. Studies in young and middle-aged [119] or older [118] adults have demonstrated that eight weeks of LC *n*-3 PUFA supplementation increased MPS rates, and the phosphorylation status of signalling proteins (mTORC1-p70S6k1 signalling) known to regulate MPS, in response to the intravenous infusion of combined amino acids and insulin. Irrespective of age, no change in basal MPS was observed with LC *n*-3 PUFA supplementation [118,119]. These data [118,119] suggest that, rather than exerting a direct anabolic effect on muscle protein, LC *n*-3 PUFA sensitise skeletal muscle to potent anabolic stimuli, such as amino acids and insulin. Moreover, a prolonged period of supplementation with LC *n*-3 PUFA was shown to enhance muscle mass and function at rest [117] and resistance training-induced improvements in muscle strength and functional capacity in older adults [116]. However, in this study [116], no measurements of SMM were collected and therefore the impact of LC *n*-3 PUFA supplementation, in combination with exercise training, on chronic changes in SMM remains unknown.

Two causal mechanisms are proposed to underpin the anabolic action of LC *n*-3 PUFA. First, LC *n*-3 PUFA may exhibit intrinsic muscle protein anabolic properties by modifying the lipid profile of the muscle phospholipid membrane [118,119]. These structural changes in membrane properties may activate membrane-bound anabolic signalling proteins, such as focal adhesion kinase (FAK) and the downstream anabolic target proteins, protein kinase B (PKB) and mechanistic target of rapamycin (mTORC1) [120]. Secondly, the potential anabolic action of LC *n*-3 PUFA supplementation also may be related to a modulated inflammatory response [121]. The next logical step for this new research topic is to investigate the role of LC *n*-3 PUFA supplementation in sensitising skeletal muscle to more physiologically relevant anabolic stimuli, such as resistance exercise and protein feeding in young and older adults.

4. Conclusions and Future Perspectives

Protein guidelines for increasing or preserving SMM are more complex than simply recommending a total daily amount of protein. We have identified several factors involved in protein nutrition, including the source, dose, timing, pattern and coingestion of other nutrients that independently, concurrently and additively influence MPS under resting and post-exercise conditions. Consequently, understanding the interaction between these aforementioned factors of protein nutrition and MPS is critical for contextualising protein recommendations for increasing or preserving SMM in healthy young and older adults.

4.1. Implications for Practice

On the basis of published literature collated in this review, we propose the following evidence-based implications for practice.

i Protein guidelines should be customised to the population (young or older adults) and situation (resting or post-exercise condition) of interest. For example, (a) the optimal dose of protein for maximal stimulation of MPS during exercise recovery is greater for older compared to young adults and (b) whey protein has been shown to stimulate a greater response of MPS compared with soy protein during exercise recovery, but not at rest.

ii Chronic periods of leucine supplementation will not necessarily facilitate long-term improvements in SMM, given that a full complement of EAA is critical for stimulating a maximal and sustained response of MPS.

iii Manipulating the leucine content of a protein source that lacks quality (*i.e.*, the protein source constitutes a low leucine composition) and/or quantity (*i.e.*, an insufficient protein dose for the maximal stimulation of MPS) effectively rescues a submaximal resting postprandial stimulation of MPS. This phenomenon has particular implications for older adults or other populations that often experience difficulties in consuming a sufficiently large dose of protein in each meal serving to stimulate a maximal response of MPS.

iv Timing protein intake in close temporal proximity to exercise is recommended, although not critical, for stimulating a maximal response of MPS.

v Coingesting CHO with a suboptimal dose of amino acids/protein may be an effective strategy for "rescuing" a submaximal response of MPS associated with a suboptimal dose of amino acids/protein. However, no additional benefit is gained from adding CHO to a dose of amino acids/protein known to saturate the response of MPS.

vi Any beneficial impact of fat coingestion on MPS is likely mediated by the anabolic action of the LC *n*-3 PUFA.

4.2. Implications for Research

Table 1 extracts from the main body of text a multitude of future academic research directions in the field of protein nutrition. This grid has been designed to illustrate the independent or interactive effects of the several factors of protein nutrition on the stimulation of muscle protein synthesis. The placement of each question is dependent on the factor of protein nutrition addressed by the question. For example, the question "Can plant-based protein sources stimulate a similar response of MPS compared with animal-based protein sources?" relates to the independent impact of *protein source* on MPS and thus fits in the protein source-protein source space. The question, "What impact does coingesting CHO with a suboptimal dose of protein have on the stimulation of MPS in young and older adults?" relates to the interactive effect of *protein dose* and *macronutrient coingestion* on MPS and thus fits in the protein dose-macronutrient coingestion space. As a general point, current protein recommendations are primarily informed by research designs whereby protein beverages are administered commonly as an isolated protein source. By characterising the response of MPS to the single and multiple bolus ingestion of mixed-macronutrient meals or supplements, it will be possible to tailor more practical and personalised nutrition advice regarding what foods/supplements should be consumed, how much of a food/supplement should be consumed and when food/supplements should be consumed on both rest and exercise training days.

In terms of future perspectives, from a methodological standpoint the field is entering an exciting period to study the role of protein nutrition in modulating muscle protein metabolism [122]. Specifically, a recently validated oral deuterium oxide isotope tracer protocol allows for the relatively non-invasive measurement of free-living, integrated rates of MPS over an intermediate time period (e.g., 1–14 days) [123,124] that, in the future, should be extended to longer time periods [125]. Hence, quantifying fraction-specific rates of MPS to represent skeletal muscle protein remodelling in response to perturbations such as resistance exercise and protein ingestion is possible over acute, intermediate and potentially chronic time periods. Such tools will inevitably expand our existing knowledge regarding protein considerations for optimising SMM in both healthy young and older adults.

As a closing remark, there are a distinct lack of data in females and middle-aged (40–55 years old) adults. Since sex-differences in the response of MPS to feeding have been reported [63,65], future studies should investigate the impact of protein feeding on MPS and SMM in cohorts of female volunteers.

Table 1. Proposed future research directions to promote understanding of how several factors of protein nutrition interact to impact the stimulation of muscle protein synthesis (MPS) at rest and during exercise recovery in young and older adults.

	Source	**Dose**	**Timing**	**Pattern**	**Coingestion**
Source	Can plant protein sources stimulate a similar response of MPS compared to animal protein sources in young and older adults? Do liquid-based forms of ingested protein stimulate a greater response of MPS compared to solid-based forms of protein foods?		What impact does protein source have on the optimal timing of protein ingestion in young adults?	What impact does protein source have on the optimal protein meal pattern for the daytime stimulation of MPS in young and older adults?	
Dose	What impact does protein source have on the optimal protein dose for stimulation of MPS in young adults?	What is the maximal effective dose of protein for the stimulation of MPS in older adults? What influence does individual lean body mass have on the optimal protein dose for stimulation of MPS?			What impact does macronutrient coingestion have on the optimal protein dose for stimulation of MPS in young adults?
Timing	How does the response of MPS during exercise recovery compare between the pre-exercise ingestion of casein *vs.* the post-exercise ingestion of whey protein?		Does the overnight stimulation of MPS with bedtime protein feeding translate into long-term gains in skeletal muscle mass?		What impact does macronutrient coingestion have on the optimal protein timing for stimulation of MPS in young and older adults?
Pattern		What impact does protein dose have on the optimal pattern of protein feeding for the aggregate daytime stimulation of MPS?		What is the impact of protein feeding pattern, combined with exercise, on the aggregate daytime stimulation of MPS in older adults?	
Coingestion		What impact does coingesting carbohydrate with a suboptimal dose of protein have on MPS in young and older adults?		Does the ingestion of protein within mixed macronutrient meals impact the optimal protein meal pattern for the daytime stimulation of MPS?	What is the impact of long chain *n-3* polyunsaturated fatty acid supplementation on the response of MPS to exercise and protein feeding in young and older adults?

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Abbreviations

The following abbreviations are used in this manuscript:

BCAA	branched chain amino acids
EAA	Essential amino acids
LBM	lean body mass
MPS	muscle protein synthesis
MPB	muscle protein breakdown
NBAL	net muscle protein balance
SMM	skeletal muscle mass

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ORIGINAL RESEARCH

Fish oil supplementation suppresses resistance exercise and feeding-induced increases in anabolic signaling without affecting myofibrillar protein synthesis in young men

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Abstract

Fish oil (FO) supplementation potentiates muscle protein synthesis (MPS) in response to a hyperaminoacidemic–hyperinsulinemic infusion. Whether FO supplementation potentiates MPS in response to protein ingestion or when protein ingestion is combined with resistance exercise (RE) remains unknown. In a randomized, parallel group design, 20 healthy males were randomized to receive 5 g/day of either FO or coconut oil control (CO) for 8 weeks. After supplementation, participants performed a bout of unilateral RE followed by ingestion of 30 g of whey protein. Skeletal muscle biopsies were obtained before and after supplementation for assessment of muscle lipid composition and relevant protein kinase activities. Infusion of L-[*ring*-$^{13}C_6$] phenylalanine was used to measure basal myofibrillar MPS at rest (REST), in a nonexercised leg following protein ingestion (FED) and following RE and protein ingestion (FEDEX). MPS was significantly elevated above REST during FEDEX in both the FO and CO groups, but there was no effect of supplementation. There was a significant increase in MPS in both groups above REST during FED but no effect of supplementation. Supplementation significantly decreased panPKB activity at REST in the FO group but not the CO group. There was a significant increase from REST at post-RE for PKB and AMPKα2 activity in the CO group but not in the FO group. In FEDEX, there was a significant increase in p70S6K1 activity from REST at 3 h in the CO group only. These data highlight that 8 weeks of FO supplementation alters kinase signaling activity in response to RE plus protein ingestion without influencing MPS.

Introduction

Muscle protein synthesis (MPS) is an important metabolic determinant of human skeletal muscle mass (Glynn et al. 2010; McGlory and Phillips 2014). Resistance exercise and provision of a source of essential amino acids (EAAs) are potent stimulators of MPS (Witard et al. 2014). Thus, repeated bouts of resistance exercise and protein feeding result in skeletal muscle hypertrophy (Cermak et al. 2012). The anabolic influence of protein ingestion and resistance exercise on skeletal muscle has led to studies examining the influence of the type (Tang et al. 2009) and dose (Witard et al. 2014) of protein on rates of MPS. Collectively, these studies have shown that in young male adults the consumption of ~0.25 g/kg of high-quality protein results in the saturation of both

rested postprandial (Witard et al. 2014) and postexercise rates of MPS (Moore et al. 2009; Witard et al. 2014) with higher doses of protein resulting in excess urea production and amino acid oxidation (Moore et al. 2009; Witard et al. 2014). The failure of protein doses above 0.25 g/kg to further enhance rates of MPS is related to the inability of the translational machinery to utilize the excess available amino acids for the purposes of protein synthesis, a phenomenon termed the muscle full effect (Bohe et al. 2001; Atherton et al. 2010).

Despite using different doses of amino acids (Moore et al. 2009; Witard et al. 2014) as well as the coingestion of carbohydrate (Staples et al. 2011), no nutritional mechanism has been shown to alter this muscle full effect. However, while the capability of carbohydrate (Staples et al. 2011) and individual amino acids (Churchward-Venne et al. 2014) to enhance the MPS response to protein ingestion has been studied in detail (Tipton and Phillips 2013), the role of fatty acids in increasing the utilization of ingested protein for the stimulation of MPS has only recently received attention. In this regard, recent studies have demonstrated that supplementation with n-3 polyunsaturated fatty acid (PUFA)-enriched fish oil (FO) also confers skeletal muscle anabolic responses. For instance, 8 weeks of FO derived n-3 PUFA supplementation was shown to potentiate rates of mixed MPS in response to a hyperaminoacidemic–hyperinsulinemic infusion in young, middle-aged (Smith et al. 2011b), and older adults (Smith et al. 2011a). Additionally, supplementing elderly women with FO during 12 weeks of resistance exercise training has been demonstrated to improve skeletal muscle strength (Rodacki et al. 2012), while one study has shown 6 months of FO supplementation, in the absence of resistance exercise, improves muscle mass and function in elderly men (Smith et al. 2015). Thus, it appears that FO supplementation enhances the n-3 PUFA composition of skeletal muscle (Smith et al. 2011a, b; McGlory et al. 2014a), which subsequently primes skeletal muscle to respond to anabolic stimulation either in the form of amino acid provision (Smith et al. 2011a, b) or mechanical stimulation (i.e., resistance exercise).

Although there is growing evidence for the efficacy of FO supplementation to enhance muscle anabolism, there remain several practical considerations that need to be addressed. First, the administration of amino acids through an intravenous infusion (Smith et al. 2011a, b) is not a viable means for the general population to consume protein. Second, it is clear that the metabolic response to infusion of amino acids differs from ingestion of an intact protein (Bohe et al. 2001). In the context of an infusion, a square-wave response of aminoacidemia results in a refractory response such that MPS declines even as aminoacidemia remains constant and elevated (Bohe et al. 2001). On the other hand, protein ingestion results in a rapid increase and subsequent decrease in aminoacidemia that stimulates a maximal postprandial response of MPS in young adults (Moore et al. 2009; Witard et al. 2014). Finally, measurements of mixed MPS have been made (Smith et al. 2011a, b), instead of the myofibrillar fraction that is critical for contractile function. Thus, whether FO supplementation potentiates rates of myofibrillar MPS in response to the oral ingestion of a dose of protein, known to stimulate a maximal response of MPS, remains unknown. In addition, it is unknown whether FO supplementation potentiates rates of myofibrillar MPS when oral protein consumption is combined with resistance exercise. Therefore, the primary aim of this study was to investigate the impact of 8 weeks of FO supplementation on the response of myofibrillar MPS to resistance exercise and protein ingestion. To address this aim, we employed a unilateral single leg resistance exercise protocol that allowed us to separate the influence of FO supplementation on the response of myofibrillar MPS to protein ingestion under resting and post exercise conditions within subject. The secondary aim was to investigate the influence of FO supplementation on the activity of kinases involved in protein feeding and resistance exercise-induced increases in myofibrillar MPS.

Materials and Methods

Participants

Twenty resistance-trained males were recruited from the University of Stirling and surrounding area to participate in the present investigation. Participant characteristics are displayed in Table 1. Prior to the commencement of the experiment each participant provided written informed consent after all procedures and risks of the

Table 1. Characteristics of participants in each group.

Parameter	Fish oil (n = 9)	Coconut oil (n = 10)
Age (yr)	24 ± 0*	21 ± 0
Body mass (kg)	87.0 ± 2.6*	80.0 ± 8.2
Lean body mass (%)	77.0 ± 1.3	76.0 ± 1.3
Body fat (%)	20.0 ± 1.5	20.0 ± 1.4
LP 1RM (kg)	143.0 ± 8.0*	134.0 ± 7.1
LP/kg/BM	2.13 ± 0.1	2.25 ± 0.1
LE 1RM (kg)	68.0 ± 2.5*	60.0 ± 2.5
LE/kg/BM	1.01 ± 0.1	1.01 ± 0.0

yr, years; kg, kilogram; LP, leg press; LE, leg extension; 1RM, one repetition maximum; BM, body mass. Values expressed as mean ± standard error of the mean.
*Denotes significantly higher than coconut oil group ($P < 0.05$).

study were fully explained in lay terms. All procedures conformed to the standards as outlined in the latest version of the Declaration of Helsinki. Following health screening, participants were excluded if they consumed any form of dietary supplementation or were taking any prescribed medication. The East of Scotland Research Ethics Service (EoSRES, Rec No: FB/12/ES/0005) approved the study procedures.

Experimental design

In a randomized, between-groups, repeated measures design, participants were assigned to either a FO (n = 10) or coconut oil condition (CO; n = 10). Due to an analytical processing error, one participant from the FO group was removed from statistical analysis (FO; n = 9). Coconut oil was chosen as a control as coconut oil does not contain any n-3 or n-6 PUFAs. Thus, coconut oil will not change the n-6/n-3 ratio as would corn oil or another PUFA. Moreover, there is no evidence that coconut oil has any impact on muscle protein metabolism.

During each visit to the laboratory, participants were verbally requested to confirm their pattern of oily fish consumption in an attempt to ensure that changes in free-living oily fish consumption did not influence muscle lipid profiles during the study. Following baseline testing for single leg 1 repetition maximum (RM) on leg press and leg extension as well as body composition using dual-energy X-ray absorptiometry (Lunar iDEXA; GE Healthcare, Hertfordshire, UK), participants reported to the laboratory in the fasted state on two separate occasions. During the initial visit a resting muscle sample was obtained for the assessment of muscle phospholipid fatty acid profiles and also for baseline activity of muscle-specific anabolic signaling kinases (70 kDa ribosomal protein S6 kinase 1 [p70S6K1], pan protein kinase B [PKB], adenosine monophosphate–activated protein kinase [AMPK] α1 and AMPKα2). Following baseline measurements, participants consumed 5 g/day of n-3 PUFA-enriched FO capsules (providing 3500 mg eicosapentaenoic acid [EPA, 20:5n-3], 900 mg docosahexaenoic acid [DHA, 22:6n-3], 100 mg docosapentaenoic acid [DPA, 22:5n-3] and 0.1 mg vitamin E; Ideal Omega-3; Glasgow Health Solutions Ltd, Glasgow, UK) for 8 weeks. Compliance to the supplementation protocol was assessed by pill count. Following the end of the supplementation period participants returned to the laboratory to participate in the experimental trial, during which the assessment of myofibrillar MPS was made to examine the influence of FO supplementation on the response of MPS to protein ingestion under resting (FED) and postexercise (FEDEX) conditions using a single leg exercise model. Participants were requested to complete a 3-day food diary questionnaire for 3 days prior to baseline testing and to repeat this pattern of consumption for 3 days leading up to the experimental trial.

Experimental trial

A schematic illustration of the experimental trial is displayed in Figure 1. On the morning of the trial, participants entered the laboratory at ~0700 h after a 10-h overnight fast. Each participant then rested in a semisupine position at which time a cannula was inserted into the forearm vein of each arm for blood sampling and L-[*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, Tewksbury, MA) infusion. After an initial baseline blood sample was drawn a primed, continuous infusion (prime: 2.0 μmol/kg; infusion ~0.05 μmol/kg/min) of L-[*ring*-$^{13}C_6$] phenylalanine was started, the arm heated, and frequent arterialized blood samples obtained. After a 3-h resting period a resting skeletal muscle biopsy was obtained as described previously (Witard et al. 2014) for the assessment of basal myofibrillar MPS rates and muscle phospholipid fatty acid composition. Following the muscle biopsy, participants performed a bout of

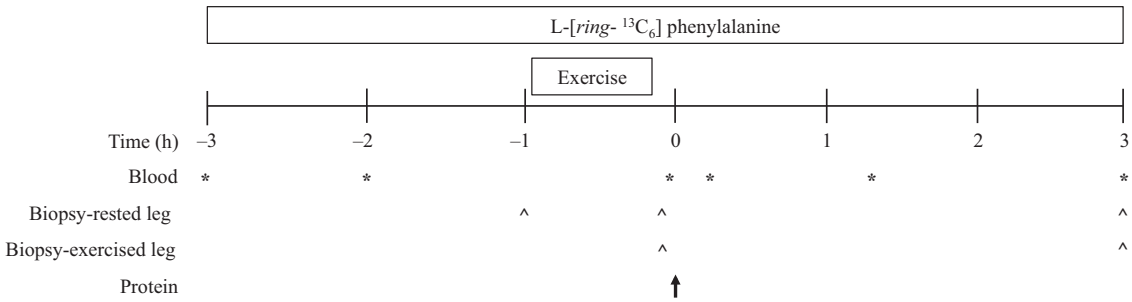

Figure 1. Schematic diagram of the experimental protocol. Initially, a baseline blood sample was drawn followed by a 3-h resting period. A muscle biopsy was then obtained followed by a bout of high-intensity unilateral resistance exercise. After the completion of the exercise bout, two muscle biopsies were extracted, one from the exercised leg and one from the rested leg immediately followed by the consumption of 30 g of whey protein. Participants were then rested in a bed for 3 h until a further muscle biopsy was obtained from each leg again.

high-intensity unilateral resistance exercise. The exercise bout consisted of 3 sets of 10 repetitions of leg press and leg extension (Cybex International Inc, Cybex International, MA) performed at 70% of individual 1 RM. A 2-min rest period was allotted between sets and a 3-min rest period between exercises. After the completion of the exercise bout, two muscle biopsies were performed, one on the exercised leg (FEDEX) and one on the rested leg (FED) immediately followed by the consumption of 30 g whey protein (~0.35 g/kg) diluted in 300 mL of water. Thereafter, participants rested in a bed for 3 h until a further muscle biopsy was obtained from each of the FEDEX and FED legs. All muscle samples were rinsed in ice-cold saline, blotted to minimize blood saturation of the sample and freed from any visible fat and/or connective tissue. Muscle samples were then frozen in liquid nitrogen and stored at −80°C until further analysis.

Analytical procedures

Blood plasma amino acid concentrations

Plasma amino acid concentrations were determined through use of the Phenomenex EZ:fast amino acid analysis kit with gas chromatography–mass spectrometry (GC Model 6890 Network, Agilent Technologies (Santa Clara, CA); MSD model 5973 Network, Agilent Technologies) as per the manufacturer's specifications.

Skeletal muscle phospholipid extraction and analysis

Total lipid content was determined by extraction of lipids from the tissue using 20 volumes of ice-cold chloroform/methanol (2:1 v/v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK) as reported previously (Folch et al. 1957). Nonlipid impurities were isolated by washing with 0.88% (w/v) KCl and the lower solvent layer containing the lipid extract dried under oxygen-free nitrogen. The phospholipid fraction was prepared from 0.5 mg of total lipid applied to a 20 × 20-cm silica gel 60 TLC plate (VWR, Lutterworth, Leicestershire, UK) and developed in isohexane–diethyl ether–acetic acid (80:20:1, by volume) before drying for ~3 min at room temperature. The plate was sprayed lightly with 2,7-dichlorofluorescein (0.1%, w/v) in 97% methanol (v/v) and the phospholipid bands then were scraped from the plate and placed in a 15-mL test tube. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transesterification in 2 mL of 1% H_2SO_4 in methanol at 50°C overnight. The samples were neutralized with 2.5 mL of 2% $KHCO_3$ and extracted with 5 mL isohexane–diethyl ether (1:1, v/v) BHT. The samples then were re-extracted with 5 mL isohexane–diethyl ether (1:1) and the combined extracts were dried and dissolved in 0.3 mL of isohexane prior to FAME analysis. FAME were separated by gas–liquid chromatography using a Thermo Fisher Trace GC 2000 (Thermo Fisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60 m × 0.32 × 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150°C at 40°C/min and then to 195°C at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were then identified according to previously published data (Tocher and Harvie 1988). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy).

Myofibrillar protein synthesis

Myofibrillar MPS was calculated using the precursor–product equation:

$$\text{myofibrillar MPS} = ([E_{2b} - E_{1b}]/[E_{ic} \times t]) \times 100$$

where E_b represents the enrichment of bound myofibrillar protein, E_{ic} is the average intracellular enrichment between two biopsies, and t is the tracer incorporation time in hours. As we employed "tracer naïve" participants (had not previously participated in a study protocol where L-[*ring*-$^{13}C_6$] phenylalanine was infused), a preinfusion blood sample was used for the calculation of resting myofibrillar MPS (Churchward-Venne et al. 2012). Myofibrillar and intracellular enrichments of L-[*ring*-$^{13}C_6$] phenylalanine were measured as described previously (Churchward-Venne et al. 2012). Briefly, for the determination of intracellular enrichments ~25 mg of muscle was homogenized in 0.6 mol/L perchloric acid and the liberated amino acids in the supernatant passed over an ion-exchange resin (Dowex 50WX8-200 resin Sigma-Aldrich, Dorset, UK) and converted to a heptaflurobutyric derivative for analysis using a gas chromatography–MS. For myofibrillar enrichment, ~50 mg of wet weight muscle tissue was homogenized on ice in buffer (10 mL/mg muscle of 25 mmol/L Tris 0.5% v:v triton X-100 and protease/phosphatase inhibitor cocktail tablets; Complete Protease Inhibitor Mini-Tabs, Roche, Indianapolis, IN; PhosSTOP, Roche Applied Science, Roche) and centrifuged at 15 000 ***g*** for 10 min at 4°C to separate the supernatant (sarcoplasmic) and pellet (myofibrillar) fractions. The myofibrillar fraction was then hydrolyzed for 72 h in 0.1 mol/L HCl and Dowex (50WX8–200 resin; Sigma-Aldrich) at 110°C and mixed on a vortex every 24 h. The free amino acids were purified with the use of

Dowex ion exchange chromatography, and the *N*-acetyl-n-propyl derivative was prepared and run on an isotope ratio MS to measure the bound enrichment of L-[*ring*-$^{13}C_6$] phenylalanine.

Kinase activity

Activity assays were conducted as described previously (McGlory et al. 2014b). Briefly, ~30 mg of human skeletal muscle tissue was homogenized by scissor mincing on ice in RIPA buffer (50 mmol/L Tris/HCl, pH 7.5; 50 mmol/L NaF; 500 mmol/L NaCl; 1 mmol/L Na vanadate; 1 mmol/L EDTA; 1% [vol/vol] triton X-100; 5 mmol/L Na pyrophosphate; 0.27 mmol/L sucrose; and 0.1% [vol/vol] 2-mercaptoethanol, and complete protease inhibitor cocktail [Roche]) followed by shaking on a shaking platform for 60 min at 4°C. Debris was removed by centrifugation at 4°C for 15 min at 13 000 ***g***. The supernatant was then removed and protein concentration determined using the BCA protein assay according to the manufacturer's instructions (Sigma-Aldrich). All assays were carried out by immunoprecipitation either for 2 h at 4°C or overnight at 4°C in homogenization buffer (AMPK [50 mmol/L Tris-HCl pH 7.25, 150 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L NaPPi, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L benzamidine, 0.1 mmol/L PMSF, 5 μg/mL soyabean trypsin inhibitor, 1% (v/v) TritonX-100] and p70S6K1/panPKB [50 mmol/L Tris-HCl pH 7.5, 0.1 mmol/L EGTA, 1 mmol/L EDTA, 1% (v/v) tritonX-100, 50 mmol/L NaF, 5 mmol/L NaPPi, 0.27 mol/L sucrose, 0.1% β-mercaptoethanol, 1 mmol/L $Na_3(OV)_4$, and 1 Complete [Roche] protease inhibitor tablet per 10 mL).

Statistical analysis

Anthropometric and 1 RM data were assessed using a between-groups Student's *t* test. All other data were analyzed using two-factor (treatment; FO vs. CO) repeated measures (time) analysis of variance (ANOVA). When a significant effect of treatment and/or time was detected a Tukey's post hoc analysis was applied to identify where differences existed. Statistical analysis was conducted using statistical package for the social sciences (SPSS) version 18.0 (IBM, Hampshire, UK). Significance was set at $\alpha < 0.05$ and all data are expressed as means ± SEM.

Results

Blood plasma amino acid concentrations

In both groups, plasma concentrations of total amino acids, EAAs, and free leucine were significantly above baseline at 15 min and 75 min following resistance exercise and protein feeding ($P < 0.05$) but returned to resting values after 180 min ($P > 0.05$; Fig. 2A–C). The increase in plasma concentrations of EAAs and leucine in the FO group were significantly higher than the CO group at 15 min postexercise and protein feeding ($P < 0.05$; Fig. 2B–C), but the plasma concentration of leucine was significantly lower in the FO group compared to the CO group at 75 min postexercise and protein feeding ($P < 0.05$; Fig. 2C).

Skeletal muscle phospholipid composition

All phospholipid profile changes in muscle are shown in Table 2. The % n-3 PUFA of total fatty acids was marginally higher before supplementation in the FO group compared to the CO group ($P < 0.05$). However, after supplementation there was a ~twofold increase in the % n-3 PUFA of total fatty acids ($P < 0.05$), whereas in the CO group % n-3 PUFA of total fatty acids remained unchanged ($P > 0.05$). In contrast, % n-6 PUFA of total fatty acids was significantly lower before supplementation in the FO group compared to the CO group ($P < 0.05$). Although the % n-6 PUFA of total fatty acids was significantly lower after supplementation in FO versus CO ($P < 0.05$), the % n-6 PUFA of total fatty acids in CO remained unchanged pre–post supplementation ($P > 0.05$). The % monounsaturated fatty acids of total fatty acids was significantly higher before supplementation in CO compared with FO ($P < 0.05$), however % monounsaturated fatty acids was reduced after supplementation in CO ($P < 0.05$) only. There was no significant difference in % saturated fatty acids of total fatty acids between groups before the intervention, however the % saturated fatty acids of total fatty acids significantly decreased after supplementation in both groups ($P < 0.05$). There was no significant difference in % dimethyl acetals of total fatty acids between groups before the intervention, however were significantly increased after supplementation in both groups ($P < 0.05$), but to a greater extent in the FO group ($P < 0.05$).

Myofibrillar protein synthesis

The response of MPS was greater in FED compared with REST in both FO (0.025 ± 0.002 to 0.069 ± 0.006% per hour, $P < 0.05$) and CO (0.024 ± 0.002 to 0.056 ± 0.005% per hour, $P < 0.05$), however no difference in the feeding-induced stimulation of MPS was observed between FO and CO ($P > 0.05$). In FEDEX, MPS was significantly elevated above REST in both FO (0.025 ± 0.002 to 0.091 ± 0.006% per hour, $P < 0.05$)

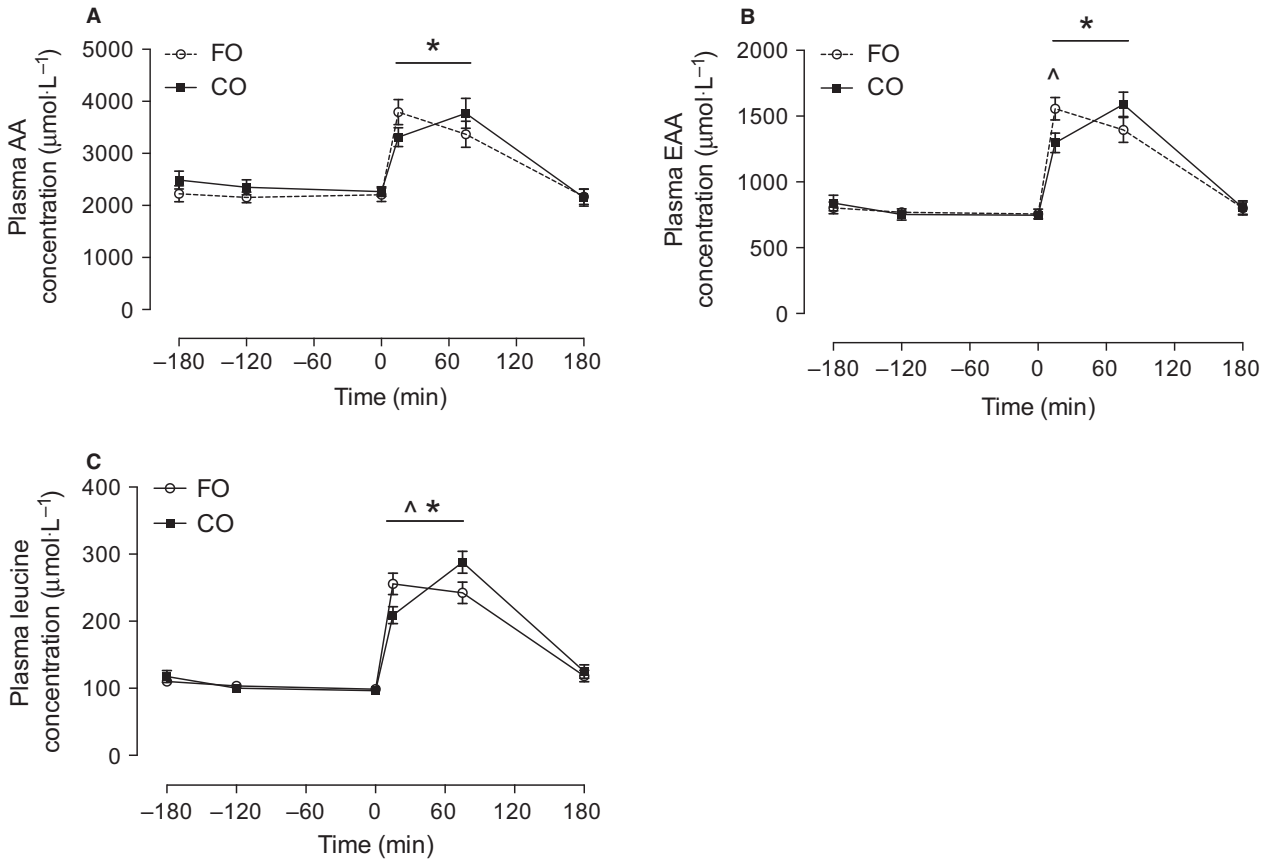

Figure 2. Blood plasma concentrations (μmol/L) of total amino acids (AA) (A), essential amino acids (EAA) (B), and leucine (C). *Denotes significantly different ($P < 0.05$) from all other time points. ^Denotes significant difference between groups. Data expressed as mean (±SEM).

and CO (0.024 ± 0.002 to 0.077 ± 0.005% per hour, $P < 0.05$), however, similar to the FED condition, no difference in the feeding plus exercise-induced stimulation of MPS was observed between FO and CO ($P > 0.05$; Fig. 3A). There also was no significant effect of supplementation on rates of myofibrillar MPS when expressed as percentage change from REST in FED or FEDEX ($P > 0.05$; Fig. 3B).

Kinase activity in response to supplementation

There were no differences between groups before supplementation in the activity of panPKB, AMPKα1, AMPKα2, or p70S6K1 ($P > 0.05$). panPKB activity was significantly suppressed ($P < 0.05$) at REST compared to before supplementation in the FO group only, indicating that 8 weeks of FO supplementation suppressed basal panPKB activity (Fig. 4A). However, there was no impact of supplementation in either group on the basal activity of AMPKα1, AMPKα2, or p70S6K1 ($P > 0.05$; data not shown).

Kinase activity in response to protein feeding (FED)

There was no impact of protein feeding on the activity of p70S6K1, AMPKα1, AMPKα2, or panPKB in either group ($P > 0.05$; data not shown).

Kinase activity in response to protein feeding and resistance exercise (FEDEX)

In response to resistance exercise pan PKB activity was significantly increased from REST at post-RE in the CO group only ($P < 0.05$; Fig. 4B). P70S6K1 activity was significantly elevated ($P < 0.05$) at 3 h FEDEX from REST in the CO group (Fig. 4C). However, there was no impact of resistance exercise and protein feeding on p70S6K1 activity post-RE or at 3 h FEDEX in the FO group ($P > 0.05$; Fig. 4C). There also was no impact of supplementation on the AMPKα1 response to resistance exercise at post-RE or at 3 h FEDEX ($P > 0.05$; Fig. 4D). However, in the CO group, in response to resistance exercise, AMPKα2 was significantly increased at post-RE from

Table 2. Muscle phospholipid fatty acid profile changes.

	Fish oil		Coconut oil	
	Before	After	Before	After
Saturated fatty acids				
14:0	0.37 ± 0.01	0.33 ± 0.02	0.32 ± 0.02	0.30 ± 0.02
15:0	0.18 ± 0.01	0.14 ± 0.00	0.15 ± 0.01	0.13 ± 0.01
16:0	18.96 ± 0.33	16.25 ± 0.10	18.87 ± 0.34	16.87 ± 0.38
18:0	14.16 ± 0.25	12.72 ± 0.13	14.10 ± 0.12	12.92 ± 0.17
20:0	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.02
22:0	0.16 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.20
24:0	0.18 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.18 ± 0.03
Total	34.09 ± 0.45[a]	29.83 ± 0.14[b]	33.83 ± 0.36[a]	30.61 ± 0.40[b]
Monounsaturated fatty acids				
16:1n-9	0.17 ± 0.01	0.19 ± 0.01	0.15 ± 0.00	0.16 ± 0.01
16:1n-7	0.37 ± 0.02	0.33 ± 0.02	0.42 ± 0.01	0.39 ± 0.02
18:1n-9	6.05 ± 0.16	4.74 ± 0.20	6.25 ± 0.21	5.92 ± 0.29
18:1n-7	2.01 ± 0.06	1.86 ± 0.06	1.94 ± 0.06	1.89 ± 0.07
20:1n-9	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
24:1n-9	0.20 ± 0.01	0.20 ± 0.01	0.22 ± 0.02	0.24 ± 0.03
Total	8.89 ± 0.14	7.35 ± 0.24	9.07 ± 0.22[a]	8.69 ± 0.36
n-6 polyunsaturated fatty acids				
18:2n-6	26.87 ± 0.59	24.17 ± 0.65	29.19 ± 0.52	28.72 ± 0.60
18:3n-6	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.09 ± 0.01
20:2n-6	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
20:3n-6	1.29 ± 0.04	1.14 ± 0.03	1.30 ± 0.09	1.48 ± 0.09
20:4n-6	13.55 ± 0.56	13.11 ± 0.38	12.67 ± 0.34	13.79 ± 0.47
22:4n-6	0.44 ± 0.03	0.30 ± 0.02	0.65 ± 0.03	0.75 ± 0.04
22:5n-6	0.35 ± 0.02	0.20 ± 0.01	0.39 ± 0.01	0.34 ± 0.02
Total	42.69 ± 0.26[a]	39.08 ± 0.43[b]	44.39 ± 0.37[c]	45.30 ± 0.45[c]
n-3 polyunsaturated fatty acids				
18:3n-3	0.25 ± 0.02	0.21 ± 0.01	0.26 ± 0.01	0.24 ± 0.01
20:5n-3	1.16 ± 0.12	4.46 ± 0.22	0.65 ± 0.05	0.69 ± 0.06
22:5n-3	1.48 ± 0.06	2.27 ± 0.08	1.29 ± 0.06	1.47 ± 0.06
22:6n-3	2.64 ± 0.18	4.22 ± 0.23	1.55 ± 0.16	1.79 ± 0.21
Total	5.53 ± 0.30[a]	11.16 ± 0.45[b]	3.74 ± 0.23[c]	4.16 ± 0.31[c]
Dimethyl acetals				
16:0DMA	5.32 ± 0.28	7.59 ± 0.12	5.35 ± 0.12	6.70 ± 0.23
18:0DMA	1.88 ± 0.11	2.68 ± 0.13	1.96 ± 0.07	2.43 ± 0.08
18:1DMA	1.62 ± 0.07	2.312 ± 0.07	1.67 ± 0.08	2.11 ± 0.09
Total	8.81 ± 0.38[a]	12.58 ± 0.13[b]	8.97 ± 0.23[a]	11.25 ± 0.29[c]

Data expressed as % total fatty acids, mean ± SEM. Means that do not share a letter are significantly different.

REST ($P < 0.05$; Fig. 4D). There was no impact of resistance exercise on AMPKα2 post-RE or resistance exercise and protein feeding at 3 h FEDEX in the FO group ($P > 0.05$; Fig. 4D).

Discussion

The novel finding from the present study is that despite a twofold increase in the n-3 PUFA composition of skeletal muscle, FO supplementation did not significantly enhance rates of myofibrillar MPS at REST nor in either FED or FEDEX condition compared to CO. However, FO supplementation did result in a reduction in resting pan PKB activity, and attenuate p70S6K1 activity at 3 h post-resistance exercise. As such, these data may suggest that FO supplementation alters anabolic signaling processes, without modulating rates of myofibrillar MPS in response to protein ingestion, or when resistance exercise precedes protein ingestion in healthy, resistance-trained young males.

Our finding that the rate of myofibrillar MPS was not significantly greater in FED in the FO-supplemented state is in contrast to previous reports in which 8 weeks of FO supplementation was shown to potentiate rates of mixed MPS in response to a hyperaminoacidemic–hyperinsuline-

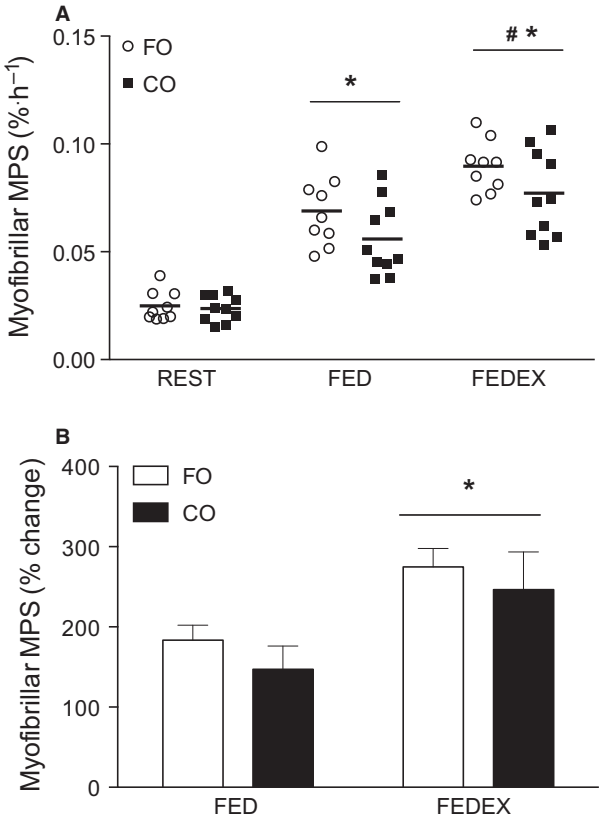

Figure 3. Rates of myofibrillar muscle protein synthesis (MPS) (% per hour) calculated during rest (REST) following protein ingestion (FED) and when protein ingestion was preceded by a bout of resistance exercise (FEDEX). *Denotes significantly different ($P < 0.05$) from REST (A) and FED (B). #Denotes significantly different ($P < 0.05$) from other time points (A). Data expressed as mean and individual responses for (A) and mean (±SEM) for (B).

mic infusion (Smith et al. 2011a, b). The lack of agreement between the findings of our study and that of the aforementioned reports (Smith et al. 2011a, b) could be due to differences in muscle fractions assessed as we measured myofibrillar MPS and previous studies (Smith et al. 2011a, b) measured protein synthesis rates of mixed muscle proteins. Additionally, the study population (resistance trained vs. untrained) or the method of amino acid administration (oral vs. intravenous) could also be a contributor to the differences. Indeed, infusion to create a condition of hyperaminoacidemia–hyperinsulinemia as used by Smith et al. (2011a, b) was such that aminoacidemia would be suboptimal for stimulating postprandial MPS. Indeed, in the studies by Smith et al. (2011a, b) plasma leucine concentrations were clamped at ~165–175 μmol/L. In contrast, we provided an oral whey protein bolus of 30 g equating to 0.35 g/kg that has been shown previously to maximally stimulate rates of myofibrillar MPS in young men (Moore et al. 2009; Witard et al. 2014), and in our study resulted in peak plasma leucine concentrations of ~250–300 μmol/L in both conditions. We propose it is possible that the ingestion of 30 g of whey protein in the current study maximized rates of myofibrillar MPS to the extent that FO supplementation would not have exerted a further anabolic influence or was undetectable. In an analogous scenario, our reasoning may explain why the addition of carbohydrate to a saturating protein dose failed to enhance rates of MPS (Staples et al. 2011). We speculate that a "potentiated" MPS response may have been observed if the protein dose our subjects ingested was less than maximally effective.

Similar to the results in the FED condition, we did not observe a significant stimulatory effect of FO supplementation on myofibrillar MPS in FEDEX. Again, we postulate that it is possible that rates of myofibrillar MPS had already been saturated with the combined effect of feeding and exercise (Witard et al. 2014), therefore preventing the detection of any potentiation of myofibrillar MPS with FO supplementation. In contrast to our results, supplementation of elderly women with FO during 12 weeks of resistance exercise training enhanced skeletal muscle strength and functional capacity (Rodacki et al. 2012). Even in the absence of resistance exercise one study has shown 6 months of FO supplementation improves muscle mass and function in elderly men (Smith et al. 2015). We do acknowledge that the present study did not assess changes in muscle strength making a direct comparison between our investigation and others (Rodacki et al. 2012; Smith et al. 2015) difficult. However, it is important to acknowledge that older individuals require a greater amount of protein to maximize rates of MPS compared to young (Yang et al. 2012; Moore et al. 2015), and older adults often fail to consume adequate amounts of protein throughout the day (Fulgoni 2008). Therefore, in these longitudinal studies (Rodacki et al. 2012; Smith et al. 2015) in which protein intake was not controlled, and we speculate, suboptimal, it is plausible that feeding and exercise-induced rates of MPS were also suboptimal, and thus a potentiation by FO supplementation on MPS and muscle mass was observed.

To examine the impact of FO supplementation on anabolic signaling molecules we employed radiolabeled $^{[\gamma-32P]}$ATP kinase assays for AMPKα2, pan PKB, and p70S6K1 (McGlory et al. 2014b) that is a quantitative readout of kinase activity. Using this method we show that 8 weeks of FO supplementation suppressed the activity of pan PKB at rest as well as AMPKα2 immediately following exercise, and p70S6K1 3 h postexercise and feeding. Since the PKB-mTORC1-p70S6K1 signaling axis has been shown to be a key phosphorylation cascade regulating MPS (Drummond et al. 2009; Dickinson et al. 2011), our finding of suppressed pan PKB and p70S6K1

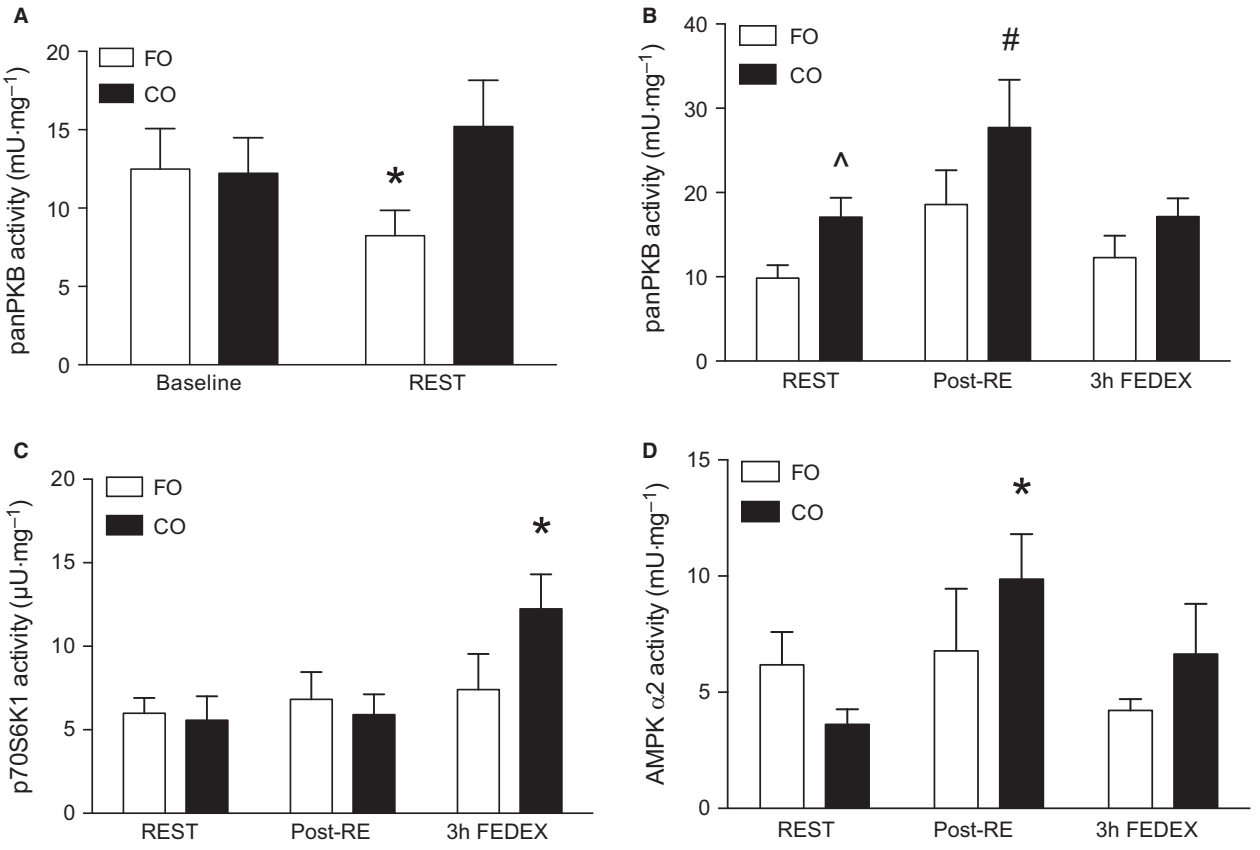

Figure 4. Changes in the activity of panPKB (mU/mg) in response to fish oil (FO) supplementation (A). Changes in the activity of panPKB (mU/mg), p70S6K1 (μU/mg), and AMPKα2 (mU/mg) immediately following resistance exercise (post-RE), and at 3 h following resistance exercise and protein feeding (FEDEX). *#Denotes significantly different ($P < 0.05$) from all other time points, ^denotes significantly different ($P < 0.05$) from the FO group at rest (REST). Data expressed as mean (±SEM).

activity without a concomitant reduction in rates of myofibrillar MPS may be considered surprising. Indeed, studies have shown that FO supplementation induces alterations in anabolic signaling phosphorylation parallel to changes in MPS in humans following amino acid infusion (Smith et al. 2011a, b) and muscle size in rodents in response to immobilization/remobilization (You et al. 2010a, b). However, a dose–response study in rodents has shown that only a small fraction of p70S6K1 phosphorylation is required to maximize rates of leucine-feeding-induced increases in MPS (Crozier et al. 2005). Moreover, other workers have shown that in humans despite the provision of large amounts of amino acids and insulin, rates of MPS remain high in the face of relatively low levels of p70S6K1 phosphorylation (Greenhaff et al. 2008). Thus, our data could be interpreted to suggest that FO supplementation leads to a shift in the relationship between kinase signaling and MPS. That is, less kinase activity is required to maximize rates of MPS in response to oral protein feeding and resistance exercise. It is also conceivable that the timing of the muscle biopsies may have contributed to the observed disconnect between static measurements of anabolic signaling responses, and the dynamic measurement of MPS; both theses remain speculative and warrant further investigation.

The main strength of our study is that we applied the physiologically relevant stimulus of oral protein feeding and resistance exercise rather than intravenous amino acid delivery to stimulate myofibrillar MPS. In addition, the present study is one of few studies in humans that have employed a direct measure of kinase activity in conjunction with the dynamic measurement of myofibrillar MPS, as opposed to semiquantitative immunoblotting. Thus, this study adds important practical information to existing proof-of-concept studies that have employed hyperaminoacidemic–hyperinsulinemic infusions (Smith et al. 2011a, b), as well as semiquantitative assessments of kinase activity (You et al. 2010a, b) to examine the impact of FO supplementation on muscle anabolism. However, some limitations of the present study must be acknowledged. Since we elected to assess FED versus FEDEX responses using a unilateral model with a view to

minimize the number of muscle biopsies performed (i.e., a between-subjects design), it is possible that we lacked the necessary statistical power to detect the influence of FO on MPS. For example, the magnitude of change in MPS with FO supplementation in the repeated measures designs of Smith et al. was ~100% in older individuals (Smith et al. 2011a) and ~50% in younger participants (Smith et al. 2011b). In our study, the difference in the change in MPS between FO and CO was smaller (~30 and 35% in FED and FEDEX, respectively). Moreover, these differences were not statistically significant. As a result, we cannot dismiss the possibility that with a greater participant number or a repeated measures design, we may have detected a statistically significant difference between FO and CO supplementation on MPS. However, any potential impact of FO in our study, even if real and undetected, is certainly much less definitive and consistent compared with Smith et al. (2011a, b). Moreover, our measurement of myofibrillar MPS was limited to 3 h postresistance exercise and feeding. Therefore, it is possible that had we extended our capture of MPS to longer than 3 h (i.e., 5 h) we may have detected an effect of FO supplementation. Finally, our participant population was healthy, resistance-trained young men, and therefore these data cannot be directly extrapolated to females or older adults. Given the relatively larger response in the older than young adults reported by Smith et al. (2011a, b), it would be interesting to repeat our study in an older population. Thus, we emphasize that our data should not be interpreted to conclude that FO supplementation does not potentiate MPS to protein feeding and resistance exercise in all populations, and situations, but rather that these data should be evaluated in the context of the experimental design.

To conclude, we show that 8 weeks of FO supplementation does not significantly enhance rates of myofibrillar MPS in response to ingestion of 30 g of whey protein in healthy, resistance-trained young men. In addition, FO supplementation did not significantly enhance rates of myofibrillar MPS when the consumption of 30 g of whey protein was preceded by a bout of high-intensity resistance exercise. Future work examining the impact of FO supplementation in conjunction with resistance exercise training on rates of MPS over a more prolonged period, or in response to suboptimal protein ingestion, in a range of populations may provide further valuable data for the literature.

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Conflict of Interest

None declared.

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CROSSTALK

Comments on the CrossTalk proposal and opposing view: The dominant mechanism causing disuse muscle atrophy is decreased protein synthesis/proteolysis

Integrating the regulation of muscle protein synthesis and degradation?

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It is with great interest that we read the cross-talk on the proposed roles that changes in protein synthesis and protein degradation play in disuse skeletal muscle atrophy (Phillips & McGlory, 2014; Reid *et al.* 2014). However, rather than adopting an 'either/or' position on this topic, we believe that a more integrated standpoint is warranted. For example, recent evidence suggests that cells/tissues can simultaneously coordinate changes in both protein synthesis and protein degradation, including in skeletal muscle (Baehr *et al.* 2014; Zhang *et al.* 2014). Moreover, recent evidence suggests that, under certain conditions, an increase in protein degradation could lead to a decrease in protein synthesis. For instance, the eukaryotic initiation factor subunit 3f (eIF3f), which plays a crucial role in protein synthesis, is targeted for degradation by the E3 ligase atrogin-1, and preventing the atrogin-1-induced poly-ubiquitination of eIF3f provides protection against starvation-induced muscle atrophy (Csibi *et al.* 2009, 2010). Similarly, myostatin-induced myotube atrophy is not only associated with an increase in the expression of components of the ubiquitin proteasome system, but also an increase in the degradation of various translation initiation and elongation factors, ribosomal proteins, and a decrease in the rate of protein synthesis (Lokireddy *et al.* 2011, 2012). While these data are derived from cell culture and animal models, we must consider the possibility that similar mechanisms may also operate in human models of disuse atrophy, whereby an increase in proteolysis could play a significant role in the decrease in protein synthesis.

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Additional information

Competing interests

None declared.

The dominant cause of disuse atrophy: a debate hindered by fallacy of evidence?

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Although both viewpoints deliver articulate arguments, to us, the notion of any singular mechanism *per se* dominating muscle atrophy responses to disuse is both premature and most likely overly simplistic. As is the case with many adaptive processes, disuse atrophy (as with hypertrophy!) follows a temporal pattern, i.e. with greater changes dominating initial phases (Wall *et al.* 2014), thereafter abating (Adams *et al.* 2003). To suggest synthesis *or* breakdown is dominant disregards the dynamic nature of protein balance and the likely scenario of each process contributing to different extents at different time-points. To us, this is as much an issue as opposing views being a cacophony 'of mice and men' (though the present authors agree with Phillips & McGlory this may explain some existing discordances). Additionally, whilst Reid *et al.* (2014) justly state: 'the dominant mechanism of disuse atrophy is not known', this does not preclude one transpiring – with appropriate time course studies. Moreover, suggestions: 'rates of proteolysis *vs.* synthesis cannot be quantified using existing methods nor can differences in these rates be measured directly', are misleading. There are ways to quantify synthesis and breakdown *rates* concomitantly in muscle, *in vivo,* using combinations of stable isotope tracers. For example, A–V balance: synthesis/breakdown, net-balance (Greenhaff *et al.* 2008); pulse-chase: fractional breakdown rates (Zhang *et al.* 1996); fractional synthesis rates via amino acid tracers or D_2O (Wilkinson *et al.* 2014). Yes, such techniques require mass-spectrometry know-how, blood/muscle sampling and temporal resolution, but surely represent the best means to arrive at *bona fide* answers to these longstanding questions.

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Additional information

Competing interests

None declared.

Disagreements move science forward

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As is often the case in an academic debate, here the affirmative and negative present arguments that do not effectively counter the alternatively presented hypothesis. This is not a critique of either group; instead we propose it is methodological in nature.

One aspect the authors have not considered in their arguments is the effect of time. We have previously reported a temporal effect in the hypertrophic response in healthy individuals following resistance training, with an acute degradative response followed by increased pro-synthesis activity (Elliott *et al.* 2012), and our current work is examining this phenomenon during muscle atrophy. Granted, this is a different research model, but it is of interest to note. We would suggest therefore that perhaps both hypotheses are correct. At this time, the temporal nature of the disuse atrophy has yet to be adequately considered.

This disparity between results that report molecular changes and those that report isotope-based results drives an artificial controversy. Natural sciences often reach such impasses when different approaches conclude differing hypotheses. We propose that a careful modelling of changes in both isotope measures of protein balance and molecular markers of pathway activity, as a function of time, will produce a clearer picture of the mechanistic control of muscle mass. Careful critique of each hypothesis will lead to the rejection of one, or the combination of the two, as suggested above. At the risk of cliché, more research is clearly needed.

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Additional information

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None declared.

Redox regulation of protein turnover during unloading-induced atrophy

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The debate articulated by Drs Phillips and McGlory (Phillips & McGlory, 2014*a*,*b*) *vs.* Drs Reid, Judge and Bodine (Reid *et al.* 2014*a*,*b*) regarding the importance of decreased protein synthesis *vs.* increased protein degradation in response to unloading/disuse is a fascinating one indeed. Skeletal muscle is a highly dynamic tissue with robust protein turnover that not only produces force and power for joint motion, but also serves as a substrate source of amino acids. Amino acid demand from skeletal muscle is enhanced for protein needs when nutrient sources are insufficient, common in our hunter–gatherer ancestors, or when injury and illness occur (Wolfe, 2006). Further, teleological arguments suggest that reduction in protein synthesis due to disuse would save energy and substrate resources.

While the mechanisms that alter protein turnover and atrophy with unloading are not fully understood, pro-oxidant (Dodd *et al.* 2010; Min *et al.* 2011) and pro-inflammatory (Judge *et al.* 2007) signalling appear to be important effectors of atrophy and fibre-type switch. Disuse increases the prevalence of oxidized proteins (Lawler *et al.* 2014). Oxidized amino acids could 'tag' proteins for degradation, particularly when levels of chaperone heat shock proteins are low (Senf *et al.* 2008). Accumulation and cross-linking of partially oxidized proteins could lead to cellular toxicity, thus providing impetus for rapid removal. Recently, reactive oxygen species (ROS) have recently been linked to (a) translocation of nNOSμ and downstream activation of FoxO3a with unloading (Lawler *et al.* 2014), and (b) suppression of Akt phosphorylation and anabolic signalling (Rahman *et al.* 2014). Future research should focus on prospective mechanisms that tie together unloading-induced responses in protein synthesis with proteolysis.

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Additional information

Competing interests

None declared.

Timing is of the essence

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Abbreviations: MPS, muscle protein synthesis; MPB, muscle protein breakdown.

We appreciated the scientific debate raised in the CrossTalk series regarding the physiological mechanisms underpinning muscle disuse atrophy (Phillips & McGlory, 2014). As stated by Phillips & McGlory, the combined efforts of multiple research groups over the past three decades have unequivocally shown a decline in post-absorptive and post-prandial muscle protein synthesis (MPS) rates accompanying uncomplicated, prolonged (≥2 weeks) disuse in humans (e.g. Gibson *et al.* 1987; Ferrando *et al.* 1996; Glover *et al.* 2008; Wall *et al.* 2013*b*). However, comparable data of how disuse affects muscle protein breakdown (MPB) in humans are simply not yet available. We have recently shown that as little as 5 days of disuse already leads to considerable muscle atrophy (Wall *et al.* 2013*a*), an impact that is unlikely attributed solely to a decline in MPS. However, to date, no *in vivo* human data of how MPB or MPS are affected during this first week of disuse have been generated.

Calculating expected muscle loss based on changes in MPS (and comparing this with observed muscle loss) as a means to indirectly infer changes (or lack thereof) in MPB is a tantalizing but challenging approach. Though relative changes in MPS or muscle mass can be assessed accurately in human disuse studies, the precise numerical value of both parameters are subject to variance depending upon methodological choices. Indirect evidence from humans concerning molecular pathways involved in proteolysis suggests that MPB rises rapidly and transiently at the onset of disuse, which may represent a mechanism to initiate the atrophy process (Wall *et al.* 2013*a*). Consequently, the appropriateness of potential countermeasures for disuse atrophy may be dependent upon the speed at which they are implemented. In this way, clinically and scientifically, timing may really be of the essence!

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Additional information

Competing interests

None declared.

Both proteolysis and decreased protein synthesis are important in causing disuse muscle atrophy

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Skeletal muscle forms the bulk of body weight and accounts for ∼50% of body mass in young adult males. In addition to its main functions of postural control and the powering of movement, skeletal muscle is important in the maintenance of body shape, structure and composition. It is also the main store of proteins in the body. As a result, skeletal muscle mass is highly regulated and is maintained by the fine balance between anabolism (protein synthesis) and catabolism (protein degradation). Both processes are closely coupled and are regulated by an intricate intracellular signalling pathway controlled by the protein kinase AKT (also known as protein kinase B) (Nader, 2005). Thus, anabolic signals such as resistive exercise, growth factors such as insulin-like growth factor (IGF) 1 and hormones such as male sex steroids lead to its activation (= phosphorylation) and in turn this leads to increased protein synthesis. In contrast, catabolic signals such as disuse, lack of weight bearing and pro-inflammatory cytokines, e.g. tumour necrosis factor (TNF) α lead to its inactivation. The inactivation of AKT is accompanied by decreased protein synthesis and the activation of the ubiquitin proteasome system. Activation of this system leads to increased protein proteolysis. Consequently, it is impossible to separate decreased protein synthesis from increased proteolysis. As a result, we suggest that disuse atrophy arises from a combination of both mechanisms and none is more dominant than the other.

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Additional information

Competing interests

None declared.

Love and marriage go together like a horse and carriage

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Both propositions fail to recognise that while the magnitude of change in protein synthesis after feeding and/or exercise is large, muscle growth and hypertrophy are slow temporal processes requiring multiple bouts of stimuli. This is particularly evident when compared with rapid muscle loss following even acute disuse. Therefore, protein degradation is perhaps a more sensitive process despite smaller fluctuations in magnitude compared with adaptive physiological responses underpinned by protein synthesis. It is worth reiterating that for muscle atrophy following disuse to occur, complex macromolecular protein structures must be dismantled and ultimately degraded. The question therefore seems not to be which is the dominant mechanism, but rather, after disuse what are the temporal regulators driving physiological change; which process comes first and informs the other; and finally, which is a more sensitive process. Paradoxically, it is worth noting that protein degradation is an important adaptive process for the post-loading response, culminating in early remodelling and repair to enable later hypertrophy. Therefore future research should extend the work that has already been undertaken regarding the cross-talk between signalling mechanisms of synthesis and degradation, which are inextricably linked, for example pro-synthetic Akt inhibition of pro-degradative FOXO and vice versa (Sandri *et al.* 2004; Edström *et al.* 2006), or the duality of function of p38 MAPK (first described by Gillespie *et al.* 2009). Therefore, we suggest degradation and synthesis orchestrate gains or losses in tandem; as the old saying goes, 'love and marriage go together like a horse and carriage/you cannot have one without the other'.

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Competing interests

None declared.

Muscle atrophy – a balanced approach

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While wary of sitting on the fence it seems to me that the dichotomy between breakdown and synthesis as 'causing' muscle atrophy is a false one. More than that, surely this presumed conflict is sterile as it does not lead to new insights for therapies. Growth or atrophy arises from an altered signal of muscle activity, which generally, but is not necessarily, a consequence of actual muscle activity. A more holistic approach asks, 'What processes are required to maintain adequate function in response to that signal?' Atrophy would follow a loss of this maintenance signal.

It is clearly the balance between synthesis and breakdown that determines muscle size. Both synthesis and breakdown may increase or decrease in response to stimulus or disuse, and changes in one may regulate changes in the other. It is the resulting balance, however, that will determine whether a muscle grows or atrophies. Reid *et al.* indicate that measuring this balance biochemically is not practical but clearly the outcome – size – can be measured using imaging. A more fundamental question is what controls that balance, as it is here that interventions are more likely to be fruitful. Control requires a signal, a monitoring process and a mechanism that takes the monitored signal and converts it into a response, ideally with a small amount of feedback. Identifying and being able to monitor/adjust this mechanism would enable intervention at a high level. Setting synthesis against breakdown is at the lowest level and risks generating heat rather than light.

Additional information

Competing interests

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Time-course studies hold the key to establishing the dominant mechanism of disuse skeletal muscle atrophy in humans

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This topical CrossTalk debate presents two opposing viewpoints concerning the predominant mechanism that governs 'uncomplicated' disuse skeletal muscle atrophy in humans. Consistent with other experts (Wall & van Loon, 2013), these viewpoints agree that both a suppression of muscle protein synthesis (MPS) and acceleration of muscle protein breakdown (MPB) likely explain the more rapid muscle loss during early ($<$5 days) compared with late ($>$10 days) phases of disuse ($\sim$1.2 *vs.* 0.5% day^{-1}). As such, to fully elucidate the predominant mechanism underpinning disuse muscle atrophy, it is necessary to characterize transient changes in muscle protein metabolism over a prolonged time-course of disuse. This challenging prospect will be facilitated by recent developments in the application of deuterium oxide (^2H_2O) isotope tracer methodology. The validation of oral ^2H_2O isotope tracer protocols allows for the

dynamic *in vivo* measurement of integrated rates of myofibrillar MPS (MacDonald *et al.* 2013; Wilkinson *et al.* 2014) and MPB (Holm *et al.* 2013) over acute (hours), intermediate (days) and chronic (weeks) time periods. Therefore, moving forward, future time-course studies (Brocca *et al.* 2012) should apply ^2H_2O tracer methodology to measure temporal changes in MPS and MPB during early (<5 days), intermediate (5–10 days) and prolonged (>10 days) phases of muscle disuse. These data will help establish the predominant mechanism that drives muscle atrophy during early through to later phases of disuse. Moreover, these important data will help inform targeted interventions (exercise, pharmaceutical, nutritional) for ameliorating human muscle atrophy and the associated hypodynamia over a prolonged time-course of muscle disuse.

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Additional information

Competing interests

None declared.

There is more to skeletal muscle disuse than a dichotomy between decreased protein synthesis or increased protein breakdown

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The topic of this crosstalk is important. Too much time has been spent on trying to simplify the driving factor of human disuse to a dichotomy between decreased protein synthesis or increased protein breakdown, digging trenches between human research and research based on animal data. Few scientists argue about the importance of a decrease in protein synthesis, yet it is increasingly difficult to overlook the emerging bulk of data demonstrating that the regulation of human muscle disuse is far more complex. Specifically, in human research we have previously overlooked the importance of investigating the very early phase of disuse/unloading (1–5 days) where the atrophy response is most strongly manifested and consequently important information has therefore been unnoticed. However, a growing number of studies show evidence of an early rise in atrogenes during human disuse (deBoer *et al.* 2007; Tesch *et al.* 2008; Abadi *et al.* 2009; Suetta *et al.* 2012) with time-course patterns similar to what have previously been demonstrated in the murine model (Sacheck *et al.* 2007).

Another important point that has been overlooked in human research for years is the age-specific way human disuse atrophy seems to be regulated (Suetta *et al.* 2012) and equally important also the phase of muscle re-growth with subsequent reloading (Suetta *et al.* 2013). Notably, this influence of ageing on muscle mass homeostasis is well documented in various animal models. Importantly, however, many links remain missing in the puzzle of human muscle plasticity, which from our point of view can be achieved only through a close interaction between human and animal research.

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Additional information

Competing interests

None declared.

The dominant mechanism causing disuse muscle atrophy is proteolysis

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One may start to address whether enhanced muscle proteolysis or protein synthesis is the dominant mechanism of disuse-induced muscle atrophy (Phillips & McGlory, 2014; Reid *et al.* 2014) with a thought-experiment.

The second law of thermodynamics implies that any complex structure left on its own is subject to decay. In buildings, for instance, broken light bulbs need replacement, preferably at a rate that matches the rate of breakdown. If replacement stops entirely the building will slowly get darker. However, one could also actively remove light bulbs (apoptosis and the proteasome require ATP) from an unused room to ensure availability in other, utilised rooms. The latter seems to occur during denervation-induced atrophy where the abundance of apoptotic and proteasome components (Sacheck *et al.* 2007) and protein breakdown (Goldspink, 1976) are increased transiently during the period of rapid atrophy. Much of the denervation-induced atrophy was prevented by inhibition of the proteasome (Beehler *et al.* 2006). Since denervation-induced atrophy is faster and more pronounced than in many other models of disuse atrophy, it will elicit qualitatively similar, but quantitatively more pronounced, changes in protein synthesis and breakdown. Rodent models are extremely helpful in this context, as the pattern of disuse-induced atrophy is similar to, but because of the higher metabolic rate much faster than, in humans. In agreement with Reid *et al.* (2014) we suggest that an increased rate of protein breakdown is the predominant factor of disuse-induced atrophy; however, potential effects of a reduced rate of protein synthesis should not be ignored.

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Additional information

Competing interests

None declared.

Caught in the crossfire?

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Whereas we appreciate that the purpose of the CrossTalk article series is to compare and contrast two opposing viewpoints on a given topic, the desire to promote one's view at the expense of all else can often undermine the value of the scientific debate. In the current set of articles, the proposal and opposing view correctly acknowledge the integral contributions of both suppressed skeletal muscle protein synthesis (MPS) and elevated skeletal muscle protein breakdown (MPB) to the atrophied state, yet both articles by default offer little consideration to the integrated action of these two processes. The complexity of skeletal muscle atrophy, and indeed physiology, is such that the determination of either party to establish a dominant mechanism appears futile when we consider all the contributing factors and mechanisms at play. Instead, we suggest that disuse atrophy should be studied using a holistic approach where value is given to MPS, MPB and their underlying regulatory mechanisms. Although the simultaneous measurement of MPS and MPB during disuse atrophy in humans is not currently possible, advances in the techniques used to accurately measure *in vivo* MPB acutely (Tuvdendorj *et al.* 2013) and over extended time periods (Holm *et al.* 2013) will facilitate such holistic study when implemented in combination with existing MPS (Burd *et al.* 2011; Wilkinson *et al.* 2014) and signalling protein activation (McGlory *et al.* 2014) measures. Validation of these techniques for use in humans remains ongoing, yet collective efforts towards the goal of such an approach will greatly enhance our existing knowledge base of disuse atrophy and provide a raised platform for stimulating interesting and beneficial discussions for the scientific community.

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Additional information

Competing interests

None declared.

Either or is digital code, a bit of both is human metabolism

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We are perplexed by the heat of the debate regarding the relative contributions of muscle protein synthesis (MPS) and breakdown (MPB) to the disuse-induced loss of muscle mass. Phillips and McGlory argue that disuse-induced muscle atrophy is almost entirely due to a reduced rate of MPS. Meanwhile, Reid, Judge and Bodine argue that MPB is the dominant mechanism and essential for disuse atrophy. Neither have definitive evidence to support their cases.

Phillips and McGlory point to a lot of data demonstrating a disuse-induced decrease in MPS *in vivo* in human subjects but do they have a case for disuse-induced changes in MPS only? We contend that they do not, largely because nothing exists until we

measure it. As such, MPB during muscle disuse was measured in only one study including seven young men (Symons *et al.* 2009) and it was found that prolonged bed rest did not significantly alter it. Phillips and McGlory use this as the case in point to dismiss any contribution of MPB to disuse atrophy – conceivably a bit premature. They further argue that the calculated rate of muscle loss based on average measured MPS rates closely (but evidently not entirely) matches the measured average loss of muscle volume or cross sectional area assessed by using MRI or CT. Thus, setting aside any uncertainties of such comparison for the sake of the argument, there could be significant contribution of accelerated MPB.

Reid, Judge and Bodine focus their arguments largely on changes in cellular factors involved in regulating proteolysis in non-human models. There is also (although sometimes conflicting) evidence for cellular adaptations indicative of temporarily upregulated proteolysis in human muscle (e.g. Jones *et al.* 2004; Urso *et al.* 2006; Suetta *et al.* 2012) but the relationship between these measures and actual rates of MPB is still uncertain. Furthermore, there is nothing to suggest that MPB is essential (i.e. required) for disuse atrophy; theoretically it is certainly possible for it to occur simply as a result of the well-established disuse-induced reduction in MPS.

Since metabolic adaptations are usually multifactorial and complex, it is conceivable that muscle disuse affects both MPS and MPB to varying degrees over time. To find out to what extent alterations in each contribute to disuse atrophy will require a comprehensive (incl. fasted and fed state) and longitudinal (early/short-term *vs.* late/prolonged) simultaneous assessment of both MPS and MPB rates.

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Additional information

Competing interests

None declared.

Ubiquitin-dependent proteolysis regulates muscle protein synthesis as well as breakdown

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As to the big concern that the dominant mechanism remains unknown in disuse skeletal muscle atrophy, our answer is that proteolysis is essential for the disuse atrophy. Unloading mechanical stress, such as microgravity, directly induced skeletal muscle atrophy (Vandenburgh *et al.* 1999), indicating that skeletal muscle cells can sense the unloading stress without hormonal and neural signalling. In the response of skeletal muscle cells to unloading conditions, muscle protein synthesis (MPS) is closely associated with muscle protein breakdown (MPB) like bone remodelling. In bone remodelling, removing old bone (bone resorption) by osteoclasts is the initiation at the remodelling site and is followed by bone formation by osteoblasts to repair damaged bone. Given that disuse atrophy is an adaptive consequence against unloading stress, we reason that removing damaged protein by MPB is the initiation.

In addition, disuse atrophy underlies the resistance of skeletal muscle cells to myotrophic IGF-1 signalling (Sandri *et al.* 2004; Stitt *et al.* 2004). We previously reported that ubiquitin ligase Cbl-b is a negative regulator for IGF-1 signalling during muscle atrophy caused by unloading (Nakao *et al.* 2009). The mechanism of Cbl-b-induced muscle atrophy is unique; it does not appear to involve the degradation of structural components of the muscle, rather it impairs muscle growth signals in response to unloading conditions. Unloading induced Cbl-b expression very quickly (less than 3 h) in skeletal muscle cells, and the IGF-1-mediated cell growth via MPS of rodent or human myocytes was not suppressed unless there was Cbl-b induction. On the basis of these findings, we propose that proteolysis, especially ubiquitin-dependent proteolysis, is the initiation for the disruption of the MPS and MPB balance.

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Motion to dismiss: insufficient evidence to mount a case against protein synthesis as the dominant mechanism causing disuse muscle atrophy in the elderly

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The well-argued positions in this CrossTalk debate (Phillips & McGlory, 2014; Reid *et al.* 2014) clearly indicate that reduced muscle protein synthesis (MPS) and elevated muscle proteolysis are *both* contributing to muscle atrophy during disuse. Concluding which is the dominant mechanism based on current evidence is difficult due to technical limitations, particularly in the direct measurement of muscle proteolysis, but also as a function of the time point of sampling, e.g. acute *vs.* chronic phases of disuse, and the population examined. Defining these mechanisms is key to translation of research into appropriate treatment. Consider the case of the elderly, a population likely to encounter periods of both acute and chronic disuse. Basal rates of MPS in the postabsorptive state are similar following 7 days of bed rest compared with pre-bed rest values in an elderly cohort, despite ∼4% loss of muscle mass (Drummond *et al.* 2012). A key regulator of muscle mass is the postprandial regulation of MPS/MPB and we know that MPS is reduced by 40% in the postprandial period following acute bed rest, which potentially accounts for this loss (Drummond *et al.* 2012). However, there are currently no data directly measuring disuse-associated changes in proteolysis in elderly individuals in either the acute or chronic phase. Thus, until the technical challenges are addressed and current markers of muscle proteolysis are embraced (e.g. MAFbx, MuRF-1, FOXO3a) it is difficult to argue a case against, in the elderly at least, decreased protein synthesis as the dominant mechanism in disuse atrophy.

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Additional information

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None declared.

The relative role of decreased protein synthesis and increased proteolysis in causing disuse muscle atrophy varies through models, muscles and species and depends on the duration of unloading

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Our works on disuse atrophy in mice slow (Cannavino *et al.* 2014) and fast (Cannavino *et al.* 2015) muscle using hind-limb unloading indicated that both an increase of proteolysis and a decrease in protein synthesis come into play early in determining muscle atrophy and to a different extent. In the early stages of disuse, atrophy in fast muscle was caused by proteolysis only, while, in slow muscle, both activation of proteolysis and reduction of protein synthesis played a role. Furthermore, the latter studies showed a good correlation between *in vitro* parameters (catabolic and synthetic markers) and the degree of muscle atrophy. In fact, preventing catabolism activation, mass was completely preserved in fast muscle and only partially in slow muscle, where synthetic parameter down-regulation persisted.

Although caution is a must when extrapolating data from animal studies to humans because of differences between muscles, species and experimental models (Pellegrino *et al.* 2011), both proteolysis and decreased protein synthesis should still be considered likely candidates in humans too.

Indeed, an early and transient induction of proteolytic markers similar to that observed in mice (Cannavino *et al.* 2014, 2015) was observed in humans (Abadi *et al.* 2009; Gustafsson *et al.* 2010). Moreover, based on our studies in mice, it appears that no activation of MurF-1 and atrogin-1 at later stages of disuse (Brocca *et al.* 2012) does not necessarily mean proteolysis plays a minor role. Therefore, we believe that the contribution of proteolysis cannot be excluded, unless the irrelevance of such a process is experimentally proved. The doubt could be dissipated if, after preventing the early induction of catabolic markers in humans, the experimental variation of muscle mass remained unaffected.

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Additional information

Competing interests

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'Measure what is measurable, and make measurable what is not so' (Galileo Galilei)

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Following years of research in muscle protein metabolism, there is strong evidence that muscle protein synthesis (MPS) is the dominant factor in simple disuse atrophy. Particularly, the theory that a decrease in MPS during this process is based on several studies involving *in vitro*, *ex vivo* as well as *in vivo* measurements of MPS (Mallinson *et al.* 2013). Conversely, even though the theory that proteolysis is the dominant mechanism underlying disuse atrophy is supported by reliable animal models, a lack in translational research makes it less credible.

However, either to confirm that MPS is the dominant mechanism during disuse atrophy in humans or to verify whether instead proteolysis is the main determinant, further research in the field is warranted.

Particularly there is a necessity to translate findings to an *in vivo* setting, using accurate direct dynamic labelling techniques able to compatibly assess MPS and proteolysis in humans. Indeed while MPS is currently determined by reliable methods, proteolysis falls behind. Recently deuterated water, a validated technique used to assess MPS (Gasier *et al.* 2010; Wilkinson *et al.* 2014), has been proposed to also measure MPB, thus allowing comparable measurements of both aspects of muscle protein turnover (Wolfe, 2011; Holm *et al.* 2013).

Therefore the development of new techniques and approaches will help researchers to link data coming from gene expression, signalling pathway and protein metabolism and thus better understanding the main mechanism lying behind disuse atrophy.

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Additional information

Competing interests

None declared.

Changes in protein degradation and synthesis are both critical in muscle atrophy

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Surprisingly, Phillips & McGlory (2014) disregard the extensive evidence that increased protein breakdown is also critically important in atrophy (Mitch & Goldberg, 1996; Glass, 2010; Reid *et al.* 2014; Cohen *et al.* 2015). Pulse-chase studies in rats first showed that atrophy induced by denervation or glucocorticoids involved accelerated proteolysis (Goldberg, 1969), and once methods were developed to measure precisely rates of degradation and synthesis in isolated muscles (Fulks *et al.* 1975; Tischler *et al.* 1982), we demonstrated that degradation rises with denervation (Furuno *et al.* 1990), disuse (Tischler *et al.* 1997), fasting (Li & Goldberg, 1976), cancer (Baracos *et al.* 1995) and acidosis (Mitch *et al.* 1994). At present, no method exists to measure accurately degradation rates in human muscles. Because proteolysis cannot be measured accurately in humans, one cannot conclude that it does not increase. Also, it is invalid to predict changes in muscle mass from measurements of overall synthesis, since individual cell proteins turnover at widely different rates, and their rates of transcription (Sacheck *et al.* 2007), synthesis (Li & Goldberg, 1976; Furuno *et al.* 1990), and degradation (Li & Goldberg, 1976; Furuno *et al.* 1990; Cohen *et al.* 2009, 2012) vary as atrophy progresses.

Phillips & McGlory (2014) dismiss 'as biased' conclusions from isolated rodent muscles. However, such preparations behave linearly for hours and are still the only method to precisely measure turnover rates. Such studies first demonstrated the ability of insulin and amino acids to suppress proteolysis (Fulks *et al.* 1975; Tischler *et al.* 1982), later confirmed in humans, and enabled the discovery of the critical adaptations during atrophy: activation of the ubiquitin–proteasome pathway (Medina *et al.* 1995; Wing *et al.* 1995) through atrogene induction (Jagoe *et al.* 2002; Lecker *et al.* 2004; Sacheck *et al.* 2007). Phillips & McGlory (2014) accept our finding (Jagoe *et al.* 2002; Lecker *et al.* 2004; Reid *et al.* 2014) that a common transcriptional programme enhances muscle proteolysis in disease states but state incorrectly that similar changes do not occur with disuse. In fact, we demonstrated that denervation and pure disuse induce the same atrophy-related changes as fasting and disease (Sacheck *et al.* 2007).

In human and animal cells, contractile activity, nutrients, and insulin activate the PI3K–AKT–mTOR pathway (Glass *et al.* 2010; Cohen *et al.* 2015), which promotes translation and inhibits proteolysis by suppressing autophagy (Efeyan *et al.* 2015)

and inactivating FoxO-mediated expression of genes for ubiquitination (Sandri *et al.* 2004; Cohen *et al.* 2015) and autophagy (Mammucari *et al.* 2007; Zhao *et al.* 2007). Thus, overall rates of protein degradation and synthesis change coordinately.

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Additional information

Competing interests

None declared.

Commentaries on Viewpoint: What is the relationship between acute measure of muscle protein synthesis and changes in muscle mass?

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Commentaries on Viewpoint: What is the relationship between acute measure of muscle protein synthesis and changes in muscle mass?

COMMENTARY RESPONSE TO VIEWPOINT: "WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?"

TO THE EDITOR: Mitchell et al. (3) critique putative links between *acute* muscle protein synthesis (MPS) and ensuing hypertrophy after resistance exercise (RE) training (RT). Just two papers have addressed this longitudinally. The first reports that young but not older individuals exhibit *acute* (fasted *mixed* muscle MPS, 24 h post-RE) increases in MPS (2); yet hypertrophy gains, assessed by DXA and fiber area, were equal. Similarly, poor quantitative MPS linkages were reported 1–6 h post-RE (*myofibrillar* muscle, fed state) with ensuing hypertrophy (4), i.e., two study's suggesting bona fide dissociation. However, for (2), prior reports of age-related temporal differences in acute MPS responses to RE (1) and lack of intra/inter age group correlations (2) mitigate such resounding conclusions. Also, in (4), RE was under fed state conditions; this is significant because consumption of protein feeds *extends* (not *amplifies*) elevations in MPS post-RE (5). Therefore, MPS responses 1–6 h post-RE were chiefly predominated by feeding, i.e., the coupling of which to RE may not aptly reflect interindividual variation (5), e.g., due to isolated mechano-auto/paracrine responses to RE. Yet, *acute* MPS can/does inform on group interventions for RT-induced muscle hypertrophy, signifying it a practicable, informative end-point. But, could *quantitative* relationships still exist and what defines "*acute*" post-RE MPS? Fasted/fed? Immediately (<6 h?)/later (>24?) What muscle fraction(s)? Is there an applicable "snapshot"? Without defining *acute* this is indiscernible. The authors justifiably raise potential technical, temporal, methodological confounder(s). Because hypertrophy is a heterogeneous and temporally dynamic process we hypothesize that (coupled to not isolating/fractionating/holistically capturing "*acute*" MPS) interindividual trajectories and *plateauing* hypertrophy in the face of fixed study end-points are the major barriers to defining quantitative links between *acute* MPS and RT-induced hypertrophy.

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COMMENT VIEWPOINT: "WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?"

TO THE EDITOR: Mitchell et al. (4) state that acute measurements of muscle protein synthesis may not necessarily reflect the magnitude of hypertrophy during long-term training studies, which we agree with. Two points deserve further comment: *1*) magnitude of muscle protein synthesis does not necessarily equate with hypertrophy, but rather remodeling, and *2*) limitations of acute measurements are a primary reason some in the field advocate for long-term measurements of synthesis. Regarding the first point, as the authors mention, synthesis is more commonly measured than breakdown and is likely the driving factor behind phenotypic change. However, it is still important to consider that breakdown can change the outcomes. If a high rate of synthesis is accompanied by equal or slightly lower rates of degradation, one would have hypertrophy, whereas the other may not. Importantly though, both are indicative of remodeling. This concept is clear when one studies endurance exercise where there is a high degree of protein remodeling, and phenotypic change, without hypertrophy (5). Regarding our second point, we have advocated for the use of deuterium oxide to measure long-term changes in protein synthesis (1, 3) because of the limitations of acute protein synthesis measurements (4). We have even emphasized this point in a previous "Viewpoint" (2). Although acute measurements of protein synthesis have value, the overall outcome of a long-term treatment or intervention should be assessed by long-term measurement techniques to understand the integrated responses over time. We hope that others, like Mitchell et al., continue to recognize this important point.

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THE POSTEXERCISE INCREASE IN MUSCLE PROTEIN SYNTHESIS RATE IS INDICATIVE OF SKELETAL MUSCLE RECONDITIONING RATHER THAN MUSCLE HYPERTROPHY PER SE

TO THE EDITOR: We regard the acute muscle protein synthetic response to exercise as an indicator of skeletal muscle reconditioning rather than predictive for muscle hypertrophy. For example, a single bout of endurance-type exercise also acutely increases muscle protein fractional synthetic rate (FSR) (1–3). The increase in the muscle protein synthetic response to endurance type exercise is generally not accompanied by substantial gains in muscle mass and, as such, is far from predictive for muscle hypertrophy in healthy, lean individuals. Instead, the postexercise increase in muscle protein synthesis is rather representative of muscle reconditioning, comprising muscle repair and remodeling. Consequently, the postexercise increase in muscle protein FSR should not be regarded as a marker for exercise training induced hypertrophy but rather as an indicator of skeletal muscle reconditioning, which comes in many different forms and measures. The muscle protein synthetic response to a single bout of resistance type exercise training may provide some insight in the extent of muscle hypertrophy observed during more prolonged resistance type exercise training but does not provide a quantitative estimation of hypertrophy in the individual (4).

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COMMENT ON VIEWPOINT: WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?

TO THE EDITOR: Mitchell and colleagues (2) present a nicely balanced viewpoint regarding the discordance between acute measurements of muscle protein synthesis (MPS) and long-term muscle mass gains in response to exercise training and nutrition. Individual genetic and other physiological characteristics, as well as methodological limitations of the endpoint measures, are correctly identified as contributing factors to the hypertrophic variability inherent in these types of training studies (1). However, the practicalities of controlling long-term training studies themselves are not addressed.

Consideration is warranted for the difficulties in controlling extended-duration intervention studies to generate appropriately valid and reliable results. Changes and variability in diet, timing of exercise in relation to meal ingestion, sleep patterns, daily stress, and compliance with the training regimen will contribute to the considerable intrinsic variability of measured changes in muscle mass (3). Small differences between interventions may easily be missed when these confounding factors are coupled with the variations outlined in the Viewpoint. Acute measurements are much easier to control and differences between interventions often can be easily detected. Therefore, any disconnect between acute metabolic studies and long-term changes in muscle mass do not necessarily reflect the worth of the metabolic studies.

There is no question that acute measurements of MPS alone should not be used to predict the training response of any given individual. However, with appropriate appreciation for their limitations, these methods can play a valuable role in acquiring information to help determine appropriate training and nutrition interventions for various populations.

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COMMENTARY RESPONSE TO VIEWPOINT: "WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?"

TO THE EDITOR: The authors raise several insightful points while describing the discordance they observe between acute measures of postexercise muscle protein synthesis (MPS) and the subsequent resistance exercise (RE) training-induced muscle hypertrophy (1, 2). Although the authors suggest that intersubject variability in the MPS response to RE is a likely major contributory factor, the impact of recruiting individuals naive to the mode of RE employed cannot be overstated. Moderate (60–75% of 1-repetition maximum) noneccentric RE in unaccustomed individuals has been shown to result in myofibrillar damage, increased inflammatory signaling (5), and induction of the unfolded protein response (4), events that are thought to modulate MPS drive. Moreover, we have observed that 24 h after a single-bout of unaccustomed concentric RE, the transcriptional "program" elicited in muscle appears dependent on whether damage has purportedly occurred and varies between individuals (3). Specifically, we observed a discord in the transcriptional regulation of gene pathways associated with MPS signaling, which could in part explain the variability observed by the authors. In contrast, the transcriptional response to a repeat session of RE performed days later was consistent across volunteers and was not punctuated by increased markers of muscle damage (3). Collectively, these observations suggest that, when attempting to translate acute, exercise-induced changes in MPS to longer term gains in muscle mass, using subjects who have already undergone a period of familiarization to laboratory RE protocols may offer a more promising approach.

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NEED FOR MEASURES OF SATELLITE CELL ACTIVATION ALONG WITH MUSCLE PROTEIN SYNTHESIS?

TO THE EDITOR: The Viewpoint by Mitchell and colleagues (4) provides a timely reminder that acute responses to exercise-nutrient interventions are not always the perfect proxy for chronic training-induced adaptations and/or changes in functional outcomes. Skeletal muscle displays remarkable plasticity with the capacity to alter the type and amount of protein in response to habitual level of contractile activity, the prevailing substrate availability, and environmental conditions (3). Such "adaptation plasticity" is common to all vertebrates but a large variation in the degree of adaptability between humans is evident, explaining the large inter-individual responses after exercise-nutrient interventions (2). The author's state that an individual's "inherited genetic predisposition, epigenetic influence, and transcriptional plasticity" are potential sources for "hypertrophic variability" after resistance training (RT). Added to that list is the possibility that satellite cell activation may underlie part of the variability in the muscle hypertrophic response. Previous work shows the acute satellite cell response to a single bout of resistance exercise is associated with the subsequent accretion of lean mass (LM) after 16 wk RT (1). Although this suggests postexercise measures of satellite cell activity could be a valid surrogate of an individual's ability to accrue LM after RT, acute measures of MPS still provide important mechanistic insight to the "anabolic" events in response to exercise-nutrient interventions. Ultimately it is clear that chronic training studies with comprehensive time-course responses of selected cellular and functional outcomes are required to provide mechanistic insight as to why training-nutrient interventions result in variable responses between individuals.

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ARE NOT INTRACELLULAR HEAT AND NEURAL ELECTRICITY STRESSES THE CAUSE OF EXERCISE-INDUCED PROTEIN SYNTHESIS?

TO THE EDITOR: Dr. Mitchell and colleagues (5) support their thesis for a possible lack of correlation between acute and chronic muscle protein synthesis (MPS) partly with their Refs. 14, 15, and 16. The conclusion for such a lack is drawn in all of these three references. However, while in Refs. 15 and 16 the quadriceps hypertrophy after 10 or 16 wk of resistance training (RT) was between 5 and 9.5% the same in Ref. 14 after 16 wk RT was 37–40%. In our view, the difference was because in Ref. 14 the load was progressively increased during the RT, while in Refs. 15 and 16 it was not. Qualitatively, it is the same difference as between MPS response after acute and chronic RT.

Myocytes are extremely vulnerable to overheating, because they can increase energy production more than 100-fold in less than a second. They do not have intracellular convective heat transfer because almost 80% of their water is entrapped in the myofibrils. The anaerobic nature of RT increases with the increase of loading because more energy is released during contraction, when muscle capillaries are compressed, preventing blood flow and cooling. Elevated myocyte temperature prevents protein synthesis, causes protein denaturation, and stimulates heat shock protein synthesis, which during postexercise repair in excess damaged protein (3, 4). This reasoning is supported by the similar effect produced by the blood flow restriction (2). Stimulation of protein synthesis by electric field (1) hints that excessive muscle neural electricity, triggered by unaccustomed loading, should have supportive MPS effect.

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TO THE EDITOR: Mitchell and colleagues have identified an important problem: can a single measurement of protein synthesis be extrapolated to provide changes in muscle mass(1)? Muscle mass is often used as a surrogate for muscle protein content, but although muscle weight reflects muscle mass, muscle weight includes muscle proteins, adipose tissue (especially, in aged or type II diabetes), and up to 70% water (3). For example loss of muscle mass in exercise reflects loss of water with or without loss of muscle protein. As suggested by Mitchell, although an increase in protein synthesis suggests increased muscle protein mass, no conclusion is possible without measurement of protein degradation. Infusion of L-(1–13C) leucine or L-(ring-2H5) phenylalanine has been used to measure whole body or skeletal muscle protein metabolism in humans (2). In animal models, rates of protein synthesis and degradation are often assayed *ex vivo* by measuring the rate of tyrosine incorporation into protein (protein synthesis) plus the release of tyrosine from muscle proteins (protein degradation) (4). This approach, however, does not allow both synthesis and degradation information to be gathered from the same animal. The rate of protein degradation is virtually always greater than the rate of protein synthesis regardless of whether undergoing muscle hypertrophy or atrophy. If the goal of measuring protein synthesis is to provide an index of changes in muscle protein mass, we suggest that investigators assess the distribution of the areas of myofibers in a cross section of muscle as described for assessing the effects of XIAP on muscle hypertrophy (5).

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SIGNIFICANCE OF LONG-TERM STUDIES IN RESISTANCE TRAINING AND MUSCLE HYPERTROPHY

TO THE EDITOR: Muscle being a postmitotic tissue is endowed with an efficient means of cell replacement to avoid muscle cell death and maintain skeletal mass. This is carried out through the dynamic balance between muscle protein synthesis and degradation (1). Muscle hypertrophy occurs when protein synthesis exceeds protein breakdown. Although the Viewpoint authors (2) agree with the importance of acute measurements in understanding mechanisms of divergent exercises and nutritional manipulations, they advocate the need for long-term studies to understand the holistic adaptations due to altered phenotype.

Muscle hypertrophy is a multifactorial process involving mechanical tension, muscle damage, and metabolic stress. In addition, many have observed numerous other factors, such as genetic predisposition, epigenetic influence, and transcriptional plasticity, age, gender, habitual physical activity, and training status, to influence the hypertrophic response to a training protocol affecting both the rate and the total gain in lean muscle mass. Hormones and cytokines, namely growth hormone, testosterone, interleukin-5, interleukin-6, etc., are also shown to play complex roles in hypertrophic process (3). Additionally, some of these hormones

have effects on immune system, bone remodeling, and extracellular fluid volume. A 20-wk-long RT revealed greater adaptability within endocrine system only in younger men (4). A discrepancy in immune responses to short-term and moderate exercise training is reported recently (5). Exercise-induced free norepinephrine concentration was reported to have effect on circulating hematopoietic stem and progenitor cell number and functionality (3). In the light of such varied complexities, it is appropriate to pursue long-term effects of exercise training on muscle hypertrophy.

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JOURNAL CLUB

Pattern of protein ingestion to maximise muscle protein synthesis after resistance exercise

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The maintenance of skeletal muscle mass is dependent upon the temporal and coordinated interaction between muscle/myofibrillar protein synthesis (MPS) and muscle protein breakdown (MPB). Resistance exercise (RE) alone elevates MPS and, to a lesser extent, MPB such that net muscle protein balance (NPB) remains negative. However, when RE is coupled with protein ingestion there is an accumulative effect on MPS resulting in a positive NPB (Phillips *et al.* 2005). Thus, repeated bouts of RE coupled with protein feeding is a viable strategy to maximise skeletal muscle hypertrophy and strength.

The impact of protein feeding on RE-induced increases in MPS has received much attention. One study has demonstrated that in young healthy males ∼20 g of high-quality protein is sufficient to maximise RE-induced rates of MPS over 4 h post-exercise (Moore *et al.* 2009). However, the interplay between the timing and quantity of protein consumed and subsequent anabolic responses throughout the course of a whole day is still poorly understood. In particular, there is a lack of data examining how the pattern of post-RE protein ingestion influences MPS later in the recovery phase (i.e. 4–12 h). A recent article published in *The Journal of Physiology* attempts to address this knowledge gap and in doing so provides valuable insights into how post-RE protein feeding strategies might be manipulated to optimise muscle anabolism. In an elegantly designed study, Areta *et al.* (2013) examined three groups of eight healthy, trained males. Participants performed a bout of bilateral leg extension RE followed by the consumption of 80 g of whey protein over 12 h of recovery ingested as either 8 × 10 g every 1.5 h, 4 × 20 g every 3 h or 2 × 40 g every 6 h. A stable isotope infusion was coupled with frequent skeletal muscle biopsy sampling to determine rates of MPS for 12 h post-RE. The data demonstrate that although all feeding strategies elevated MPS during the 12 h recovery period, consuming 20 g of whey protein every 3 h was the superior strategy for stimulating MPS rates. The authors concluded that these findings have the potential to maximise outcomes of resistance training designed to elicit a maximal hypertrophic response.

The data of Areta *et al.* show that manipulating the pattern of protein ingestion following RE can have a significant impact on the subsequent muscle anabolic response. The divergent feeding strategies of Areta *et al.* were used to mimic possible patterns of protein intake commonly observed in resistance-trained athletes. That is, 8 × 10 g every 1.5 h represents a 'grazing' approach, whereas 2 × 40 g every 6 h relates to the 'three square meals per day' approach. Yet, both of these strategies were inferior for stimulating MPS over 12 h of post-RE recovery compared with 4 × 20 g ingested every 3 h. However, it is important to note that this response was characterised when protein was ingested alone, and as the authors acknowledge, this finding cannot be evaluated in the context of a mixed meal. Indeed, it is commonplace to consume protein in the form of a mixed-macronutrient meal. Therefore, it is reasonable to postulate that macronutrient co-ingestion could alter intestinal transit, thus influencing amino acid absorption kinetics (Deutz *et al.* 1995) and perhaps MPS. Moreover, this study used high-quality whey protein and it remains to be seen if a similar pattern of MPS post-RE would be observed using the same feeding strategies with a slow-release protein such as casein. Such information may be valuable to individuals who choose not to (or are unable to) ingest high-quality protein in supplemental form following exercise, but instead consume whole-food protein sources.

Areta *et al.* should be highly commended for underlining the importance of not only the quantity, but particularly the pattern of post-RE protein ingestion to maximise the rate of MPS over 12 h. However, as a note of caution, their findings are limited to a healthy young male population. In this regard, recent evidence demonstrates that the elderly require more protein (40 to > 20 g) to elicit optimal increases in RE-induced rates of MPS than the young (Yang *et al.* 2012). It is therefore reasonable to consider whether the temporal influence of post-RE protein feeding on elderly muscle could be different compared to that of young. In this regard, the next logical step is to apply the model of Areta *et al.* in elderly and other populations, in whom maintenance of muscle mass is a critical determinant of longevity and quality of life. Yet, it should be acknowledged that Areta *et al.* afford data pertaining to only 12 h of recovery from RE. Hence, whether the acute responses of MPS to RE and protein feeding translate into a long-term functional response remains unknown.

The findings of Areta *et al.* will no doubt also grasp the attention of coaches and athletes alike. As such, some may cite the use of a bilateral exercise stimulus and absence of participants with large amounts of lean mass (>75 kg) as issues that preclude full applicability in a 'real-world' setting. To date, it is unclear whether exercising a greater volume of muscle mass is limiting for MPS in response to a given protein dose. Therefore, individuals with greater muscle mass or those engaged in whole-body RE training sessions may require ingestion of a greater protein dose to stimulate MPS maximally. With regard to the notion of applicability to the 'real-world' setting, it also may be significant that the participants entered the experimental trial in the fasted state. As a result the authors are unable to identify whether a pre-exercise meal would influence the MPS response to RE and various feeding strategies. This point becomes more relevant when considering the impact of insulin on MPB with regard to the true *growth* response and therefore the long-term applicability of the findings. Future studies assessing MPS and MPB in both the clinical and the athletic setting following RE and feeding are now required.

The study by Areta *et al.* also reveals novel nutrient–exercise interactions in cellular signalling. Phosphorylated mTOR^{Ser2448} was ∼2- to ∼6-fold above resting values throughout the 12 h recovery period independent of protein feeding strategy. Phosphorylation of p70S6K^{Thr389} was also increased above baseline, again in all feeding

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strategies. However, there was discordance between the degree of p70S6K^{Thr389} phosphorylation and the MPS response. In fact, the magnitude of phosphorylated p70S6K^{Thr389} displayed a 2×40 g to $> 4 \times 20$ g to $> 8 \times 10$ g pattern at 1 and 7 h post-RE. This finding is surprising given that phosphorylated p70S6K^{Thr389} is a key player in protein synthesis yet it was the 4×20 g strategy that induced the most favourable influence on MPS but median impact on phosphorylated p70S6K^{Thr389}. However, it is important to recognise that the timing of the biopsies at 1 and 7 h coincided with a greater volume of protein consumed prior to those biopsies for the 2×40 g condition, which may explain the discordance between p70S6K^{Thr389} signalling and MPS.

The common method employed to assay protein phosphorylation, a proxy of activity, in an exercise science setting, and in the present investigation, is Western blotting (WB). In contrast to the quantitative and reproducible techniques used to measure MPS, WB is a semi-quantitative method. Additionally, phosphorylated p70S6K^{Thr389} is recognised as a key controller of ribosomal biogenesis. So although the phosphorylation of p70S6K^{Thr389} post-RE does not correspond to the greatest acute MPS response it may in fact be leading to greater levels of ribosomal transcription. Interestingly, phosphorylation of p70S6K following RE often occurs in the nucleus, where ribosomal biogenesis commences. A caveat of the field is that no study has employed cellular fractionation techniques to reveal whether different RE and feedings strategies alter the ratio of nuclear to cytoplasmic phosphorylated p70S6K in human skeletal muscle. Hence, the lack of concordance between the MPS and signalling response in this and numerous other works emphasises the need for the development of new measures regarding readouts of ribosomal biogenesis in addition to fully quantitative methods to ascertain signalling activity following RE and nutrition.

To conclude, the study by Areta *et al.* contributes novel data to the body of literature highlighting the importance of the timing and quantity of protein consumed post-RE for muscle anabolism. By mimicking the habitual feeding strategies of many athletes engaged in resistance training, the authors move closer to bridging the gap between science and the applied setting. Future work that identifies the impact of different macronutrients consumed in combination, i.e. fat, carbohydrate, protein and fibre, on MPS in both elderly and young is warranted. Furthermore, there is growing interest in whether having greater amounts of muscle mass, or indeed exercising muscle mass involved in trainin impact RE-induced rates of MPS. Thus, future studies that examine the MPS response in individuals with large muscle mass, performing real-world RE, may provide informative data for clinical and athletic practice.

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