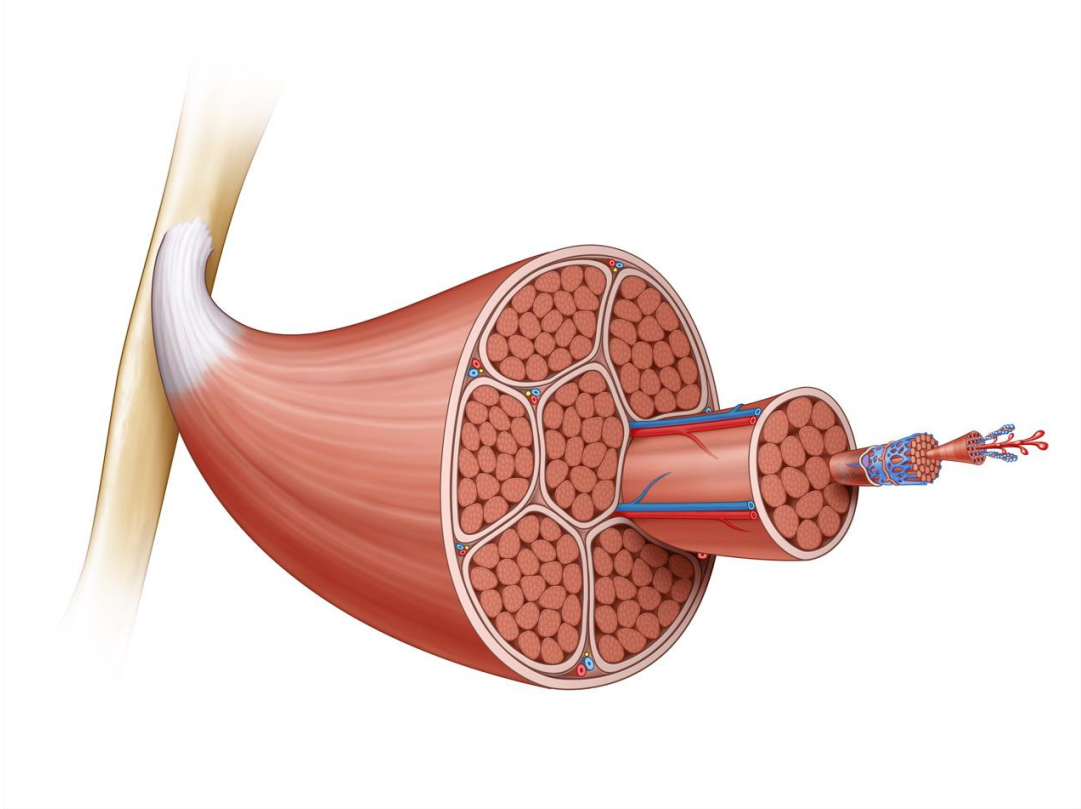
# The impact of short term disuse and dietary protein intake on skeletal muscle mass and protein synthesis rates in humans

Sean Kilroe



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The work presented in this thesis was performed within NUTRIM, School of Nutrition and Translational Research in Metabolism, at the department of Sport and Health Science at Exeter University, United Kingdom.



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DISSERTATION

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# Chapter 1

General Introduction

**Overview**

Skeletal muscle performs a variety of critical roles within the body including providing ambulation, postural control and participating in the regulation of whole body metabolism (1). It is a highly plastic organ that is very adaptable to its environment; contraction, nutrition e.g. protein ingestion and hormones can all alter skeletal muscle mass and function. Skeletal muscle usually accounts for more than 50% of total body mass and it is a major contributor to basal metabolic rate, with ~85% of post-prandial glucose disposal occurring in skeletal muscle (2). Muscle mass and strength are both positively correlated with quality of life (3) and functional capacity (4), which is of particular importance in older age. Low muscle mass and strength are correlated with reduced physical function (5) and ultimately premature death (6). In modern society physical activity level and dietary intake are two main factors that affect skeletal muscle mass, function and metabolic capacity. The prevalence of sedentary behavior has increased in modern society (7) and this reduction in physical activity has contributed to muscle atrophy. Muscle atrophy contributes to multiple negative health effects including a loss of muscle strength (8), a decline in basal metabolic rate, and the development of insulin resistance (9). The loss of muscle mass plays a key role in the progression of certain non-communicable diseases such as obesity and type 2 diabetes. Therefore, the maintenance of muscle mass, as well as strength, throughout the lifespan is of critical importance to reduce the prevalence of these non-communicable diseases and maintain good health into older age. Skeletal muscle is a highly plastic organ and responds to the load placed upon it. Short periods of muscle inactivity that can occur due to injury or illness can result in an accelerated loss of muscle mass (10,11). These short periods of muscle disuse, that typically last 7 days of less, have also been linked to the accelerated loss of skeletal muscle mass over the lifespan (12). Thus, a better understanding of how muscle mass is regulated during periods of disuse is required before effective interventions to counter the loss of skeletal muscle mass and function during a period of muscle disuse can be developed.

*Skeletal muscle disuse*

Skeletal muscle disuse is the absence of contraction of skeletal muscle. Periods of muscle disuse are a common phenomenon in modern society, at some point in their lives it is likely that most people will undergo at least one if not more periods of skeletal muscle disuse. Some common forms of disuse occur because of injury (e.g. leg fracture) or hospitalisation (e.g. after elective orthopedic surgery). The Canadian Institute of Health reported that in 8.2% of older adults (~3,100,000) were admitted to hospital and required at least a short period of bed rest. The prevalence of leg immobilisation was 0.35% of the Canadian population (~130,000) for hip and knee replacements combined, but would be higher if all cases of immobilisation were included (13). A more recent review, (14) has suggested that repeated brief periods of muscle disuse across the life span may play a role in the development of the age related loss of muscle mass (Sarcopenia) and function (Dynapenia). Which in turn can have substantial socio-economic impact, including the lack of independence in the elderly. In a clinical setting the duration of disuse will depend on the severity of the injury of illness. In older adult patients who were admitted to hospital for elective surgery, the time from surgery to discharge was on average 6 days (10). The average length of stay for more serious conditions that require admission to the intensive care unit (ICU) was 12 days (15) but will vary depending on the medical condition of each patient. Overall the average length of stay in

hospitals for all ages and conditions in Europe is 7 days (*Hospital discharges and length of stay statistics - Statistics Explained*, 2017). As just 7 days is the average length of stay in hospital this highlights the importance of the potential impact of such short term periods of disuse. We need to assess the importance of characterizing changes in muscle mass, function and metabolism during these shorter term periods and develop countermeasures.

*Experimental models of skeletal muscle disuse in humans.*

Over a number of decades different models of skeletal muscle disuse have been employed to assess the effects of muscle disuse on skeletal muscle health, mass and metabolism. The models of disuse can generally be classified into two groups; whole body disuse, where all muscle groups of the body are affected. Common examples of this include bed rest (9,17,18), dry water immersion (19,20) and partial gravity/spaceflight (21). The second model of disuse is local immobilisation, which implements muscle disuse to a single muscle group or small group of muscles. Examples include the use of leg casts or braces with the use of crutches to immobilise and prevent muscle contractions of a single leg (11,22–24) with the contralateral leg serving as a control. Whole body bed rest where patients can sit upright in bed is a highly clinically relevant model of disuse as it mimics hospitalization (25) and offers a controlled environment for assessing muscle disuse (i.e. it allows for the easy provision of a controlled diet). In contrast leg immobilisation offers greater practicality to the participant as they can carry out their daily activities outside of the lab and maintain their habitual dietary intake. One important difference between the two models of muscle disuse is that whole body disuse in the form of bed rest invokes changes in systemic factors such as increased systemic inflammation (26) and increased whole body insulin resistance (9) which could influence muscle mass changes during bed rest. In contrast, leg immobilisation does not result in these systemic changes and thus represents a good model to investigate the effect of disuse *per se* on muscle mass. A second important point is the length at which bed rest and leg immobilisation immobilise the quadriceps and hamstring muscle groups. Bed rest immobilises the quadriceps at a shorter length than leg immobilisation and the opposite is true for the hamstring muscle. Previous research in animal models has observed greater muscle mass loss when a muscle is immobilised at a greater length compared to a shorter length.

*The influence of muscle disuse on muscle mass and function.*

Muscle disuse involves the removal of muscle contraction. Due to this lack of contraction skeletal muscle strength (27,28), as well as mass (9,11,27) will decrease during a period of disuse. This reduction in muscle mass and strength has negative consequences for overall health. The reduction in strength after a period of disuse is particularly problematic in older adults, as they will typically have lower strength before a period of disuse, and further decreases in muscle function can impair mobility that will have knock on effects on their sociological and psychological well-being.

There are a variety of techniques that have been applied to assess how the mass of skeletal muscle responds to a period of disuse. Computed tomography scans (CT), dual x-ray absorptiometry scanning (DEXA) and magnetic resonance imaging (MRI) are the most commonly used techniques with the quadriceps being the most studied muscle group. The rate of quadriceps muscle atrophy is the highest during the early stages of disuse where Wall et al., (2015) reported a 0.7% per day rate of muscle loss during 5 days of leg immobilisation. This rate of muscle loss reduces to 0.4% per day over 6 weeks of leg

immobilisation (27). This adaption in the rate of muscle loss is presumably a response of the body to preserve skeletal muscle.

Despite the effect of muscle disuse on quadriceps muscle mass being well studied there is a lack of data on the impact of disuse on the individual muscles that make up the thigh (e.g. semitendinosus and vastus lateralis). Current evidence on individual thigh muscle atrophy is highly detailed in response to bed rest, especially long term best rest (e.g. 14 to 56 days). Detailed measurements of the individual muscles of the thigh have been measured, primarily using MRI scanning which provides an increased resolution to do this compared to other methods that have been used to measure muscle atrophy, for example computed tomography (29,30). After long term bed rest, the vastii muscles atrophied the most of all the thigh muscles and significantly more than the rectus femoris, adductor magnus, biceps femoris short head and gracilis after 8 weeks (30). This is due to the vastii muscle group being the predominate muscle group used during walking and many other common activities such as standing and sitting (31). However, details regarding how individual muscles of the thigh respond to unilateral leg immobilisation where the quadriceps are immobilised at a longer length than the hamstrings are currently lacking during short term disuse. This thesis will focus on how different muscle groups e.g. quadriceps and hamstrings, respond to unilateral leg immobilisation during short term disuse.

Besides assessing the effects of muscle disuse on muscle mass, the muscle function response to disuse has also been extensively studied. These findings have mostly focused on the quadriceps with reductions in leg extension strength being reported following both bed rest (9,32,33) and leg immobilisation (11,34,35). As highlighted above, the reduction in muscle strength is more rapid during the early stages of disuse. Whereas the decline in leg strength may be as high as 2% per day during the first week of disuse (9,32), it is reduced down to 0.4% per day when assessed over a more prolonged period of bed rest (17). Interestingly, the magnitude and rate of the reduction in strength appears to be dependent on the disuse model and the muscle and type of contraction that is being assessed. When comparing previous work using bed rest (22,23) and leg immobilisation (36,37) models over a 2 to 3 week period, leg immobilisation appears to be accompanied by a slightly greater loss of quadriceps strength when compared to bed rest (1.2 vs 1.6% per day). This may be attributed to the efficacy of both models to reduce the level of muscle contractions.

There are fewer data available on how disuse affects the function of different muscles of the legs. Previous work has found no differences in the reduction in concentric strength of the quadriceps and hamstrings (~12% for both) after a 10-14 day period of bed rest (36,37). Future research should also focus on how different types of muscle contraction e.g. concentric vs eccentric of the quadriceps and hamstrings muscles are affected by disuse. Such data may also help us to design and target more effective rehabilitation strategies.

*The effect of muscle disuse on muscle protein synthesis and breakdown rates.*

Two main factors are involved in the regulation of skeletal muscle mass; the synthesis of skeletal muscle proteins, termed muscle protein synthesis (MPS) and the breakdown of skeletal muscle proteins, termed muscle protein breakdown (MPB). The rates of these two metabolic processes are influenced by multiple factors and fluctuate strongly throughout the day. The result of them combined is the net protein balance, if one is persistently higher than the other will determine if skeletal muscle proteins are lost or accrued. During muscle disuse muscle tissue is lost due to a net negative protein balance where muscle protein

breakdown rates are higher than muscle protein synthesis rates.

Early work on how leg immobilisation alters muscle protein synthesis rates was conducted in patient groups who underwent a period of leg immobilisation to recover from a tibial fracture (8,27). Patients were immobilised for ~5 weeks which reduced post-absorptive MPS rates by ~26% compared to the control, non-immobilised leg. However, it is difficult to ascertain whether the injury and subsequent inflammation may have influenced this reduction in post-absorptive MPS rates. Subsequent work has predominantly displayed that leg immobilisation and bed rest will reduce both post-absorptive and post-prandial MPS rates, providing a conclusive evidence base for this argument (11,22,23). This decline in MPS rates is rapid as after just 5 days of leg immobilisation both post absorptive and post-prandial MPS rates were reduced to a similar extent, by 41 and 53 % respectively (11). However, this reduction in MPS rates plateaus over time during disuse, as previous research has shown that there is no further decline in post-absorptive MPS rates when comparing 10 and 21 days of leg immobilisation (22).

The role or contribution of MPB rates to disuse induced muscle atrophy is far less well understood. This is largely attributed to the fact that measuring MPB rates *in vivo* is technically more difficult than measuring MPS rates. There are multiple methods to measure MPB rates, reviewed in (38), including two and three pool models with the use of stable isotope tracers to measure a dilution in steady state in the blood by unlabeled amino acid efflux from the muscle and calculate a fractional breakdown rate (39). One of the main differences when using stable isotope tracers to measure MPS and MPB is that when measuring MPS rates it is possible to measure the direct incorporation of the tracer into muscle protein as the endpoint. In contrast when using stable isotope tracers to measure MPB the endpoint is the rate of unlabeled amino acid efflux from muscle tissue (38). From the small number of studies that have assessed the effect of disuse on MPB rates there are conflicting findings. Tesch *et al.*, (2008) report an 44% increase in 3-methylhistidine excretion from muscle, a measure of MPB measured by dialysis after 3 days of unilateral leg immobilisation, conversely (41) observed no changes in MPB rate after short term bed rest. Differences may be due to the time point at which the measurements are made, Tesch *et al*., (2008) measured MPB after 3 days, whereas Symons *et al*., (2009) measured MPB rate after 21 days, a time where previous reviews suggest MPB rates would have been reduced to pre disuse levels (42).

With the current data available it is difficult to ascertain to what role reductions in post-absorptive and post-prandial MPS rates contribute to changes in net protein balance during disuse. Wall et al., (2013) suggested that reductions in post-prandial MPS rates accounted for over 40% of muscle mass lost over 2 weeks of leg immobilisation, and ~80% by post-absorptive and post-prandial combined. Due to both post-absorptive and post-prandial MPS rates declining after a short term period of leg immobilisation other authors have argued that the reduction in MPS can fully explain the muscle atrophy observed during disuse (8,43). Depending on whether MPB rates change during disuse and contribute to changes in net protein balance will determine how much of a role reductions MPS rates may contribute to changes in net protein balance during disuse. However, these current calculations rely on a number of assumptions, most importantly that measuring myofibrillar protein synthesis rates after a period of disuse reflect the changes in chronic myofibrillar protein synthesis rates that occur throughout the entire period of disuse, and inferring muscle mass from single slice cross sectional measurements. The application of more modern techniques, such as applying deuterium oxide ($D_2O$) to measure myofibrillar protein synthesis rates throughout a period of disuse, have now been successfully applied (44). In combination with other methods to measure muscle mass, like magnetic resonance imaging (MRI), these studies should allow us

to improve our understanding of the role and contribution of MPS and MPB rates to disuse induced muscle atrophy.

*Protein intake and muscle disuse*

Recovery from illness or injury often requires a period of muscle disuse, which typically occurs in the form of bed rest (e.g. during hospitalisation) or limb immobilisation (e.g. following injury). Previous work displays clear evidence that muscle disuse in both bed rest and leg immobilisation models will result in leg muscle atrophy and are accompanied by reductions in muscle protein synthesis rates (11,22,23,45). Dietary protein ingestion stimulates protein synthesis rates and inhibits protein breakdown rates which, under normal conditions, allows for postprandial net protein accretion within muscle tissue (46). As a consequence it has been speculated that maintaining or increasing dietary protein consumption during a period of disuse may alleviate the loss of muscle mass and function (47,48). Surprisingly, there has been little research investigating how different amounts of protein intake may affect muscle protein synthesis rates and modulate concomitant muscle atrophy during disuse. This is interesting as under free living conditions a protein intake of around 20 g stimulates MPS rates (49) and over time leads to increases in muscle hypertrophy when combined with resistance training (50). It must be noted that anabolic resistance to protein ingestion has been demonstrated even after a short period of disuse (11), thus if it is possible to overcome this impairment in MPS rates in response to protein ingestion, higher protein intakes are likely to be required (51).

The small number of studies that have controlled protein intake or have provided protein supplementation to participants undergoing a period of disuse have presented conflicting findings. Protein supplementation with 20 g protein provided twice daily in the elderly was ineffective at reducing muscle atrophy during 5 days of leg immobilisation (52). Although the participants' diet was not controlled protein supplementation was increased to 1.6 $g \cdot kg \cdot d^{-1}$ compared to a control group of 1.1 $g \cdot kg \cdot d^{-1}$. In comparison, during one week of bed rest a diet that contained just 1.0 $g \cdot kg \cdot d^{-1}$ of protein was able to maintain net nitrogen balance from pre-bed rest values compared to a diet that had a protein intake of 0.6 $g \cdot kg \cdot d^{-1}$ (53). Unfortunately, no direct measures of muscle mass were made. In contrast, over long term bed rest a study that fully controlled dietary intake and compared 1.6 vs 1.0 $g \cdot kg \cdot d^{-1}$ found that the higher protein intakes actually resulted in a numerically (although not statistically) greater muscle mass loss after 60 days of bed rest (54). Currently the role of different amounts of protein intake during disuse and its influence on muscle atrophy and muscle protein synthesis rates during disuse remains poorly understood. Future work should aim to understand what the optimal amount of protein intake should be to attenuate the loss of muscle mass and function during a period of disuse.

*The role of non-animal derived protein sources on muscle anabolism*

A large body of research has investigated the anabolic potential of ingesting different animal based proteins, such as proteins derived from meat, fish, eggs and milk (55). The primary examples are whey (49,51,56), casein (57), egg (58) and milk (59) proteins, all of which have been shown to stimulate muscle protein synthesis rates. There is a smaller but growing body of research that has investigated the anabolic effects of consuming non-animal based proteins. It has been assumed that the anabolic potential of these protein sources is lower compared to animal based proteins due to their slower digestibility and lower essential amino acid content (55). With the increasing popularity of vegan and vegetarian diets, more

work has assessed the anabolic potential of plant based proteins. Alongside the increasing popularity of plant based proteins the projected increase in the worldwide population to approximately 9 billion by 2050 (60) further places pressure on the need to identify high-quality plant based proteins. This is of particular importance due to the sustainability of plant based proteins which are reported to require between 50 to 150 times less water and 30 to 300 times less land to produce (61) when compared to animal based proteins.

Wheat protein is the most abundant plant based protein and is found in many common foods for example, bread and cereal and compromises around 40% of dietary protein intake in the average US adult (62). Despite reduced digestibility compared to animal based proteins, wheat protein has been shown to increase muscle protein synthesis rates when ingested in higher quantities (e.g. 60 g) (63). Alongside wheat protein other sustainable protein sources, including potato protein (64), also increase post-prandial muscle protein synthesis rates. Further research should continue to find other sustainable protein sources that have strong anabolic properties. One of such protein sources could be mycoprotein. Mycoprotein is a sustainable whole food source that has a high protein content (45% of total mass) and is produced by the cultivation of the fungus *Fusarium venenatum*. Mycoprotein was developed in the 1960's and can be textured (via freezing) and flavored into different products e.g. chicken flavoured burgers or beef flavoured mince (65). Like other non-animal derived protein sources the production of mycoprotein is environmentally friendly and emits less than one fifth of the greenhouse gasses compared to the same quantity of beef protein (Carbon trust, 2014). It has a complete amino acid profile, is high in fibre, and is well tolerated when consumed by humans (66). To date research on the anabolic potential of mycoprotein is limited and thus this will be investigated in Chapter 4 of this thesis.

*Outline of the thesis*

This thesis outlines a number of studies that detail how skeletal muscle disuse affects muscle size, function and metabolism, and investigates how nutritional strategies, particularly the role of protein ingestion, may be used to alleviate the negative effects of muscle disuse on skeletal muscle health. In **Chapter 2** I used serial MRI scans to provide a detailed and temporal picture of upper leg muscle atrophy during short-term (one week) unilateral leg immobilisation in thirteen healthy, young men. I hypothesized that a decrease in thigh muscle volume would already be evident after two days of immobilisation, and that further declines would be seen after a week of immobilisation. Furthermore, I hypothesized that individual muscle groups (and constituent muscles (e.g. *M. quadriceps, M. vastus medialis* and *M. vastus lateralis*) that perform more of a role in habitual physical activities would experience significantly greater reductions in muscle volume when compared with muscles that undertake less habitual loading (e.g. *M. hamstrings*, *M. biceps femoris short head*, *M. adductor* group, muscles). In line with this, I hypothesized that leg extension strength would decline to a greater extent than leg flexion strength after seven days of unilateral leg immobilisation.

Recently the application of deuterated water as a means to measure muscle protein synthesis rates has regained popularity in the field due to its capacity to assess free living muscle protein synthesis rates during interventions lasting days (67–69) or weeks (44,70). This methodology has recently been applied to assess cumulative (71) and temporal (70) changes in free living muscle protein synthesis rates throughout resistance training programs of varying durations. Few studies have applied this method in muscle disuse atrophy research (44,72). In **Chapter 3** I applied oral deuterated water dosing methods to

assess the temporal impact of disuse on myofibrillar protein synthesis rates during one week of limb immobilisation in healthy, young men. I hypothesized that disuse would reduce myofibrillar protein synthesis rates within 2 days, and to a greater extent than after one week, and this would primarily explain the observed changes in the rate of muscle disuse atrophy.

In **Chapter 4** I investigated the effectiveness of a sustainable non-animal derived protein source called mycoprotein to increase circulating amino acid levels *in vivo* in humans. Mycoprotein is a protein source that is created by the continuous fermentation of a fungi called *Fusarium Venenatum*. With the environmental concerns of some forms of animal based protein sources, particularly the carbon emissions and water usage involved in the production of animal based proteins, mycoprotein represents a more environmentally friendly protein source. In Chapter 4, we investigated the amino acid bioavailability and anabolic potential of mycoprotein in comparison to a reference animal derived protein source.

In **Chapter 5** I conducted a dose-response study comparing how high (1.6 g·kg bm·$d^{-1}$), low (0.5 g·kg bm·$d^{-1}$) and negligible (0.15 g·kg bm·$d^{-1}$) daily dietary protein intakes influenced daily MPS rates determined using the deuterated water approach (73) and muscle mass loss determined via MRI (73) during a three day period of unilateral leg immobilisation in healthy, young men. I hypothesised that declining dietary protein intakes would lead to a greater decline in daily myofibrillar protein synthesis rates and a greater muscle loss.

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# Chapter 2

## Temporal and muscle specific disuse atrophy during one week of leg immobilisation

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## *Abstract*

Background Musculoskeletal injuries necessitate periods of disuse (i.e. limb immobilisation) during which rapid skeletal muscle atrophy occurs. The relative susceptibility of different muscles of the thigh to disuse atrophy remains uninvestigated. I assessed muscle disuse atrophy of individual thigh muscles throughout one week of unilateral knee immobilisation.

Methods Thirteen healthy, young (20.2±0.6 y) men underwent 7 days of unilateral leg immobilisation via knee bracing. MRI scans were performed bilaterally prior to, and following 2 and 7 days of immobilisation to determine the volume and anatomical cross-sectional area (aCSA) of the individual muscle groups of the upper legs.

Results In contrast to the control leg, total thigh muscle volume had decreased by 1.7±0.3 ($P$<0.01) and 5.5±0.6% ($P$<0.001) in the immobilised leg after 2 and 7 days of disuse, respectively. Muscle loss was significantly greater in the *M. quadriceps* (day 2; 1.7±0.3 ($P$<0.05) and day 7; 6.7±0.6%) when compared with the *M. hamstrings* (day 2; 1.4±0.2% ($P$<0.01) and day 7; 3.5±0.3%) following 7 days of disuse ($P$<0.001). Individual muscles of the thigh exhibited different atrophy rates with the *M. vastus lateralis* aCSA showing the greater (2.6±0.4 and 7.2±0.8%), and the *M. gracilis* the lesser (1.1±0.7 and 2.3±1.0%) decline following 2 and 7 days of immobilisation, respectively ($P$<0.01).

Conclusion Thigh muscle disuse atrophy occurs rapidly and is already evident within 2 days of leg immobilisation and progresses at a similar rate over the next 5 days (~0.8% muscle loss per day). *M. quadriceps* muscle shows more atrophy when compared with the *M. hamstrings*.

## *Introduction*

Periods of skeletal muscle disuse can occur for a variety of reasons, such as hospitalization that may mandate bed-rest or due to injury which may require limb immobilisation. The average hospital stay is 5-7 days when admitted with acute illness (1) and most periods of disuse requiring home-based recovery (illness or mild injury) also last less than one week (2). Consequently, the clinical relevance of such short-term disuse has recently attracted considerable research attention (3, 4, 5). Studies have shown that 5-7 days of disuse results in marked declines in muscle size (6), muscle fiber cross sectional area (CSA) (4), strength (3, 6), and insulin sensitivity (3, 7) and induces anabolic resistance of skeletal muscle tissue (8, 9). This potent deconditioning effect of short-term disuse also lends support for previous suggestions that short periods of disuse may be additive over the lifespan and act as the key driver of age-related muscle loss (10, 11).

To date, research on short-term disuse atrophy has mainly assessed limb or whole body fat free mass (FFM) using dual-energy x-ray absorptiometry (DEXA), particularly for whole body disuse (12, 13), or muscle anatomical CSA (aCSA) of large muscle groups (for example mid- thigh or *M. quadriceps*) using computed tomography (CT) generally when a unilateral limb immobilisation model is used (6, 9). Applying magnetic resonance imaging (MRI) to obtain serial images of a muscle group allows for the quantification of muscle volume of an entire muscle group, and allows for the calculation of changes in the individual constituent muscles (14, 15). Furthermore, by utilizing MRI to calculate muscle volume and by assuming or calculating muscle density, the determination of muscle mass is possible (16). Therefore, MRI may provide a more sensitive tool to assess time dependent changes in muscle mass during short periods of disuse.

In the present study we used serial MRI scans to provide a detailed and temporal picture of upper leg muscle atrophy in combination with unilateral leg strength testing during short-term (one week) unilateral leg immobilisation in thirteen healthy, young men. We hypothesized that a decrease in thigh, *M. quadriceps* and *M. hamstrings* muscle volume would already be evident after two days of immobilisation, and that further declines would be seen after a further five days of immobilisation. Furthermore, we hypothesized that individual muscle groups (and constituent muscles (e.g. *M. quadriceps, M. vastus medialis* and *M. vastus lateralis*) that perform more of a role in habitual physical activities would experience significantly greater reductions in muscle volume and aCSA when compared with muscles that undertake less habitual loading (e.g. *M. hamstrings*, *M. biceps femoris short head*, *M. adductor* group, *M. sartorius* and *M. gracilis* muscles). In line with this, we hypothesized that *M. quadriceps* strength would decline to a greater extent than *M. hamstrings* strength after seven days of unilateral leg immobilisation.

## *Materials and methods*

### *Subjects*

Thirteen young, healthy males (age = 20±1 y; body mass index (BMI) = 23.4±0.9 kg·m$^{-2}$; body fat % = 15±2; lean mass = 63±2 kg) volunteered to take part in this study. Prior to inclusion in the study all subjects underwent a routine medical screening to ensure no adverse health conditions were present and that they met the eligibility criteria for the study. Only young males were included in the present study since both age (17) and sex (18) can influence the rate of muscle disuse atrophy, and our goal was to maintain a homogenous population for the current study. Exclusion criteria included; a personal or family history of thrombosis, musculoskeletal/metabolic disorders (e.g. type 2 diabetes), BMI <18.5 or >28, any musculoskeletal injury of the legs in the 12 months before starting the study, any contraindications to MRI scanning (e.g. metallic implants), participation in a structured resistance type exercise training program within 6 months prior to the study, consumption of any nutritional supplements prior to and during the study. During the screening visit subjects completed a general health questionnaire, body composition was assessed by air displacement plethysmography (BODPOD; Life Measurement, Inc. CA, USA), height, weight and blood pressure were measured. Subjects were also familiarized to the unilateral one-repetition maximum (1-RM) strength testing technique for each exercise with the dominant (i.e. stronger) leg being identified. All subjects were informed of the nature and possible risks of the experimental procedures before providing written informed consent. The study was approved by The Sport and Health Science Ethics committee of the University of Exeter (150221/B/02), in accordance with the guidelines set out in the Declaration of Helsinki. This study was registered as a clinical trial with clinicaltrials.gov (NCT02984332).

### *Experimental protocol*

A schematic of the experimental design is shown in **Figure 2.1**. After screening, eligible subjects reported to the laboratory at ~0900 h in the fasted state for a pre-testing visit where 1-RM strength testing was performed. One week after the pre-testing visit subjects returned to the laboratory (subjects were instructed to refrain from vigorous exercise during this period) and underwent a pre-immobilisation magnetic resonance imaging (MRI) scan of both thighs at ~0800 h before starting the immobilisation period at ~0900 h. After 2 days of immobilisation the subjects returned to the laboratory and underwent a second identical MRI scan at ~0900 h, during which the brace was removed by the experimenter for the MRI scan and was re-fitted in the exact same position immediately post-MRI. The subject was assisted onto the MRI bed using a wheelchair to ensure no weight bearing of the immobilised leg occurred. After 7 days of immobilisation subjects completed the final study visit, which consisted of a final MRI scan at ~0900 h followed immediately by post-immobilisation unilateral 1-RM strength testing. Subjects were only allowed to bear weight on the immobilised leg following completion of the strength testing.

### *Immobilisation protocol*

A schematic of the experimental design is shown in **Figure 2.1**. After screening, eligible subjects reported

to the laboratory at ~0900 h in the fasted state for a pre-testing visit where 1-RM strength testing was performed. One week after the pre-testing visit subjects returned to the laboratory (subjects were instructed to refrain from vigorous exercise during this period) and underwent a pre-immobilisation magnetic resonance imaging (MRI) scan of both thighs at ~0800 h before starting the immobilisation period at ~0900 h. After 2 days of immobilisation the subjects returned to the laboratory and underwent a second identical MRI scan at ~0900 h, during which the brace was removed by the experimenter for the MRI scan and was re-fitted in the exact same position immediately post-MRI. The subject was assisted onto the MRI bed using a wheelchair to ensure no weight bearing of the immobilised leg occurred. After 7 days of immobilisation subjects completed the final study visit, which consisted of a final MRI scan at ~0900 h followed immediately by post-immobilisation unilateral 1-RM strength testing. Subjects were only allowed to bear weight on the immobilised leg following completion of the strength testing.

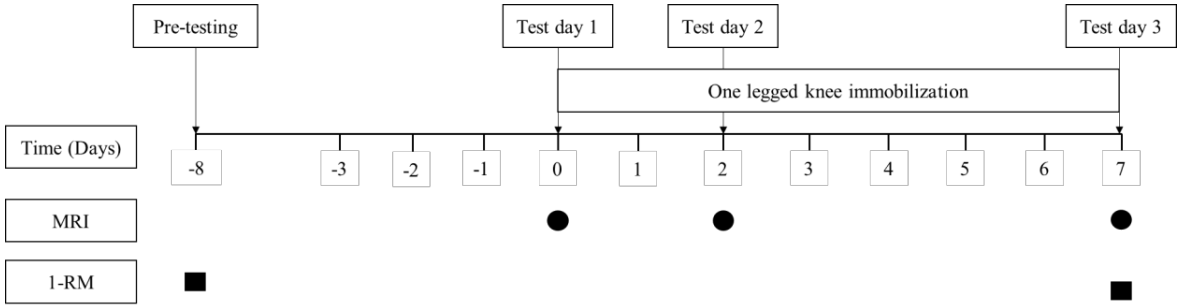


***Figure 2.1*** Schematic representation of the experimental design. Thirteen healthy young males underwent 7 days of unilateral leg immobilisation via knee brace. MRI, Magnetic resonance imaging, 1-RM, unilateral 1-repetition maximum strength testing.

*Determination of muscle volume and anatomical cross-sectional areas (aCSA)*

A 1.5 tesla (T) MRI scanner (Intera, Phillips, The Netherlands) was used to obtain images of both thighs in the axial plane over the full length of the femur. A T1-weighted 3D turbo spin echo sequence was used (field of view 500 x 500 mm, reconstructed matrix 512 x 512 mm, echo time 15 ms, repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the supine position. A 4-element sense body radiofrequency (RF) coil was wrapped around both thighs. During the pre-immobilisation scan a specified distance from a bony landmark (femoral condoyle) on the immobilised leg in the frontal plane was used to center the axial plane images (19). This same distance was used on all subsequent MRI scans to ensure the axial images were in the same location along the length of the thigh on all scans.

Philips on-line MRI software was used to analyze the images obtained in the axial plane (the same experimenter performed all manual segmentation of the images). Starting at the most distal image (approximately mid patella) where each muscle group could be fully delineated, the muscle of interest was manually segmented to calculate slice anatomical cross-sectional area (aCSA). Moving proximally every third image was analyzed until complete delineation of the muscle groups of interest could no longer be identified (the femoral head for the *M. quadriceps* and thigh muscle groups and the ischial tuberosity (*M. hamstrings* origin) for the *M. hamstrings*). This was on average 16, 15 and 14 images for the thigh, *M. quadriceps* and *M. hamstrings* volume measurements, respectively (see **Figure 2.2** for

representative images). This method for calculating muscle volume has previously been reported to give accurate and reliable results (20). We also internally validated this approach where we compared data from analyzing every image (i.e. 45 images) along the length of the muscle with the analyzes of every third image (i.e. 15 images; for 3 subjects) which elicited equivalent calculations of muscle volume (bias = -21.6 $cm^3$, standard deviation = 7.7, lower limits of agreement = -36.7 $cm^3$, upper limits of agreement -6.4 $cm^3$). Muscle volume was calculated using a previously published method (16) where the total aCSA for all images was calculated and multiplied by the slice thickness plus the distance between slices (linear interpolation) (in this case a total of 3 cm, 5 mm slice thickness, 25 mm slice gap), summarized by the following equation:

$$muscle\ volume = \sum_{aCSA} \cdot (slice\ thickness + slice\ gap)$$

Mid *M. quadriceps* aCSA was calculated by segmenting the *M. quadriceps* muscle in a single MR image 15 cm above the top of the patella to aid comparisons to previous studies (9, 19, 21). aCSA was calculated for all the individual muscles of the thigh that could be completely delineated (eleven individual muscles; *M. rectus femoris*, *M. vastus lateralis*, *M. vastus intermedius*, *M. vastus medialis*, *M. semitendinosus, M.*

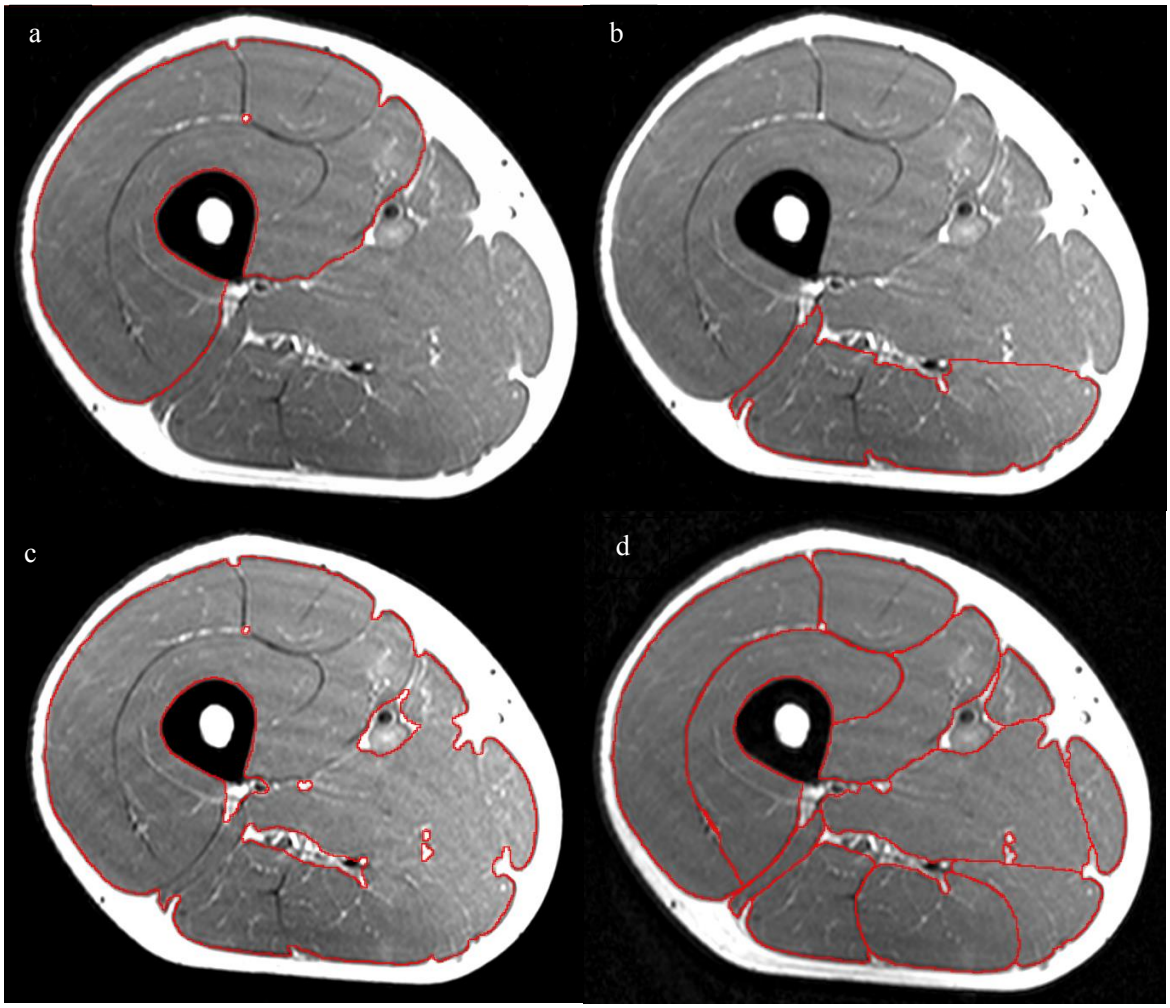


***Figure 2.2*** Representative MR images in the middle of the upper leg with delineations of; a) *M. quadriceps* muscle, b) *M. hamstrings* muscle, c) thigh muscle and d) 11 individual muscles.

*semimembranosus, M. biceps femoris short head*, *M. biceps femoris long head*, *M. adductor* group (3 *M. adductor* muscles combined), *M. sartorius*, *M. gracilis*) of both the immobilised and control legs (muscles such as the *M. pectineus*, *M. psoas* and *M. iliopsoas* were not analyzed as they could not be adequately visualized for quantification). The single axial MR image that had the largest aCSA for each muscle was located (at a specific location along the length of the thigh) and the muscle of interest was manually segmented as described above. This same corresponding image (on the same location of the thigh) was analyzed on scans 2 and 3 to assess for changes in aCSA over the immobilisation period.

### *Calculations of muscle mass and muscle atrophy related to anatomical longitude*

Muscle volumes calculated from the MRI scans were used to calculate muscle mass to understand the weight of muscle tissue lost during immobilisation. Muscle mass was calculated by multiplying muscle volume by 1.04 $g \cdot cm^{-3}$ (the reported density of leg muscle tissue) (22) in line with previous work (16). To assess if different longitudinal sections of a muscle group atrophied to differing extents, the length of each muscle group (*M. quadriceps*, *M. hamstrings*, thigh) was first calculated by summing the thickness of all the slices and slice gaps between the most proximal and distal images analyzed. Subsequently, starting from the knee (the most distally analyzed image = 0%) the distances at 10% intervals up to the femoral head (the most proximally analyzed image = 100%) were calculated for each muscle and each subject to allow for comparisons between subjects of different heights and leg lengths. Then the aCSA analyzed at each 10% distance along the length of the muscle (or the aCSA analyzed that was closest to each 10% distance) for each muscle group and each subject was compared between pre- and post- immobilisation (i.e. day 0 and 7) for the immobilised leg.

### *Strength testing*

Unilateral leg extension, leg curl, leg press and calf raise exercises were performed in the stated order and for both legs separately using standard gym equipment (Life Fitness, Cambridge, UK). 1-RM strength testing was assessed using an incremental multiple repetition testing procedure that was carried out individually for each leg with the leg identified to be immobilised always being tested first. After two warm up sets of 8 and 4 repetitions at self-determined 25% and 50% of 1-RM, respectively, single repetitions at 1-RM were attempted. The weight was increased incrementally until no further weight could be lifted, with each attempt separated by a 2-min rest. The final 1-RM lift was taken as the heaviest repetition that was successfully completed with correct technique where full range of motion was achieved.

### *Statistical analyses*

All data are presented as means±SEM and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Two-way repeated measures ANOVAs with leg (immobilised and control) and time (pre and post (for strength); or day 0, day 2 and day 7 (for MRI)) as within subjects factors were used to compare differences in strength, muscle volume and aCSA. For the immobilised leg only, a two-way ANOVA with muscle group and time as within subject factors was used

to assess if different muscle groups responded differently to immobilisation. Pearson's correlation analyses were used to assess the relationship between initial muscle size (*M. quadriceps* and *M. hamstrings*) or pre-immobilisation muscle strength (leg extension and leg curl) with the absolute amount of muscle volume lost after 7 days for each muscle group. A Pearson's correlation was also used to assess the relationship between pre-immobilisation aCSA along the longitude of each muscle (thigh, *M. quadriceps* and *M. hamstrings*) and the aCSA lost along the longitude of each muscle after 7 days of immobilisation. For all ANOVAs, when a significant interaction was found Bonferroni post-hoc tests were applied to locate individual differences. Statistical significance was set at $P<0.05$.

## *Results*

### *Muscle group volumes and mass*

Muscle volume of the thigh, *M. quadriceps*, and *M. hamstrings* muscle groups as computed from MRI scans are displayed in **Figure 2.3** There were no differences in muscle volume between the legs pre-immobilisation for any of the muscle groups ((thigh: control leg = 4651±222 $cm^3$, immobilised leg = 4697±237 $cm^3$ ($P$>0.05); *M. quadriceps*: control leg = 2315±120 $cm^3$, immobilised leg = 2342±129 $cm^3$ ($P$>0.05); *M. hamstrings*: control leg = 833±40 $cm^3$, immobilised leg = 830±38 $cm^3$ ($P$>0.05)). In the control leg muscle volume remained unchanged at all time points in all muscle groups (all $P$>0.05). Significant time *x* leg interactions were detected for thigh ($P$<0.001), *M. quadriceps* ($P$<0.001) and *M. hamstrings* ($P$<0.001) such that the immobilised leg thigh muscle volume decreased by 1.7±0.3% (to 4617±236 $cm^3$, $P$<0.001), comprising a 1.7±0.3% decline in *M. quadriceps* volume (to 2301±127 $cm^3$; $P$<0.01) and 1.4±0.2% decline in *M. hamstrings* muscle volume (to 818±37 $cm^3$; $P$<0.001)) after 2 days. Similarly, after 7 days of immobilisation thigh, *M. quadriceps* and *M. hamstrings* muscle volumes had decreased by a further 3.9±0.4, 5.0±0.6 and 2.1±0.2%, respectively (to 4438±223, 2186±117 and 801±37 $cm^3$, respectively). This resulted in a total muscle volume loss of 5.5±0.6, 6.7±0.6 and 3.5±0.3% from thigh, *M. quadriceps* and *M. hamstrings*, respectively, over the entire week. The contribution of *M. quadriceps*, *M. hamstrings* and remaining (e.g. *M. adductor* group, *M. sartorius*, *M. gracilis*) muscle volume lost to total thigh muscle volume lost after 7 days is shown in **Figure 2.3a**. The relative loss of muscle volume was not different between the *M. hamstrings* and *M. quadriceps* during the first 2 days of immobilisation, but was significantly greater in the *M. quadriceps* between 2 and 7 days ($P$<0.01) and over the entire week ($P$<0.001). When expressed as muscle mass, similar changes in the leg muscles were observed with immobilisation. Specifically, there were no differences in thigh (4837±231 g and 4885±246 g; $P$>0.05), *M. quadriceps* (2407±124 g and 2435±134 g; $P$>0.05) or *M. hamstrings* (866±41 g and 863±40 g; $P$>0.05) muscle mass between control and immobilised legs, respectively, and no significant changes were observed with time in any of the muscle groups in the control leg. In the immobilised leg thigh, *M. quadriceps*, and *M. hamstrings* muscle groups lost 83±14 g *(P*<0.01), 42±8 g ($P$<0.01) and 12±2 g ($P$<0.001) of muscle mass, respectively, after 2 days of immobilisation. After 7 days of immobilisation the immobilised leg had lost 269±32 g from the thigh muscle ($P$<0.001), which was attributed to a *M. quadriceps* muscle mass loss of 162±19 g ($P$<0.001) and *M. hamstrings* muscle mass loss of 30±3 g ($P$<0.001). After 7 (day 7 *M. quadriceps* to *M. hamstrings* mass difference = 132 g, $P$<0.001) but not 2 (day 2 *M. quadriceps* to *M. hamstrings* mass difference 30 g, $P$>0.05) days of immobilisation the *M. quadriceps* lost more muscle mass than the *M. hamstrings*.

### *Mid- M. quadriceps and individual muscle anatomical cross-sectional areas (aCSA)*

There were no differences in mid- *M. quadriceps* aCSA at the pre-immobilisation timepoint (control leg = 7940±306 $mm^2$, immobilised leg = 8068±326 $mm^2$ ($P$>0.05)). In the control leg mid- *M. quadriceps* aCSA remained unchanged at all time points (all $P$>0.05). Significant time *x* leg interactions were detected for mid- *M. quadriceps* aCSA ($P$<0.001) such that the immobilised leg mid- *M. quadriceps* aCSA decreased

after 2 days by 1.6±0.3% (to 7935±331 $mm^2$, $P$<0.05) and 7 days by 6.3±0.5% (to 7556±300 $mm^2$, $P$<0.001). Individual muscle aCSA for the control and immobilised legs during immobilisation are presented in **Figure 2.5a and b**. For all the individual muscles there were no differences between the control and immobilised leg pre-immobilisation ($P$>0.05). No changes were observed in any individual muscle aCSA during immobilisation in the control leg ($P$>0.05) with the exception of the adductor muscle group which decreased after 7 days (1.3±0.5%, $P$<0.05). Significant time *x* leg interactions were found for all individual muscles (all $P$<0.001 except adductor group $P$<0.01) except the sartorius ($P$=0.056) and gracilis ($P$>0.05). After two days of immobilisation the following individual muscles' aCSA decreased; *M. vastus lateralis* by 2.6±0.4% ($P$<0.001), *M. vastus medialis* by 2.0±0.5% ($P$<0.01), *M. vastus intermedius* by 2.5±0.4% ($P$<0.05), *M. rectus femoris* by 1.4±0.4% ($P$<0.05), *M. semitendinosus* by 1.7±0.5% ($P$<0.01), and *M. adductor* group by 1.4±0.4% ($P$<0.05). After 7 days of immobilisation all individual muscles except the *M. gracilis* and *M. sartorius* had decreased in the immobilised leg; *M. vastus lateralis* by 7.2±0.8%, *M. vastus medialis* by 7.0±0.7%, *M. vastus intermedius* by 6.9±1.2%, *M. rectus femoris* by 4.5±0.6%, *M. biceps femoris long head* by 5.4±0.6%, *M. biceps femoris short head* by 2.9±0.6%, *M. semimembranosus* by 5.0±0.6%, *M. semitendinosus* by 4.6±0.6%, *M. adductor* group by 3.5±0.5% (all $P$<0.001). The contribution of each of the 11 individual muscle CSA lost to total thigh muscle CSA lost after 7 days is shown in **Figure 2.5b.** There were differences in relative muscle loss between individual muscles over the entire week of immobilisation ($P$<0.001). Specifically, the *M. vastus lateralis* lost significantly more relative aCSA than the *M. adductor* group ($P$<0.05), *M. biceps femoris short head* ($P$<0.01), *M. sartorius* ($P$<0.001) and *M. gracilis* ($P$<0.001). The *M. vastus intermedius* reduced in aCSA to a greater degree than the *M. biceps femoris short head* ($P$<0.05), *M. sartorius* ($P$<0.01) and the *M. gracilis* ($P$<0.001) muscles. The *M. vastus medialis* lost significantly more aCSA compared with the *M. biceps femoris short head* ($P$<0.01), the *M. sartorius* ($P$<0.01) and the *M. gracilis* ($P$<0.001) muscles.

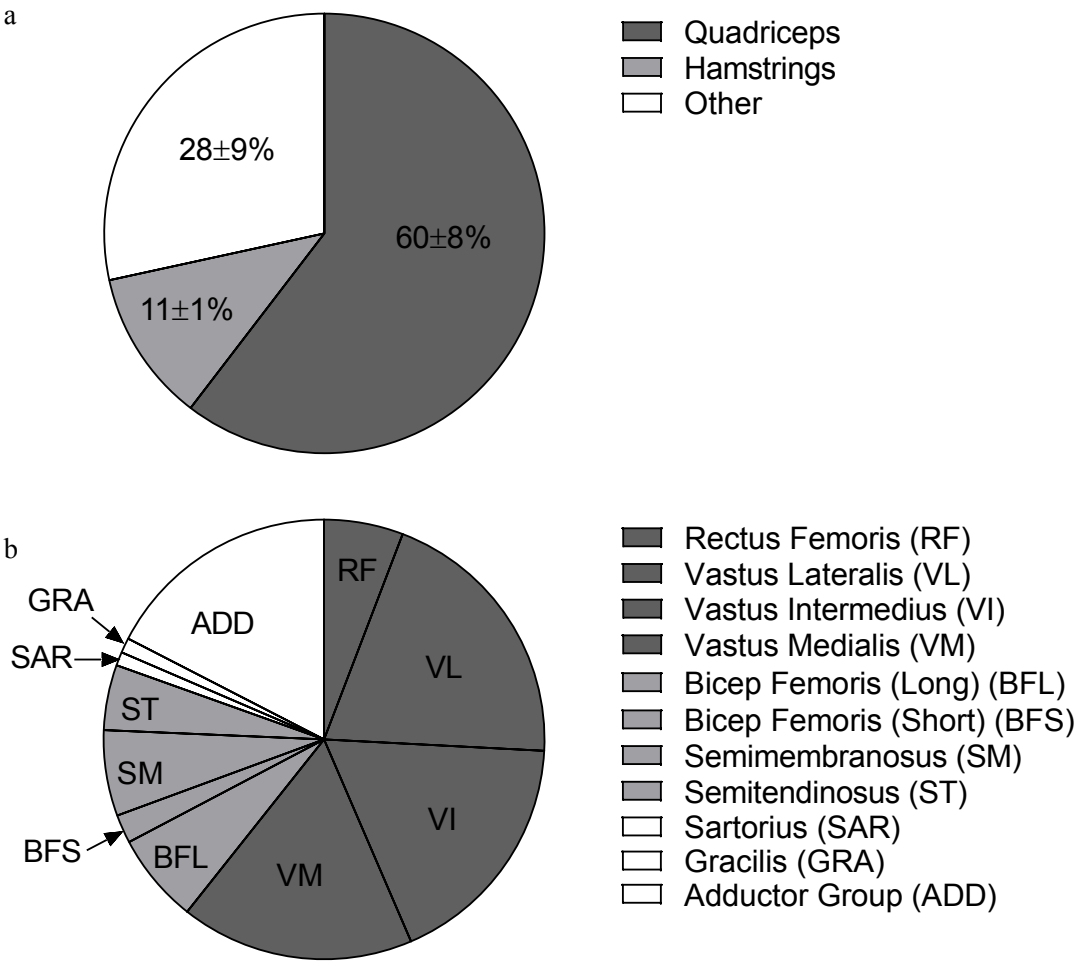


***Figure 2.4*** a) Pie chart depicting the contribution of absolute *M. quadriceps*, *M. hamstrings* and other (e.g. *M. adductor* group, *M. sartorius*, *M. gracilis* volume) muscle group volume lost as a proportion of absolute thigh volume lost in the immobilised leg after 7 days of immobilisation. Note absolute total thigh volume lost expressed as 100%, and *M. quadriceps, M. hamstrings,* and other muscle volume contributions expressed as percentages (means±SEM, displayed on each segment). b) Pie chart depicting the contribution of each of the 11 measured individual muscles absolute aCSA lost as a proportion of absolute thigh aCSA lost after 7 days of immobilisation in the immobilised leg. Note absolute total thigh aCSA lost expressed as 100% and the contribution of each individual muscle are expressed as percentages, as follows; *M. rectus femoris* contribution = 5.8±0.8%, *M. vastus lateralis* contribution = 20.0±2.6%, *M. vastus intermedius* contribution = 17.7±2.5%, *M. vastus medialis* contribution = 17.1±1.5%, *M. biceps femoris long head* contribution = 6.7±0.8%, *M. biceps femoris short head* contribution = 2.1±0.6%, *M. semimembranosus* contribution = 6.3±0.7%, *M. semitendinosus* contribution = 4.8±0.6%, *M. sartorius* contribution = 1.0±0.3%, *M. gracilis* contribution = 1.1±0.5%, *M. adductor* group contribution = 17.4±2.1%. For both figure 4a and b, dark grey segments represents *M. quadriceps* volume and *M. quadriceps* individual muscles respectively, light grey segments represent *M. hamstrings* volume and individual *M. hamstrings* muscles respectively, and white segments represents other/remaining muscle volume and individual muscles respectively. Data that make the segments represent means, *n*=13. Data in the legend represents means±SEM, *n*=13.

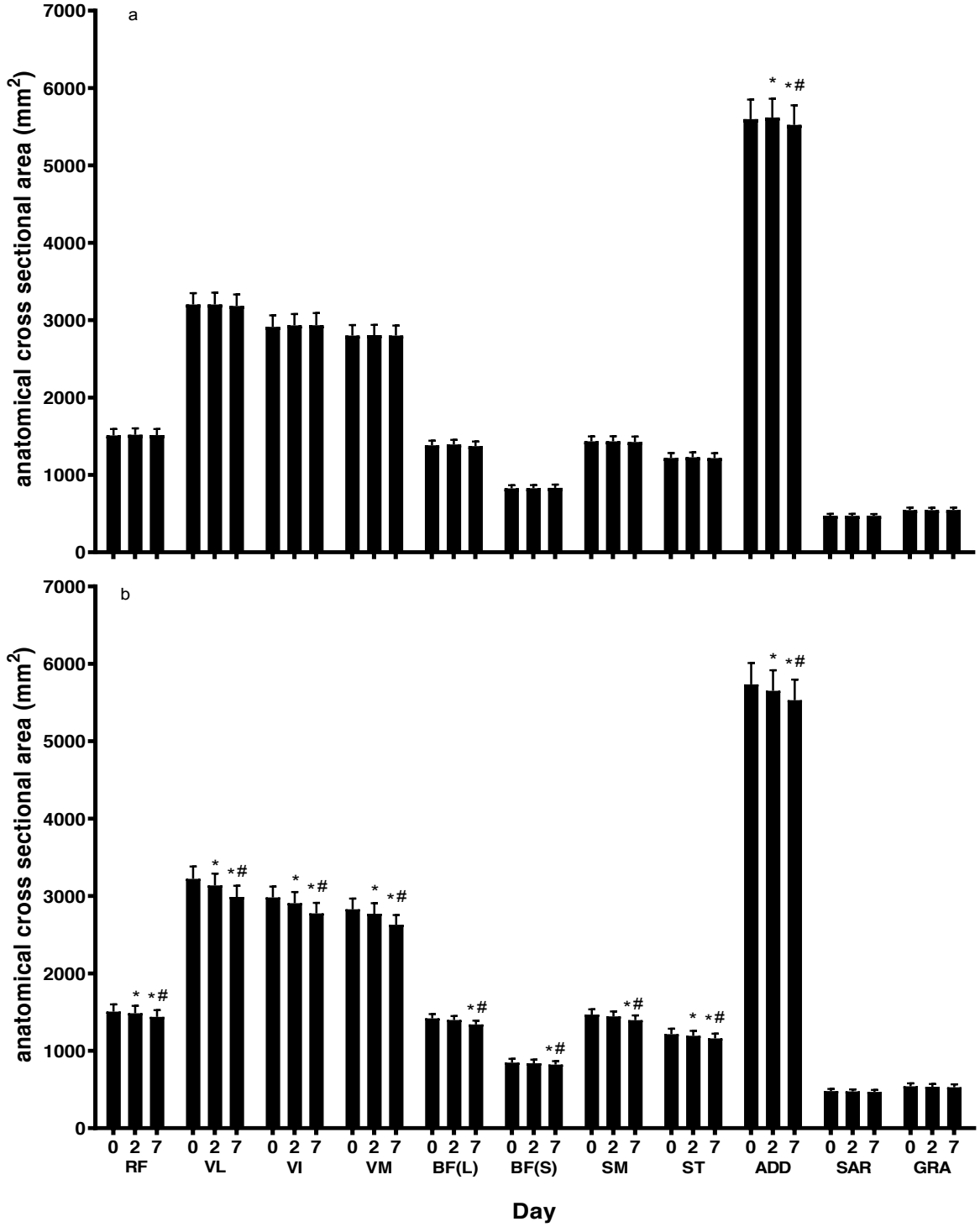


***Figure 2.5*** Individual muscle anatomical cross-sectional area of 11 muscles of the thigh of both the control leg (a) and the immobilised leg (b) before (day 0) and after 2 and 7 days of immobilisation, individual muscle cross sectional area was measured using a 1.5 T MRI scanner. The initials of each muscle are noted along the x axis (RF = rectus femoris, VL = vastus lateralis, VI = vastus intermedius, VM = vastus medialis, BF(L) = biceps femoris long head, BF(S) = biceps femoris short head, SM = semimembranosus, ST = semitendinosus, ADD = adductor group, SAR = sartorius, GRA = gracilis. A two-way repeated measures ANOVA (leg x time) was conducted for each muscle group, where a significant interaction was detected a Bonferroni post hoc test was conducted, * denotes $P<0.05$ significant difference from day 0 within the same leg, # denotes $P<0.05$ significant difference from day 2 within the same leg. Data presented are means±SEM, $n=13$.

*Muscle atrophy related to anatomical longitude*

The data presented in **Figure 2.6**, show the absolute and relative muscle volume for the thigh (a and b), *M. quadriceps* (c and d) and *M. hamstrings* (e and f) muscle groups for the immobilised leg expressed as declines in aCSA along the length of the muscle (i.e. separated into 10% segments from knee to hip). After 7 days of immobilisation the thigh, *M. quadriceps* and *M. hamstrings* experienced heterogenous absolute reduction in aCSA along the length of the muscle (Figure 2.6a, c and e; all $P<0.001$) which was still the case for the thigh and *M. quadriceps* when expressed as relative loss ($P<0.001$), but not the *M. hamstrings* ($P>0.05$) (Figure 2.6b, d and f). For the thigh, the central portion of the muscle (middle 10%, 50-60%) lost aCSA to a greater absolute extent (50% = 972±139 $mm^2$ and 60% = 1141±144 $mm^2$) compared with the most proximal 10% (90-100% of thigh length, hip joint. 90% = 335±126 $mm^2$ and 100% = -28±82 $mm^2$) and the most distal 10% (0-10% of thigh length, knee joint. 0% = -1±21 $mm^2$, 10% = 84±103 $mm^2$) sections of the thigh muscle. All 10% increments from 30% to 70% along the length of the thigh muscle reduced in aCSA in comparison to 0% (all $P<0.001$ except 30% compared with 0%, $P<0.05$) and 100% (all $P<0.001$, except 30% compared to 100%, $P<0.01$) where no change in aCSA was found between 0 and 7 days of immobilisation ($P>0.05$). When looking at the relative change in aCSA along the length of the thigh muscle, the only differences were 50% (-6.0±0.8%, $P<0.05$) and 60% (-6.5±0.7%, $P<0.05$) sections which declined to a greater extent than the 0% section (1.1±2.5%). Similar to the thigh, the *M. quadriceps* also experienced largest absolute reduction in aCSA in the central portion of the muscle (Figure 2.6c), where 40-70% area above the knee reduced in aCSA significantly more than 0 and 100% (all $P<0.001$). Although there were fewer significant differences in relative *M. quadriceps* decreases in aCSA (Figure 2.6d), again the central area of the *M. quadriceps* (40-70%) decreased in aCSA more than 0% ($P<0.05$) but not 100% ($P>0.05$). In contrast, the *M. hamstrings* experienced less variation in absolute reductions in aCSA (Figure 2.6e) where only 40% and 50% areas above the knee declined more than 0 and 100% (both $P<0.05$), and no differences were found along the length of the *M. hamstrings* muscle when relative declines in aCSA was assessed (Figure 2.6f).

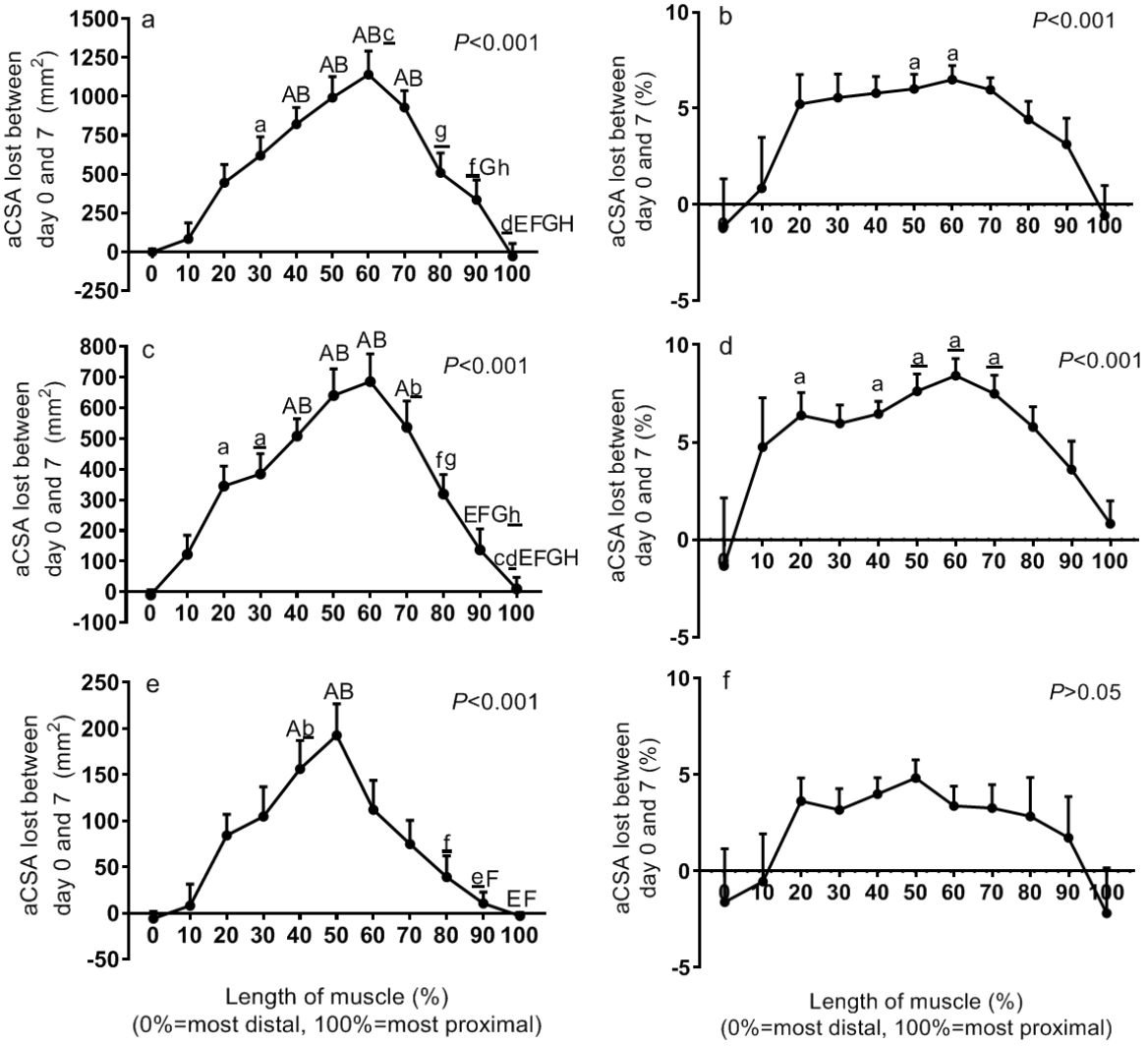


***Figure 2.6*** Muscle atrophy related to anatomical longitude of the muscle groups measured between days 0 and 7 of immobilization in the immobilized leg. 0% represents the most distal MRI image along the length of the leg that was analyzed e.g. lateral femoral condyle at the knee joint. 100% represents the most proximal MRI image along the length of the leg that was analyzed e.g. the greater trochanter at the hip joint. Graph a) = absolute aCSA lost along the length of the thigh muscle, graph b) = % aCSA lost along the length of the thigh muscle, graph c) = absolute aCSA lost along the length of the *M. quadriceps* muscle, graph d) = % aCSA lost along the length of the *M. quadriceps* muscle, graph e) = absolute aCSA lost along the length of the *M. hamstrings* muscle, graph f) = % aCSA lost along the length of the *M. hamstrings* muscle. A one-way ANOVA was used for each muscle group to assess if different locations along the length of the thigh atrophied more than others, *P* values represented above each graph, where a significant test was found Bonferroni post hoc tests were used to assess where differences occurred, these are displayed on the graph. a, a and A denote *P*<0.05, *P*<0.01, *P*<0.001 significant difference from 0%. b and B denote *P*<0.01, *P*<0.001 significant difference from 10%. c and c denote *P*<0.05 and *P*<0.01 significant difference from 20%. d denote *P*<0.01 significant difference from 30%. e and E denote *P*<0.01 and *P*<0.001 significant difference from 40%. f, f and F denote *P*<0.05, *P*<0.01, *P*<0.001 significant difference from 50%. g, g and G denote *P*<0.05, *P*<0.01, *P*<0.001 significant difference from 60%. h, h and H denote *P*<0.05, *P*<0.01, *P*<0.001 significant difference from 70%. Data presented are means±SEM, *n*=13.

### *Leg strength*

Unilateral leg strength data are presented in Table 1.1. Maximum strength decreased for leg extension (by 18.7±1.8%, *P*<0.001), leg press (by 21.0±3.5%, *P*<0.001) and calf raises (by 8.3±1.5%, *P*<0.001) in the immobilised leg, with no changes in the control leg. Neither the immobilised nor control leg changed in maximum strength for the leg curl exercise after 7 days of immobilisation (*P*>0.05).

| Leg | Exercise (single leg) | Pre Immobilisation (kg) | Post Immobilisation (kg) | Percentage change (%) |
|---|---|---|---|---|
| Control | Leg Extension | 89±5 | 88±5 | -1±2 |
| | Leg Press | 133±8 | 138±9 | +3±2 |
| | Leg Curl | 49±3 | 50±3 | +0.4±2 |
| | Calf Raise | 106±6 | 104±6 | -2±2 |
| Immobilised | Leg Extension | 92±6 | 74±4* | -19±2[b] |
| | Leg Press | 139±10 | 109±9* | -21±4[b] |
| | Leg Curl | 49±3 | 47±3 | -4±2 |
| | Calf Raise | 106±7 | 97±7* | -9±2[a] |

***Table 2.1*** Values represent means±SEM, *n*=13. *=significant difference from pre-immobilization value, *P*<0.001. [a]=significant difference from control leg for same exercise *P*<0.05, [b]=significant difference from control leg for same exercise *P*<0.001.

### *Correlational analyzes*

Correlational analyzes are shown in **Figure 2.7**. Pre-immobilisation muscle volume showed a moderate but significant negative correlation with the absolute (but not relative) amount of muscle volume lost for both the *M. quadriceps* (absolute, r=-0.679, *P*<0.01; relative, r=-0.15, *P*>0.05) and *M. hamstrings* muscle groups (r=-0.592, *P*<0.05; relative, r=-0.10, *P*>0.05), with the thigh muscle showing a trend for the same response (r=-0.501, *P*=0.081; relative, r=-0.06, *P*>0.05). The absolute change in *M. quadriceps* volume had a moderate positive correlation with the absolute change in leg extension strength (r=0.563, *P*<0.05), though no significant correlation was observed between the absolute loss of *M. hamstrings* volume and absolute change in leg curl strength after 7 days of immobilisation (r=-0.592, *P*<0.05). Pre-immobilisation aCSA along the length of the thigh, *M. quadriceps* and *M. hamstrings* muscles all had a positive correlation with aCSA lost along the length of each muscle respectively after 7 days of immobilisation (thigh, r=0.718 *P*<0.001, *M. quadriceps*, r=0.741 *P*<0.001, *M. hamstrings*, r=0.596 *P*<0.001) (**Figure 2.7**).

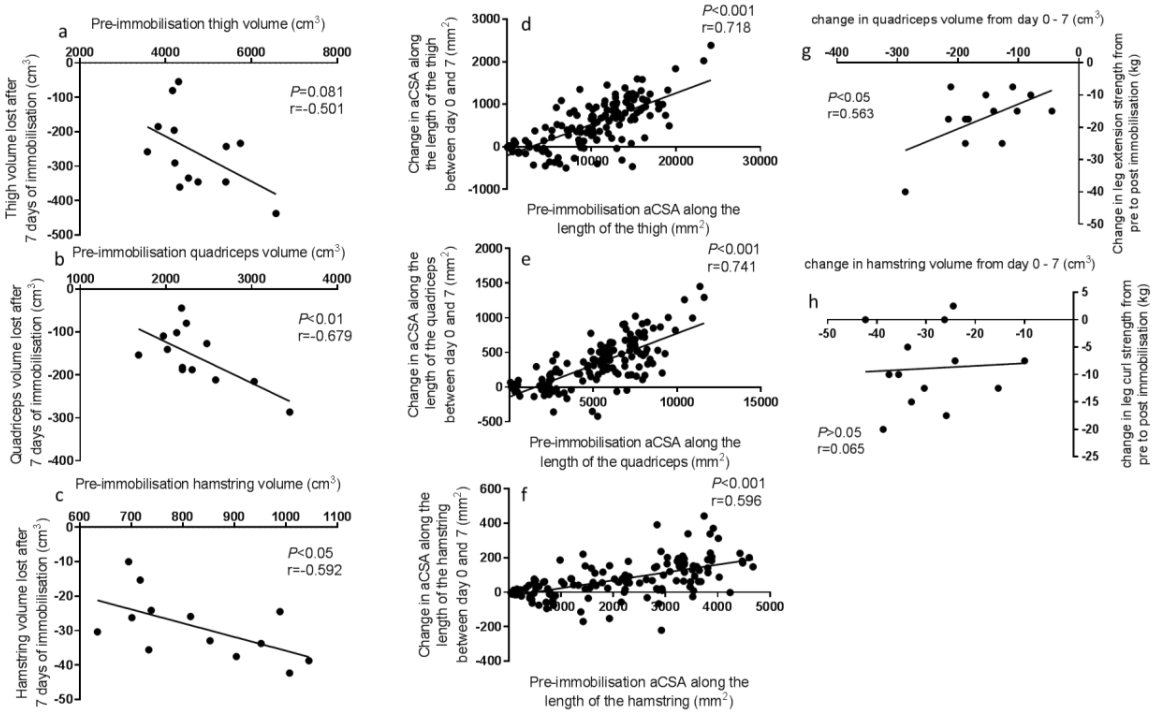


***Figure 2.7*** Correlational analyses. Left column of the panel shows correlations between initial muscle volume and muscle volume lost after 7 days of leg immobilization; for a) thigh, b) *M. quadriceps* and c) *M. hamstrings*, muscle groups, respectively. The middle column shows atrophy (by aCSA) after 7 days of immobilization along the longitude of the following muscle groups; d) thigh, e) *M. quadriceps* and f) *M. hamstrings*, respectively, correlated with pre-immobilization muscle aCSA along the longitude of each of the muscle groups. The column on the right are correlations between; g) *M. quadriceps* volume lost vs leg extension strength lost and h) *M. hamstrings* volume lost vs leg curl strength lost, both after 7 days of immobilization. For each individual graph data were analyzed using a Pearson's correlation coefficient, *P* and r values for each correlation are displayed on each graph respectively. Statistical significance was set at $P<0.05$. Data presented are means±SEM, $n=13$.

## *Discussion*

The present study demonstrates that thigh muscle atrophy occurs rapidly (within 2 days) following the onset of muscle disuse (limb immobilisation via leg brace) and continues at a similar rate for at least one week (by approximately 1% per day). The majority of thigh muscle loss during one week of immobilisation was mainly attributed to the loss of *M. quadriceps* volume rather than loss of *M. hamstrings* volume, and was reflected by a greater decline in leg extension compared with leg flexion strength. Of the 11 individual thigh muscle groups, the *M. vastus lateralis* was the most susceptible to short-term disuse atrophy.

The study of short-term (< one week) muscle disuse atrophy has direct applications to clinical practice and rehabilitation where physical inactivity is mandated (21, 23). Here, we report a detailed characterization of thigh muscle atrophy during one week of leg immobilisation. We report a basic rate of upper leg muscle atrophy over one week in line with previous comparable studies. For example, we observed thigh muscle aCSA at the mid-part of the upper leg (15 cm above the patella) atrophied by ~6%, largely explained by a ~6% loss of *M. quadriceps* aCSA (with only a ~3% loss of *M. hamstrings* aCSA), which is in keeping with previous reports of upper leg muscle atrophy (generally *M. quadriceps* only) determined by single slice CT scan during one week of leg immobilisation (leg cast) (i.e. ~6% atrophy) (24, 25). The use of MRI in the present study also allowed us to extend on these findings by taking multiple images (~45, of which ~15 were manually segmented) across the whole upper leg allowing us to report thigh muscle, *M. quadriceps* and *M. hamstrings* volumes temporally across one week of unilateral leg immobilisation. This approach allowed the striking observation that muscle volume of the thigh, *M. quadriceps* and *M. hamstrings* had already decreased substantially (by 1.7±0.3%, 1.7±0.3% and 1.4±0.2%, respectively) following merely 2 days of immobilisation. The *M. quadriceps* had atrophied to a greater extent (6.7 vs 3.5 %) when compared with the *M. hamstrings* after seven days (see Figure 2.3 b and c). Given that the *M. quadriceps* muscle comprises the largest muscle group of the upper leg, it is clear that *M. quadriceps* atrophy largely explains thigh disuse atrophy during short term immobilisation (see Figure 2.3a). Indeed, the susceptibility of the *M. quadriceps* to atrophy to a greater extent than the *M. hamstrings* could, at least in part, be explained by its larger mass (15, 9). In support, we report both initial *M. quadriceps* and *M. hamstrings* volumes (Figure 2.7 b and c), as well as the larger longitudinal areas of the muscle (Figure 2.7 e and f), correlate with the volume of muscle lost and the aCSA lost along the longitude of each muscle, after 7 days of immobilisation, respectively. However, it is also likely that the degree of atrophy experienced by the two constituent muscle groups of the thigh is also influenced by other factors such as the brace model utilized, where the *M. quadriceps* are immobilised at a longer length (of the muscle fascicles and overall muscle length e.g. distance between origin and insertion) than the *M. hamstrings*. Previous research has shown that the length at which a muscle is immobilised affects its rate of atrophy (26), which also impacts upon its basal tone throughout disuse (27) which may also modulate the rate of muscle atrophy. Furthermore, other factors such as differing muscle fiber composition (28, 29), differing 'training' status (i.e. habitual gravitational loading) (30), or differential expression of regulatory genes/proteins across muscle groups (28, 31), may contribute to the differential atrophy observed across muscle groups. Worthy of note is that this divergent disuse atrophy response of the *M. quadriceps* and *M. hamstrings* may be a transient phenomenon given that longer term (14 days) studies (albeit during bedrest) do not report any differences between the rate of *M. quadriceps* (-6.5%) and *M. hamstrings* volume (-6.1%) decline (29).

We also used our muscle volume measurements to estimate muscle mass. This allowed for the

quantification of actual muscle tissue lost as a result of disuse, which amounted to 83 g from the thigh muscle after 2 days of immobilisation (*M. quadriceps* = 42 g and *M. hamstrings* = 12 g). Over the full week of disuse the thigh muscles atrophied by 269 g, consisting of 162 g loss specifically from the *M. quadriceps* and 30 g from the *M. hamstrings*. Interestingly, these measurements slightly exceed previous estimations extrapolated from computed tomography (CT) approaches (33) which report ~220 g lost from a whole leg, or estimated changes of mass based on alterations in muscle protein turnover from stable isotope experiments which have estimated 150-200 g (9, 21). These data highlight the rapid *and* substantial muscle mass loss consequent with disuse, and underlines the importance of understanding the underlying mechanisms and the development of effective interventions (34, 35, 36), for combatting functional and metabolic decline in multiple populations.

The resolution of MRI allows for the assessment of the aCSA of the individual muscles of the thigh, *M. quadriceps* and *M. hamstrings*. In this respect, we present the first full assessment of how each of the constituent individual muscles of the thigh temporally respond to one week of immobilisation (see Figure 2.5 a and b). The rapid muscle disuse atrophy seen at two days of immobilisation at the level of the whole thigh was detectable by the majority of the individual muscles, with the exceptions of the *M. biceps femoris long head*, *M. biceps femoris short head*, *M. semimembranosus*, *M. gracilis* and *M. sartorius* (see Figure 2.5b). After 7 days of immobilisation, only the *M. gracilis* and *M. sartorius* did not exhibit decreased aCSA. The *M. biceps femoris short head*, *M. adductor* group, *M. sartorius* and *M. gracilis* typically atrophied to a lesser extent than the *M. vastii* muscles (Figure 2.5b). These data are in line with previous (longer term) bed-rest studies (15, 37) which also report that the *M. gracilis* and *M. sartorius* are more resistant to disuse atrophy, though this has not been seen in other studies (38), however due to the complexity of bed rest studies a limited number of participants (*n*=6) were included. Pooling data from multiple bed rest or leg immobilisation studies may lead to better determination of possible atrophy resistant muscle groups (particularly of smaller muscle groups e.g. *M. gracilis*). Overall evidence suggests that the *M. gracilis* and *M. sartorius* are more resistant to atrophy. This is likely to be due to the low gravitational load placed upon these muscles (30) and the low activation of these muscles during common habitual activities e.g. walking (39).

The heterogeneous rates of muscle disuse atrophy that we observed throughout the thigh were reflected by divergent effects on muscle strength when activating those muscle groups. Specifically, leg extension strength, during which the force generation is provided almost exclusively by the activation of the *M. quadriceps*, declined considerably more when compared with the leg curl (where the *M. hamstrings* is the primary agonist) (Table 1). This mirroring supports the contention that the rate of muscle loss is a primary factor determining the loss of function during disuse (17). Indeed, we also observed a correlation between the amount of mass lost from the *M. quadriceps* and the magnitude of functional decline (Figure 2.7g). Though not determined in the present study, it is also clear that such a decline in muscle strength would also impact on muscle power, as has previously been noted (17). This provides an additional challenge specifically for the injured athlete when considering the multifactorial rehabilitation that may be required to return to full functional capacity. Though previous research has repeatedly shown unilateral leg immobilisation results in a reduction in *M. quadriceps* strength (21, 17, 41), fewer studies have assessed the impact on *M. hamstrings* strength. However, two weeks of bed rest has been shown to reduce *M. quadriceps* and *M. hamstrings* strength (isometric MVC) to a similar degree (12-13%) (42) implying that our data showing *M. hamstrings* to be remarkably resistant to disuse induced declines in function may be

attributable to the model of disuse which necessitates that the two muscle groups are immobilised at different positions (e.g. *M. quadriceps* at a longer length than the *M. hamstrings*).

The rapid (i.e. within two days) and muscle (group) specific decline in mass and function that we report during immobilisation has important practical implications. For example, following a sports injury or in a more clinical setting, these data should encourage practitioners to begin appropriate interventions (e.g. neuromuscular electrical stimulation (NMES) (35, 43) or higher protein intakes; to counteract muscle decline as soon as it is safe to do so. If an injury or illness is sustained and a subsequent period of non-weight bearing is mandated, interventions should focus on larger muscle groups (e.g. *M. quadriceps*) as these are more susceptible to atrophy and functional decline (partly due to greater habitual gravitational loading). In immobilisation (not bed rest) the *M. quadriceps* seems uniquely resistant to declines in function with the *M. hamstrings* being resistant after one week. Such interventions may be beneficial since the data (44) indicate that reducing muscle loss and the metabolic decline of muscle tissue during disuse will aid in a faster return to pre-injury strength level and return to play in sports (44). A limitation of the present study is that only males were included. While this allowed for a more homogenous population to allow us to precisely measure and describe temporal, muscle specific disuse atrophy, it prevents the data being generalizable to females. Given that sex-based differences in the rate of muscle disuse atrophy likely exist (18), it is of importance that future work assesses whether similar results are demonstrable in females.

In conclusion, we report in this study that thigh muscle atrophy occurs rapidly (within just 2 days) and at a sustained rate (by approximately 0.8% per day) during one week of immobilisation, and this atrophy is mainly attributed to the loss of *M. quadriceps* tissue mass. Furthermore, the constituent muscles of the thigh atrophy during immobilisation at markedly differing rates (~0.4%-1.0% per day). The preponderance towards *M. quadriceps* rather than *M. hamstrings* atrophy during immobilisation is accompanied by functional declines manifesting distinctly within leg extension movements.

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# Chapter 3

## Short-term muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates

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## *Abstract*

Background Short-term muscle disuse has been reported to lower both post-absorptive and post-prandial myofibrillar protein synthesis rates. This study assessed the impact of disuse on daily myofibrillar protein synthesis rates following short-term (2 and 7 days) muscle disuse under free living conditions.

Methods Thirteen healthy young men (age, 20±1 y; BMI, 23±1 $kg \cdot m^{-2}$) underwent 7 days of unilateral leg immobilisation via a knee brace with the non-immobilised leg acting as a control. Four days prior to immobilisation participants ingested 400 mL 70% deuterated water, with 50 mL doses consumed daily thereafter. Upper leg bilateral MRI scans and muscle biopsies were collected before, and after 2 and 7 days of immobilisation to determine quadriceps volume and daily myofibrillar protein synthesis rates.

Results Immobilisation reduced quadriceps volume in the immobilised leg by 1.7±0.3 and 6.7±0.6 % after 2 and 7 days, respectively, with no changes in the control leg. Over the one week immobilisation period myofibrillar protein synthesis rates were 36±4% lower in the immobilised (0.81±0.04%·$d^{-1}$) compared with the control (1.26±0.04%·$d^{-1}$) leg ($P$<0.001). Myofibrillar protein synthesis rates in the control leg did not change over time ($P$=0.775), but in the immobilised leg were numerically lower during the 0-2 day period (16±6%, 1.11±0.09%·$d^{-1}$, $P$=0.153) and were significantly lower during the 2-7 day period (44±5%, 0.70±0.06%·$d^{-1}$, $P$<0.001) when compared with the control leg.

Conclusions One week of muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates in healthy young men.

## *Introduction*

The recovery from injury or illness requires otherwise healthy individuals to undergo a period of short term (≤ one week) muscle disuse and/or physical inactivity, during which rapid skeletal muscle atrophy and declines in functional and metabolic capacity occur (42,97) . It has also been proposed that the accumulation of such short periods of disuse may contribute to the development of age related sarcopenia (103) . Despite the clinical relevance, the physiological mechanisms responsible for muscle disuse atrophy are yet to be fully elucidated.

A loss of skeletal muscle mass must ultimately be explained by a chronic alteration in muscle protein synthesis and/or breakdown rates. We (11) and others (8,22,104,105) have shown that experimental muscle disuse results in a decline in post-absorptive muscle protein synthesis rates, without any apparent change in muscle protein breakdown rates (8). More recently, we (11) and others (45) have also reported that disuse brings about a resistance to the anabolic properties of protein ingestion. Consequently, it has been suggested that impairments in post-absorptive and post-prandial muscle protein synthesis rates largely explain (uncomplicated) muscle disuse atrophy in humans (43,106,107).

To date, data concerning disuse induced alterations in muscle protein synthesis rates have relied on the continuous infusion of stable isotope labelled amino acids, under laboratory conditions, and their subsequent incorporation into serial muscle biopsies to capture hour-by-hour muscle protein synthesis rates at limited time points before, during or after a disuse intervention. While this allows the controlled assessment of muscle protein synthesis rates, these measurements can only be made over a few hours before potential tracer recycling (i.e. protein bound tracers being released back into the precursor pools) becomes a confounding variable. As a result, these measurements capture only a small snapshot of time (e.g. 1-2%) that a volunteer spends undergoing muscle disuse, and do not account for the combined effects of all lifestyle factors that may contribute to chronically altered muscle protein synthesis rates (e.g. repeated and varied mixed meal ingestion, whole body and altered physical activity patterns, hormonal and diurnal metabolic fluctuations, etc.). Consequently, it is of importance to establish whether measurements of how disuse affects acute muscle protein synthesis rates translate to daily and free living muscle protein synthesis rates and, therefore, can be quantitatively predictive of muscle disuse atrophy. Moreover, the difficulties in obtaining multiple acute measures of muscle protein synthesis rates within the same individual explains the lack of data fully detailing how muscle protein turnover is temporally regulated during short-term disuse. This is of interest since it has been suggested that both the rate of atrophy and the contribution of alterations in muscle protein synthesis rates may differ in the first two/three days of disuse compared with subsequent time points (108).

Recently the regular ingestion of deuterated water as a means to measure muscle protein synthesis rates has regained popularity in the field due to its capacity to assess free living muscle protein synthesis rates during an intervention lasting days (67,109,110) or weeks (24,70). This methodology has recently been applied to assess cumulative (109) and temporal (70) changes in free living muscle protein synthesis rates throughout resistance training programs of varying durations but, to date, has only minimally been directed at muscle disuse atrophy research (24,44).

In the present work we applied oral deuterated water dosing methods to assess the temporal impact of disuse on free living and daily myofibrillar protein synthesis rates during one week of limb immobilisation in healthy young men. We hypothesized that disuse would reduce myofibrillar protein synthesis rates

within 2 days, and to a greater extent than after one week, and this would primarily explain the observed rate of muscle disuse atrophy.

## *Materials and methods*

### *Subjects*

Thirteen healthy young men (age=20±1 y, BMI=23.4±0.9 kg·m-2) were included in the present study. Only young males were included in the present study since both age (20) and sex (21) can influence the rate of muscle disuse atrophy, and our goal was to maintain a homogeneous population for the current study. Participants attended the laboratory for a routine medical screening and completed a general medical questionnaire to assess their eligibility for participation, and to ensure no adverse health conditions were present. Exclusion criteria included; a (family) history of deep vein thrombosis/cardiovascular disease, metabolic disorders (e.g. type 2 diabetes), musculoskeletal/orthopedic disorders, a body mass index of above 28.5 kg·m-2 or below 18.5 kg·m-2, participation in a structured resistance training program within 6 months prior to the study, any musculoskeletal injury of the legs within 12 months before the study, use of anticoagulants, consumption of any nutritional supplement prior to and during the study. During the screening participants height, body mass and blood pressure were measured, body composition was also assessed by air displacement plethysmography (BODPOD; Life Measurement, Inc. CA, USA). All subjects were informed of the nature and possible risks of the experimental procedures before providing written informed consent. The study was part of a wider study assessing muscle disuse atrophy that was approved by The Sport and Health Science Ethics committee of the University of Exeter (151021/B/02), in accordance with the guidelines set out in the Declaration of Helsinki. This study was registered as a clinical trial with clinicaltrials.gov (NCT02984332).

***Table 3.1*** Dietary intake and physical activity levels during a habitual period and during one week of single leg immobilisation.

| | Pre-immobilisation | During immobilisation |
|---|---|---|
| Energy intake (MJ·d$^{-1}$) | 11.1±0.7 | 11.3±1.0 |
| (Kcal·d$^{-1}$) | (2647±167) | (2689±288) |
| Protein intake (g·d$^{-1}$) | 119±7 | 115±12 |
| Protein intake (g·kg$^{-1}$·d$^{-1}$) | 1.6±0.1 | 1.6±0.1 |
| Protein intake (En%) | 18±1 | 17.3±1 |
| Carbohydrate intake (g·d$^{-1}$) | 298±25 | 308±42 |
| Carbohydrate intake (En%) | 45±2 | 46±2 |
| Fat intake (g·d$^{-1}$) | 102±10 | 108±11 |
| Fat intake (En%) | 35±2 | 36±2 |
| Light physical activity (h·d$^{-1}$) | 1.4±0.2 | 1.0±0.1 |
| Moderate physical activity (h·d$^{-1}$) | 2.6±0.4 | 1.7±0.2* |
| Vigorous physical activity (h·d$^{-1}$) | 0.3±0.1 | 0.1±0.1 |
| Total physical activity (h·d$^{-1}$) | 4.3±0.5 | 2.8±0.2* |

*Values represent means±SEM, n=13. *=significant difference from pre-immobilisation value, P<0.05.*

### *Experimental design*

A graphical representation of the experimental study design can be seen in **Figure 1**. Following successful completion of the screening visit eligible participants attended the laboratory for 5 experimental visits across 11 days and this included a 7 day period of unilateral leg immobilisation using a leg brace and ambulation via the use of crutches. To measure daily myofibrillar protein synthesis rates throughout the immobilisation period participants underwent a deuterium oxide dosing protocol (described below). This

protocol was designed to achieve and maintain 0.8-1.0% body water deuterium enrichment during the measurement periods in line with our previous work (109). Participants arrived at the laboratory on day 5 of the experimental protocol at ~0800 h for the first of three experimental test days. During this visit, bilateral muscle biopsies were collected from the *m. vastus lateralis* and an MRI scan was conducted of both thigh muscles (at ~0830 h; participants were transported to and from the MRI scanner via a wheelchair to ensure no contraction or weight bearing occurred before/after the MRI scans and biopsies). Thereafter, a 7 day immobilisation period was started at ~0900 h. After 2 and 7 days of immobilisation subjects returned to the laboratory at ~0800 h for an identical test day. Muscle biopsies were obtained under local anesthesia, using the percutaneous Bergstrom needle biopsy technique (111), from the *m. vastus lateralis* of both legs ~15 cm above the patella and ~3 cm below the fascia. Immediately following a muscle biopsy the muscle tissue was quickly assessed and any blood or non-muscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

### *Immobilisation protocol*

The model of immobilisation used was a unilateral leg brace (X-ACT Donjoy brace, DJO global, Vista, CA, USA) with the participant ambulating on crutches (after receiving instruction) throughout the immobilisation period. The immobilised leg was randomized and was counterbalanced for leg dominance with the non-immobilised leg acting as a within-subject control. Using the hinge of the brace the knee was fixed at an angle of 40° flexion (full knee extension = 0°) to ensure no weight bearing occurred. Subjects were instructed that all ground contact, and muscle contraction (except for ankle rotation exercises twice per day to activate the venous muscle pump), in the immobilised leg were forbidden. Adhesive tape with the experimenter's signature inscribed was placed around the straps of the brace. Breaking of the tape would indicate that the brace had been altered and resulted in exclusion from the study (23), no participants were excluded. A plastic shower cover was provided to the participants to wear over the brace when showering. Daily contact was maintained with the subject throughout the study to ensure proper compliance.

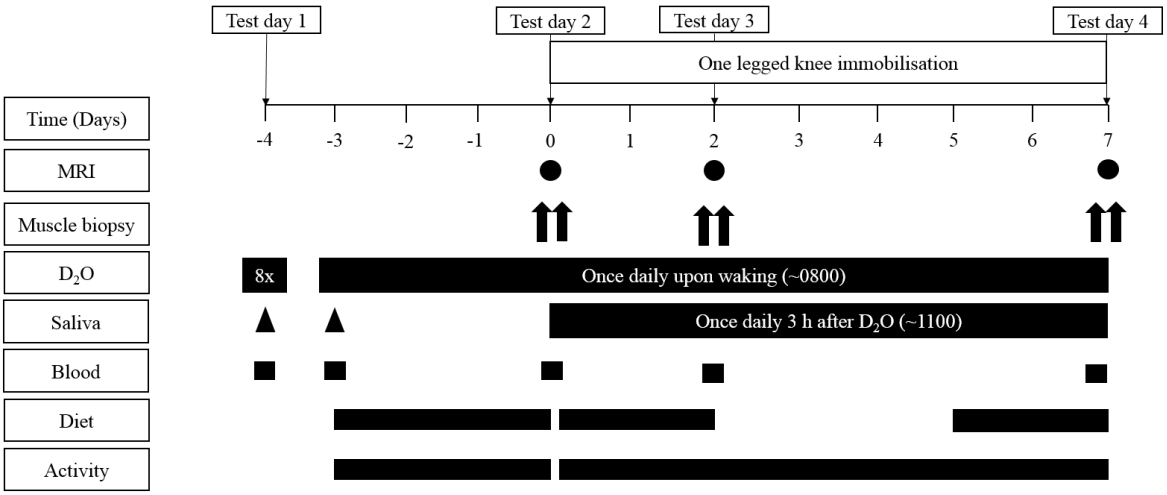


***Figure 3.1*** Study Schematic. Thirteen healthy young males underwent 7 days of unilateral leg immobilization via knee brace. MRI, Magnetic resonance imaging. $D_2O$, deuterated water ingestion. Activity, physical activity measured continuously by GENEactiv wrist watch accelerometry. Diet, habitual dietary intake recorded by self-reported written diet diary. Blood, venous blood sample. Arrows represent bilateral *m. vastus lateralis* muscle biopsies, (i.e. taken from the control and immobilized legs).

### *Deuterated water dosing protocol*

The deuterated water dosing protocol was based on our previous work (15). Day 1 of the experimental protocol acted as a D2O loading day where participants consumed 400 mL 70% D2O separated over the day as 8 x 50 mL boluses (CK Isotopes Ltd, Leicestershire, UK). Upon arrival at the laboratory (0730 h) background blood and saliva samples were collected before the first bolus of D2O was ingested. The first dose of D2O was consumed at ~0800 h with the remaining doses being consumed every 1.5 h. Participants stayed at the university until 4 out of the 8 loading day D2O doses had been consumed, with the remaining D2O doses being consumed at home under instruction of timings (i.e. leaving 1.5 h between each). Every day following the loading day participants consumed a maintenance dose of D2O (50 mL) upon waking (~0800 h). One participant reported some mild feelings of vertigo and dizziness during the loading day which passed after approx. 2 h. Three hours (~1100 h) after the daily D2O maintenance dose a daily saliva sample was collected using a cotton mouth swab (Celluron, Hartmann, Germany) which the participant lightly chewed for ~1 min until saturated with saliva. The saturated sponge was placed into an empty syringe where the swab was squeezed to release the saliva into a collection tube. The saliva samples were used to assess the body water 2H enrichment and were stored in the participant's freezer until they were returned at the next study visit. Additional blood samples were collected during the test days (i.e. day 5 [pre], 7 [after 2 days of immobilisation] and 12 [post]). Venous blood samples were collected from the antecubital vein via venipuncture technique and collected into EDTA-containing vacutainers which were centrifuged at 4,000 rpm for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C until further analysis took place. To ensure uniformity and compliance with the D2O protocol participants were provided with a log to record the times they consumed the D2O and were provided with enough doses to last until their next study visit, at which point containers were returned, counted and subsequent doses were provided.

*Diet and physical activity*

For 3 days prior to immobilisation subjects' physical activity was measured using an accelerometer (GENEactiv, Activinsights, Cambridgeshire, UK) that was attached to the non-dominant wrist. Subjects were instructed to wear the accelerometer continuously with data being collected at a 60 Hz sampling frequency. Subjects' physical activity was measured again throughout the 7 day immobilisation period. Subjects' were instructed to refrain from vigorous physical activity during immobilisation but to attempt maintain their habitual activity levels despite using crutches for ambulation (to avoid whole body sedentariness during immobilisation). Physical activity data from the GENEActiv accelerometers were converted into 60 s epochs and used to estimate time spent performing total physical activity (all intensities) using standard cut-off points (24). Subjects' diets were recorded for 3 days (two week days and one weekend day) prior to immobilisation by a self-reported written diet diary following detailed instructions and advice from a member of the research team. Subjects were asked to refrain from alcohol intake and maintain a similar diet during and throughout the immobilisation period and this was assessed by two further 2 day diet diaries in the first and last two days of immobilisation, these were averaged to create an 'immobilisation diet'. Dietary analyses for the calculation of energy and macronutrient intakes were completed using specialized nutrition software (Nutritics Professional Nutritional Analysis Software; Swords, Co. Dublin).

*Magnetic resonance imaging and quadriceps volume calculation*

MRI scan methodology of the upper legs for the determination of quadriceps volume has been described in detail previously (25). In brief, a 1.5 tesla (T) MRI scanner was used to make axial plane images over the full length of the femur. A T1-weighted 3D turbo spin echo sequence was used (field of view 500 x 500 mm, reconstructed matrix 512 x 512 mm, echo time 15 ms, repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the supine position, a 4-element sense body radiofrequency coil was wrapped around both thighs. Philips on-line MRI software was used to analyze the images obtained in the axial plane (the same experimenter performed all manual segmentation of the images). Starting at the most distal image (approximately mid patella) where each muscle group could be fully delineated, the muscle of interest was manually segmented to calculate slice cross sectional area. Moving proximally every third image was analyzed until complete delineation of the muscle groups of interest could no longer be identified (the greater trochanter). Muscle volume was calculated using a previously published method (26) where the total CSA for all images was calculated and multiplied by the slice gap plus the distance between slices (linear interpolation) (in this case a total 3 cm, 5 mm slice thickness, 25 mm slice gap), summarized by the following equation:

$$muscle\ volume = \sum_{aCSA} \cdot\ (slice\ thickness + slice\ gap)$$

*Body water deuterium enrichment*

Body water deuterium enrichment was measured using the saliva samples collected daily throughout the course of the study. All saliva samples were centrifuged at 10,000 g and were subsequently diluted 70-fold with ddH2O to lower deuterium enrichments so that they were in the detection range of the isotope ratio mass spectrometer (IRMS). Following the dilution step, samples were prepared for analysis on the IRMS using the protocol described by Scrimgeour et al., (27). In summary, small plastic cups holding 4 mg of 5% platinum on alumina, 325 mesh (Sigma-Aldrich, St. Louis, MO) were placed inside 3 ml glass vials (Labco Exetainer; Labco, Lampeter, UK). Subsequently 300 µL of the diluted saliva samples was placed into the vials, vials were then sealed using rubber septums and a screw cap. The air within each vial was simultaneously evacuated and replaced by hydrogen gas. Vials were then left for 24 h at 21°C for deuterium equilibration between the hydrogen gas and saliva samples to occur. The deuterium enrichment of the hydrogen gas was then measured in duplicate on a IRMS (Micromass Optima IRMS fitted with a multiprep and Gilson auto-injector; Micromass, Manchester, UK). Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for deuterium loss during equilibration.

*Plasma free [$^2$H]alanine enrichments*

Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 5975C MSD & 7890A GC, Wilmington, USA). First the plasma samples were deproteinized using dry 5-sulfosalicylic acid. Subsequently free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, mesh size 100-200 µm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA). The purified amino acids were converted to their *tert*-butyldimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA before analysis via GC-MS. The plasma free alanine mass isotopomers (M and M+1) were measured using selective ion monitoring at *m/z* 232 and 233. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation.

*Myofibrillar bound $^2$H alanine enrichments*

Myofibrillar protein-enriched fraction was extracted from ~50 mg of wet weight muscle tissue by hand-homogenization on ice using a pestle in a standard extraction buffer (10 µl/mg). The samples were centrifuged at 2,500 g for 5 min at 4°C and the pellet was then washed with 500 µl of ddH2O and again centrifuged at 2,500 g for 10 min at 4°C. The myofibrillar protein was solubilized by adding 1 ml of 0.3 M NaOH and heating for 30 min at 50°C with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 9,500 g and 4°C, the supernatant containing the myofibrillar protein was kept and the collagen protein pellet was discarded. The myofibrillar proteins were precipitated by the addition of 1 ml of 1 M PCA and spun at 700 g and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolyzed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated at 120°C.

The free amino acids were subsequently dissolved in 25% acetic acid solution and passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA) and eluted with 2 M NH4OH. Following this the eluted amino acids were dried and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (28). The derivatized were measured using a gas-chromatograph-isotope ratio mass spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE) and a 5 m precolumn. Ion masses 2 and 3 were analyzed to determine the 2H/1H ratios of muscle protein-bound alanine. A series of known standards was used to assess the linearity of the mass spectrometer and to control for the loss of tracer.

*Skeletal muscle mRNA analyses*

Skeletal muscle mRNA expression of 48 genes involved in the regulation of skeletal muscle mass and metabolism were analyzed (gene names and symbols are listed in Table 2) as described previously (24). In brief, total RNA was isolated from ~20 mg of frozen muscle tissue using TRIzol Reagent (Life Technologies, Invitrogen) according to the supplier's instructions. Total RNA was quantified spectrophotometrically at 260 nm and RNA purity was assessed as the ratio of readings at 260/280 nm (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific). Subsequently first strand cDNA was synthesized from 150 ng of RNA using a SuperScript III cDNA synthesis kit (cat. no. 11752-050; Invitrogen, Life Technologies, CA, USA). Taqman low-density custom designed array cards (ABI Applied Biosystems, Foster City, CA, USA) were used for the relative quantification of expression of the 48 selected genes. Each card ran eight samples simultaneously against 48 Taqman gene expression assay probes that were pre-fixed into each well on the card. In summary, 50 µL of Taqman universal master mix (ABI, Applied Biosystems) was added to 150 ng of RNA equivalent cDNA in an RNase free Eppendorf with RNase free water being added to a make a final reaction volume of 100 µL. Samples were vortexed and centrifuged (briefly) then pipetted into sample reservoirs on the micro fluidic array cards, following this the array cards were centrifuged (1000 rpm for 1 min) (Hereaus 3 S-R Microfuge, Thermo Fisher Scientific, Waltham, MA, USA) and then run on a QuantStudio 12K Flex Real-Time PCR system (ABI, Applied Biosystems). Relative quantification of the genes was performed using the delta delta Ct method (2-ΔΔCt), data were normalized to the geometric mean of GAPDH and 18s genes (4).

***Table 3.2*** Names and symbols of the gene expression assay targets preloaded upon PCR microfluidic cards.

| Gene Name (human skeletal muscle) | Symbol |
|---|---|
| Insulin receptor substrate 1 | *IRS1* |
| Phosphoinositide-3-kinase regulatory subunit 1 | *PI3K* |
| AKT serine/threonine kinase 2 | *AKT2* |
| Mechanistic target of rapamycin | *mTOR* |
| Ribosomal protein S6 kinase B2 | *RPS6KB1 (P70s6k)* |
| Eukaryotic translation initiation factor 4E binding protein 1 | *EIF4EBP1 (4E-BP1)* |
| Solute carrier family 7 member 5 | *SLC7A5 (LAT1)* |
| Solute carrier family 38 member 2 | *SLC38A2 (SNAT2)* |
| Solute carrier family 36 member 1 | *SLC36A1 (PAT1)* |
| DNA damage inducible transcript 4 | *DDIT4 (REDD1)* |
| Eukaryotic 18S rRNA | *18s rRNA* |
| Forkhead box O1 | *FOXO1* |
| Forkhead box O3 | *FOXO3* |
| Calpain 1 | *CLPN1* |
| Calpain 3 | *CLPN3* |
| Caspase 3 | *CASP3* |
| Proteasome subunit beta 1 | *PSMB1* |
| Nuclear factor kappa B subunit 1 | *NFKB* |
| F-box protein 32 | *MAFbx* |
| Tripartite motif containing 63 | *TRIM63 (MuRF1)* |
| Beclin 1 | *BECN1* |
| Myostatin | *MTSN* |
| Growth differentiation factor 11 | *GDF11* |
| Solute carrier family 2 member 4 | *GLUT4* |
| Glycogen Phosphorylase (muscle) | *PYGM* |
| Glycogen synthase 1 | *GS1* |
| Hexokinase 2 | *HK2* |
| Pyruvate dehydrogenase kinase 4 | *PDK4* |
| CD36 molecule | *CD36* |
| Fatty acid synthase | *FASN* |
| Sterol regulatory element binding transcription factor 1 | *SHREBP1* |
| Carnitine palmitoyltransferase 1B, | *CPT1B* |
| Acetyl-CoA carboxylase beta | *ACACA* |
| Peroxisome proliferator activated receptor alpha | *PPARα* |
| PPARG coactivator 1 alpha | *PGC1α* |
| Mitofusin 1 | *MFN1* |
| Dynamin 1 like protein | *DNM1L* |
| Transcription factor A, mitochondrial | *TFAM* |
| Dystrophin | *DMD* |
| Sarcoglycan alpha | *SGCA* |
| Laminin subunit alpha 2 | *LAMA2* |
| Integrin subunit beta 1 | *ITGB1* |
| Actinin alpha 3 | *ACTN1* |
| Desmin | *DES* |
| Vascular endothelial growth factor A | *VEGF* |
| Hypoxia inducible factor 1 alpha subunit | *HIF-1* |
| Angiopoietin 1 | *ANGPT1* |
| Glyceraldehyde-3-phosphate dehydrogenase | *GAPDH* |

*Symbols in brackets represent gene synonyms.*

*Calculations*

Myofibrillar protein fractional synthesis rates (FSR) were calculated based on the incorporation of [$^2$H] alanine into myofibrillar protein and either the mean free plasma [$^2$H] alanine or the mean body water deuterium enrichment throughout the time period as precursors. FSR was calculated using the standard precursor-product method expressed as daily rates as follows:

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

where $E_{m1}$ and $E_{m2}$ are the myofibrillar muscle protein-bound enrichments on either day 0 and 2, 0 and 7, and 2 and 7 respectively. $E_{precursor}$ represents either mean plasma free [$^2$H] alanine or mean body water deuterium enrichment corrected by a factor of 3.7 based on deuterium labelling of alanine during *de novo* synthesis (15, 24) (mean enrichment between day 0-2, 0-7 or 2-7). *t* represents the time between biopsies (day 0-2, 0-7 or 2-7).

*Statistics*

All data are presented as means±SEM and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). A paired samples *t*-test was used to compare myofibrillar protein synthesis rates in the control vs immobilised legs between days 0 and 7, and physical activity and dietary intake between pre and during immobilisation. Two-way repeated measures ANOVAs with leg (immobilised and control) and time (day 0-2 and day 2-7 [for myofibrillar protein synthesis rates] and days 0, 2 and 7 [for MRI]) as within subjects factors were used to compare differences in myofibrillar protein synthesis rates over time, gene expression and quadriceps volume. Pearson's correlation coefficient was used to assess the relationship between tracer precursor pools. For all ANOVAs, when a significant interaction was found Bonferroni post-hoc tests were applied to locate individual differences. Statistical significance was set at $P<0.05$.

## *Results*

### *Diet and physical activity analyses*

Table 1 shows participants' habitual dietary intake and physical activity data averaged for 3 days preceding, and for 4 (for dietary intake) and 7 days (for physical activity) during the immobilisation period. No differences in energy or macronutrient intake (fat, carbohydrate and protein) were observed between pre and during immobilisation (all $P>0.05$). Light and vigorous physical activity were not different between pre and during immobilisation ($P>0.05$). Moderate physical activity reduced from pre to during immobilisation ($P=0.032$).

### *Quadriceps muscle volume*

Quadriceps muscle volumes of the control and immobilised legs were determined by MRI (previously reported in detail in Kilroe et al, 2019). There were no difference in quadriceps volume between legs pre-immobilisation (control leg = 2315±120 $cm^3$, immobilised leg = 2342±129 $cm^3$ [$P=0.993$]) and the control leg remained unchanged throughout the study ($P=0.981$). However, a significant time *x* leg interaction was detected ($P<0.001$) such that quadriceps volume of the immobilised leg had decreased by 1.7±0.3% after 2 days (to 2301±127 $cm^3$; $P=0.007$) and by a further 5.0±0.6% between days 2 and 7 days (to 2186±117 $cm^3$; $P<0.001$), resulting in a total decrease in quadriceps muscle volume of 6.7±0.6% ($P<0.001$) over the full week of immobilisation.

### *Precursor pool enrichments*

Saliva deuterium enrichments (**Figure 3.2A**) reached 0.69±0.02% on day 0 (start of immobilisation), and averaged 0.71±0.02%, 0.78±0.03% and 0.76±0.02% over 0-2, 2-7 and 0-7 time points, respectively. Saliva deuterium enrichments showed a modest increase over the immobilisation period (main effect of time, $P<0.001$) such that enrichments at day 6 (0.80±0.02%, $P=0.024$) and 7 (0.83±0.03%, $P<0.001$) were elevated above day 0, and day 7 enrichments were higher than day 1 (0.70±0.02%, $P=0.003$). Plasma free [$^2$H] alanine enrichments (Figure 2A) reached 2.8±0.1 mole percent excess (MPE) on day 0 and averaged 3.1±0.2 MPE over the week of immobilisation. Plasma free [$^2$H] alanine enrichments also showed a slight increase over the immobilisation period (main effect of time, $P<0.001$) such that day 2 (3.1±0.1 MPE, $P=0.017$) and day 7 (3.5±0.1 MPE, $P<0.001$) enrichments were higher than day 0 (2.8±0.1 MPE). A Pearson's correlation coefficient showed that the saliva deuterium and the plasma [$^2$H] alanine precursor pools were strongly correlated (data from all time points, 0-2, 2-7 and 0-7 collapsed into one test) ($r=0.959$, $P<0.001$) (**Figure 3.2B**). Plasma free [$^2$H] alanine enrichments were, on average, 3.93-fold greater than saliva deuterium enrichments (**Figure 3.2C**). It should be acknowledged that we have not determined muscle intracellular free [$^2$H] alanine enrichments in the present work. It is conceivable that our intervention may have influenced global muscle alanine metabolism divergently across legs which could affect local precursor enrichments. However, our (15) and others' (31) previous observations that muscle intracellular free [$^2$H] alanine does not differ substantially across legs even when unilateral leg

interventions are applied (e.g. daily resistance-type exercise) and correlate tightly with plasma free [$^2$H] alanine enrichments, suggest the latter to be a valid precursor pool.

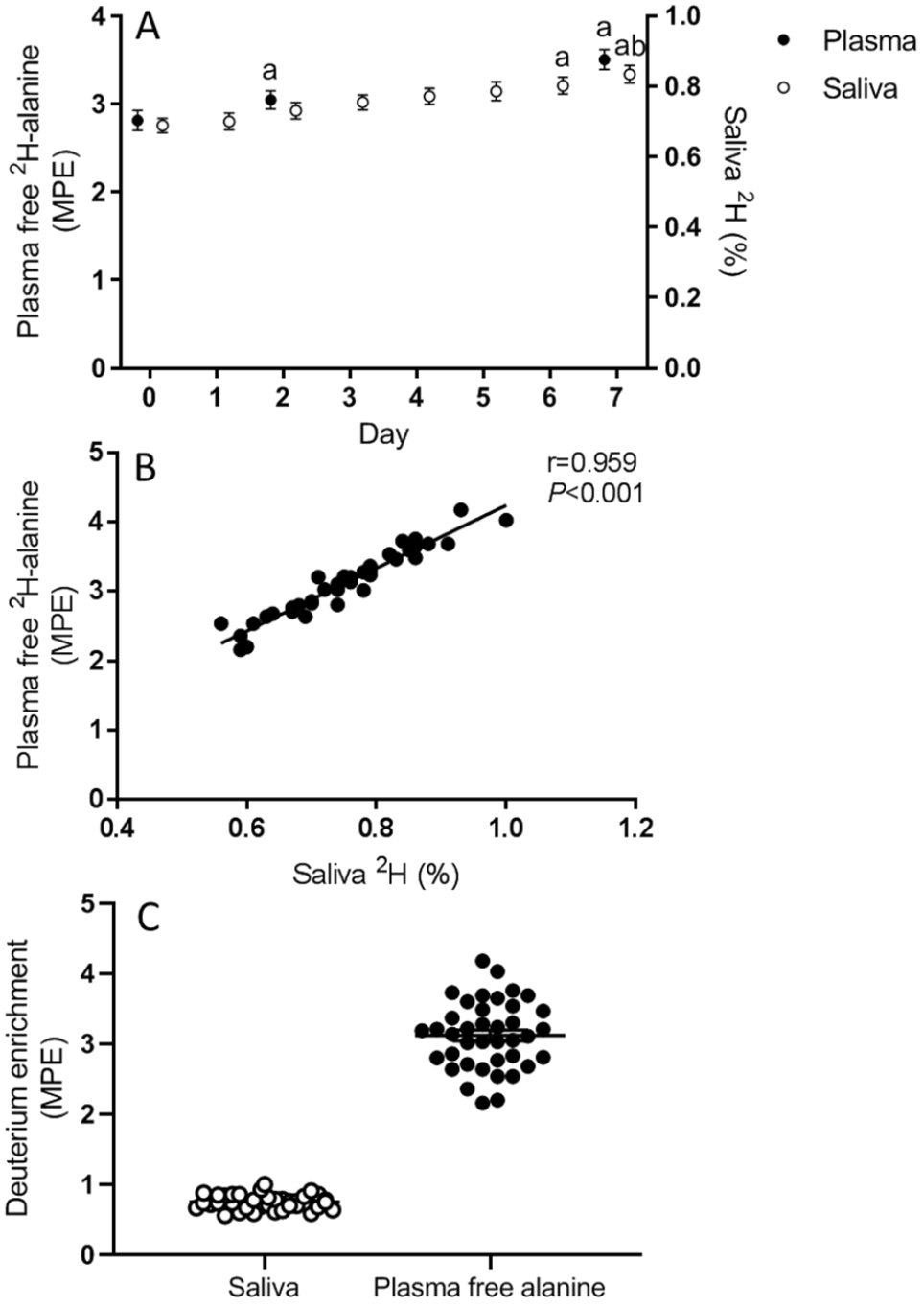


***Figure 3.2*** Stable isotope precursor pool data. Plasma free $^2$H-alanine (MPE) (left y-axis) and saliva $^2$H (%) (right y-axis) enrichments displayed over the week of immobilization (A), with data analyzed using one-way repeated measures ANOVAs, and the correlation (B) between saliva $^2$H (%) and plasma free $^2$H-alanine (MPE) analyzed by a Pearsons' correlation analysis. C, displays comparison of individual participant saliva enrichment (%) and plasma free $^2$H-alanine (MPE) enrichments at day 0, 2 and 7 timepoints. a = significant difference from day 0 in corresponding precursor pool $P$<0.05, b = significant difference from day 1 in saliva precursor pool $P$<0.05. Data are means±SEM, $n$=13.

*Daily myofibrillar protein synthesis rates*

Myofibrillar protein bound [$^2$H] alanine enrichments showed significant time (*P*<0.001), leg (*P*<0.001) and time *x* leg interaction (*P*<0.001) effects. After 2 days of immobilisation, myofibrillar protein bound [$^2$H] alanine enrichments increased by 20±10 % more in the control (0.0695±0.0045 MPE) compared with the immobilised (0.0579±0.0042 MPE) leg (*P*=0.511). After 7 days, myofibrillar bound protein [$^2$H] alanine enrichments increased over time in both legs but by 53±11 % more in the control (0.2448±0.0096 MPE) compared with the immobilised (0.1596±0.0079 MPE) leg (*P*<0.001). Daily myofibrillar FSRs (%·d$^{-1}$) were calculated using mean (of each individual's data during that time period) saliva deuterium enrichments ((corrected by a factor of 3.7 to account for the labeling coefficient between saliva and plasma; (15, 25)) and then separately using mean plasma free [$^2$H] alanine enrichments (again, an individual's own data averaged over the period of interest) as precursor pools over the 0-2, 2-7 and 0-7 time-points of immobilisation (**Figure 3.3**). Over the entire week of immobilisation daily myofibrillar FSR, calculated using either the saliva deuterium enrichment (control leg = 1.26±0.05%·d$^{-1}$ and immobilised leg = 0.81±0.04%·d$^{-1}$) or plasma free [$^2$H] alanine enrichment (control leg = 1.14±0.05%·d$^{-1}$; immobilised leg = 0.71±0.04%·d$^{-1}$) as the precursor pools were 36±4% (*P*<0.001) and 37±4% (*P*<0.001) lower, respectively, in the immobilised compared with the control leg. A Pearson's r product moment correlation showed a strong correlation between FSR calculated from the two precursor pools with data collapsed for both legs over the full week of immobilisation (r = 0.982, *P*<0.001 [Figure 3C]).

When assessing the temporal changes in myofibrillar FSR over the early (i.e. 0-2 days) and late (i.e. 2-7 days) immobilisation periods and using the saliva deuterium enrichment precursor, significant main effects of time (*P*=0.020), condition (*P*<0.001) and a time x condition interaction *(P*=0.046) were detected. Specifically, myofibrillar FSR was 16±6% (*P*=0.153) and 44±5% (*P*<0.001) lower at 0-2 (control leg = 1.32±0.08%·d$^{-1}$; immobilised leg = 1.11±0.09%·d$^{-1}$) and 2-7 days (control leg = 1.24±0.07%·d$^{-1}$; immobilised leg = 0.70±0.06%·d$^{-1}$) in the immobilised compared with the control leg. Myofibrillar FSR remained unchanged over time in the control leg but declined by 37±11% from 0-2 to 2-7 days (*P*=0.005) in the immobilised leg. When using the plasma free [$^2$H] alanine enrichment to assess temporal changes in myofibrillar FSR over the early (i.e. 0-2 days) and late (i.e. 2-7 days) immobilisation period, significant main effects of time (*P*=0.013), condition (*P*<0.001) and a time x condition interaction *(P*=0.048) were detected. Specifically, myofibrillar FSR was 16±6% (*P*=0.159) and 44±5% (*P*<0.001) lower at 0-2 (control leg = 1.19±0.07%·d$^{-1}$; immobilised leg = 1.01±0.08%·d$^{-1}$) and 2-7 days (control leg = 1.11±0.07%·d$^{-1}$; immobilised leg = 0.62±0.05%·d$^{-1}$) in the immobilised compared with the control leg. Myofibrillar FSR remained unchanged over time in the control leg but declined by 38±11 % from 0-2 to 2-7 days (*P*=0.003) in the immobilised leg. A Pearson's r product moment correlation showed a strong correlation between the two precursor pools for both legs over both the 0-2 and 2-7 day time points (both legs and time periods collapsed into one test) (r=0.995, *P*<0.001 [Figure 3F]).

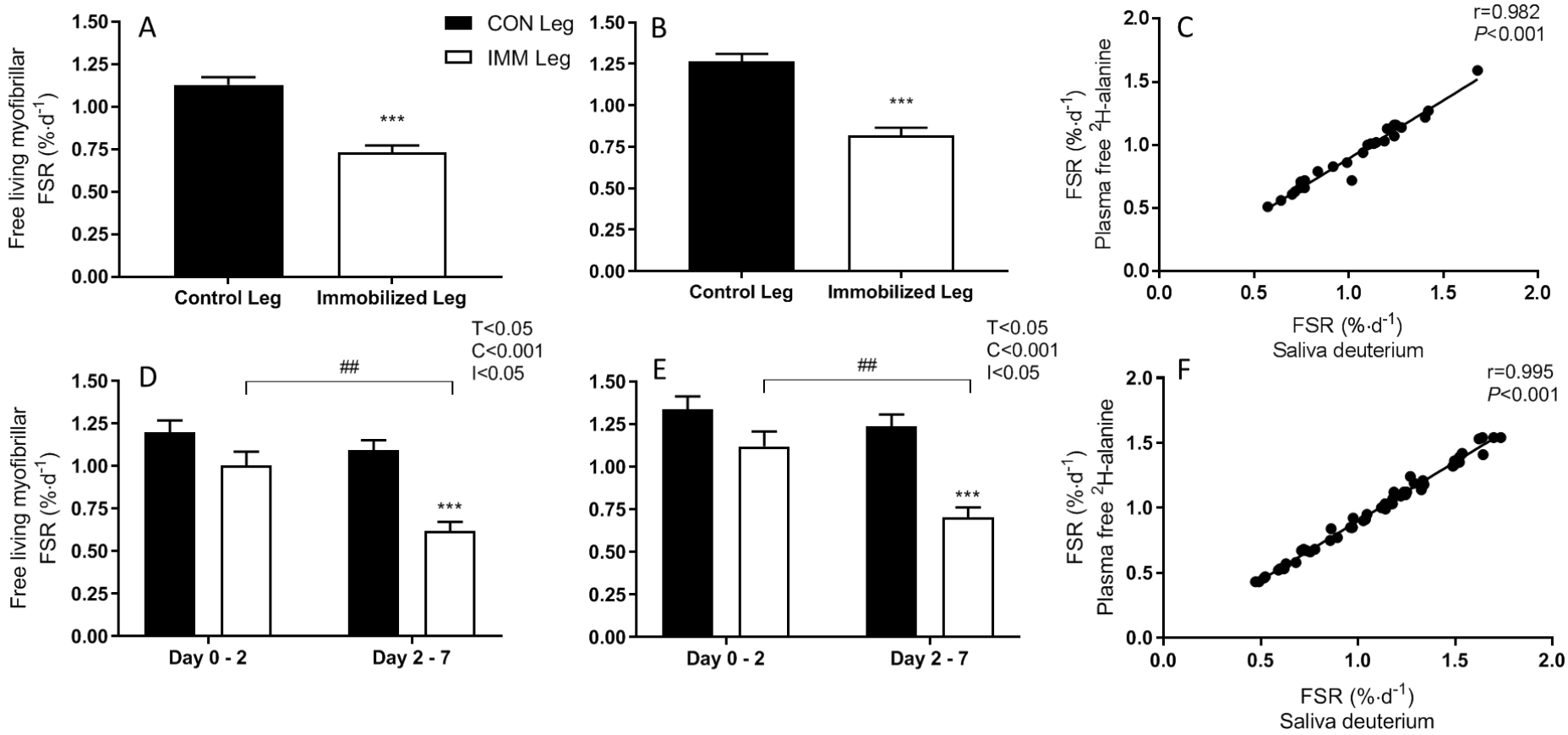


***Figure 3.3*** Free living myofibrillar fractional synthesis rates (%·d$^{-1}$). Graphs A and B show free living myofibrillar FSR (%·d$^{-1}$) over the full week calculated from plasma and saliva precursor pools, respectively, as individually assessed by paired samples t-test. Graphs D and E show free living myofibrillar FSR between days 0 – 2 and 2 - 7 of immobilization calculated from plasma and saliva precursor pools, respectively, as assessed by two-way repeated measures ANOVA (leg x time), with time (T), condition (C) and interaction (I) effects displayed above each graph. Where a significant time x treatment interaction was detected Bonferroni post hoc tests were conducted to locate individual differences; *** denotes $P$<0.001 significant difference between legs at corresponding timepoint, ## denotes $P$<0.01 significant difference within leg between 0-2 and 2-7 timepoints. Graphs C and F show correlations between myofibrillar FSRs calculated from the two different precursors between day 0-7 (C) and 0-2 and 2-7 days combined (F), analyzed by Pearson's correlation analyses. Data are means±SEM for the non-correlation graphs, $n$=13.

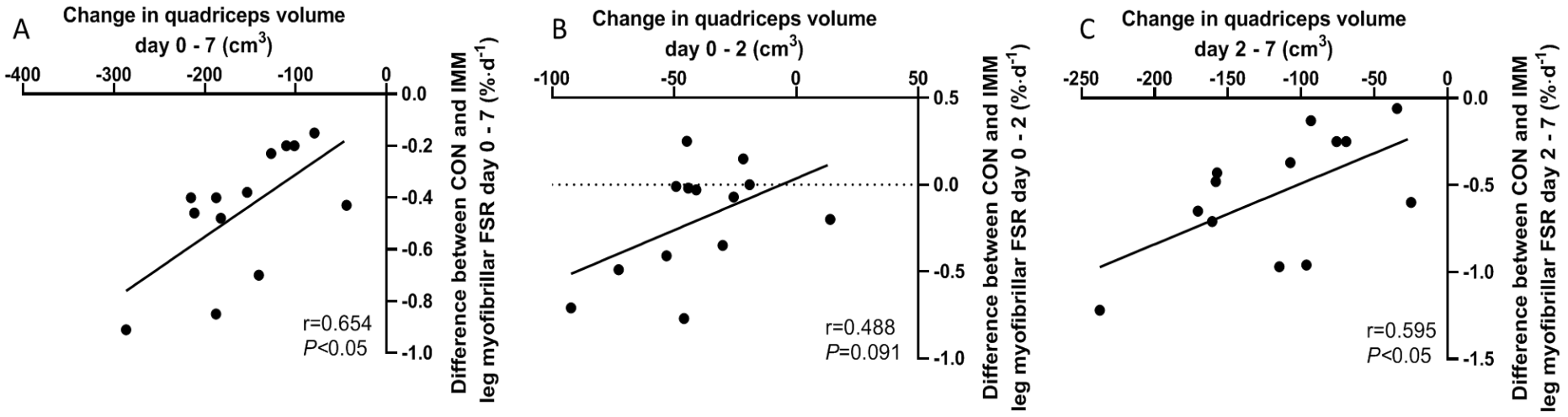


***Figure 3.4*** Correlations between the change in quadriceps volume in the immobilized leg and the difference between control and immobilized leg myofibrillar FSR across the following timepoints; A) days 0 – 7, B) days 0 – 2 and C) days 2 – 7. Data analyzed by Pearson's correlation analyses, r and *P* values displayed on each graph, *n*=13.

*Skeletal muscle gene expression*

The skeletal muscle expression of genes involved in insulin signaling and muscle protein synthesis/growth factors, muscle protein breakdown, carbohydrate and lipid metabolism, and mitochondrial metabolism/angiogenesis/force transduction are shown in **Figures 3.5, 3.6, 3.7 and 3.8,** respectively. The remaining genes of the 46 analyzed are shown in **Figure 3.9**. Thirteen of the analyzed genes (*P70S6K*, *EIF-4EBP1*, *REDD1*, *MTSN*, *GS1*, *HK2*, *FAT*, *CPT1B*, *PPARα*, *DNML1*, *TFAM*, *SCGA*, *ANGPT1*) showed no changes throughout the experiment (time, condition and interaction effects all $P>0.05$). Twenty genes exhibited a time effect (all $P<0.05$) only, such that they changed to an equivalent extent (either an increase [*AKT2*, *LAT1*, *PAT1*, *CLPN1*, *GDF11*, *FASN*, *MFN1*, *ITGB1*, *HIF1α*, *DES*, *mTOR*, *SHREBP1*, *BECN1*, *PDK4*, *FOXO1* and *SNAT2*] or a decrease [*PYGM*]) in both the control and immobilised legs. Some of these genes (*ACABα*, *DMD* and *VEGFα*) also exhibited a treatment effect (all $P<0.05$) without any interaction (all $P>0.05$). Thirteen genes (*MAFbx*, *PGC1α*, *PSMB1*, *NFKb*, *IRS1*, *ACTN3*, *PI3K*, *MuRF1*, *FOXO3*, *CASP3*, *CD36*, *CLPN3*, *GLUT4*) displayed an interaction effect such that divergent responses occurred between legs over time (all $P<0.05$). With one exception (*PI3K*) these responses were restricted to 7, but not 2, days of immobilisation. For example, after 7 days of immobilisation the relative expression of *PSMB1* (Figure 3.6F) and *MAFBx* (Figure 3.6I) increased by 83±15% and 123±26 % (both $P<0.001$), respectively, in the immobilised leg only. *NFKb* (Figure 3.6G) increased in both legs at 7 days but to a greater extent in the immobilised compared with the control leg (115±24 vs 59±23 %; $P=0.027$). Muscle mRNA expression of *IRS1* (Figure 3.5A), *ACNT3* (Figure 3.8I) and *PGC1α* (Figure 3.8B) remained unchanged in the control leg ($P>0.05$), but reduced by 54±9, 77±7 and 52±6 %, respectively, in the immobilised leg after 7 days (all, $P<0.001$). Only *PI3K* (Figure 3.5B) expression increased after both 2 (87±24%, $P<0.001$) and 7 (181±17%, $P<0.001$) days in the immobilised leg with no change in the control leg ($P=0.774$). *MuRF1* (Figure 3.6J), *FOXO3* (Figure 6B), *CASP3* (Figure 3.6D), *CD36* (Figure 3.7E) and *CLPN3* (Figure 3.6E) all displayed trends for a divergent response in relative expression between legs over the immobilisation period (interaction effects all $P<0.10$). The relative expression of *MuRF1* and *FOXO3* did not change after 2 days but displayed a trend to increase by 175±51 and 112±22 %, respectively, and *CLPN3* a trend to decrease by 37±5%, after 7 days in the immobilised leg (all, $P<0.05$]), with no changes in the control leg (all, $P>0.05$). *CASP3* increased in both legs at 7 days but to a greater extent in the immobilised compared with control leg (179±34 vs 92±40 %; $P=0.313$). Muscle *CD36* expression displayed a trend to increase in the control leg after 2 (73±51%) and 7 (65±49%) days, with no change in the immobilised leg ($P=0.994$).

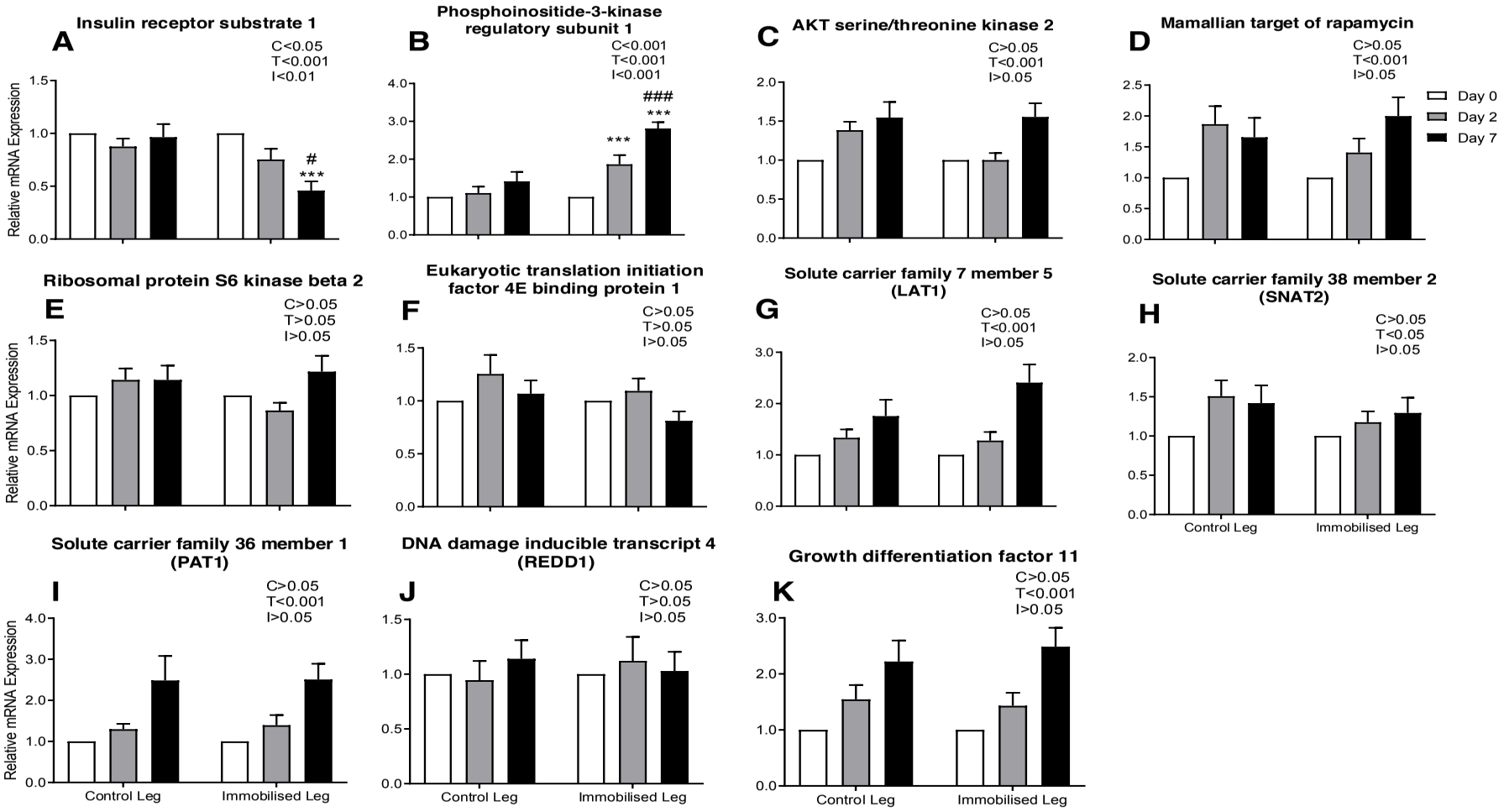


***Figure 3.5*** Skeletal muscle mRNA expression of genes involved in muscle protein synthesis and/or identified as growth factors, in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, *, ** and *** denotes *P*<0.05, *P*<0.01 and *P*<0.001 significant differences from day 0 within the same leg, #, ## and ### denotes *P*<0.05, *P*<0.01 and *P*<0.001 significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

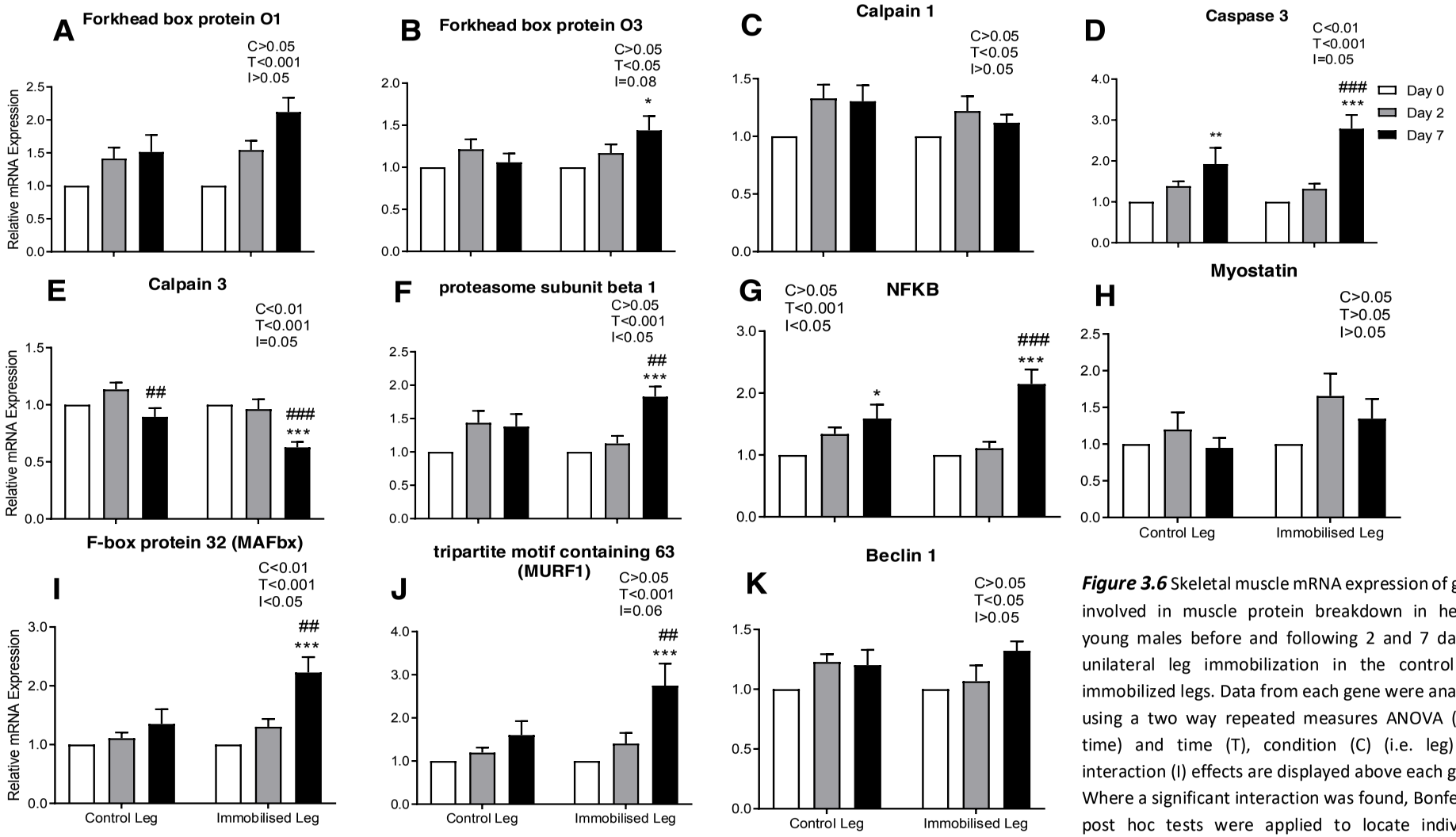


***Figure 3.6*** Skeletal muscle mRNA expression of genes involved in muscle protein breakdown in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, * and *** denotes $P<0.05$ and $P<0.001$ significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, $n=13$.

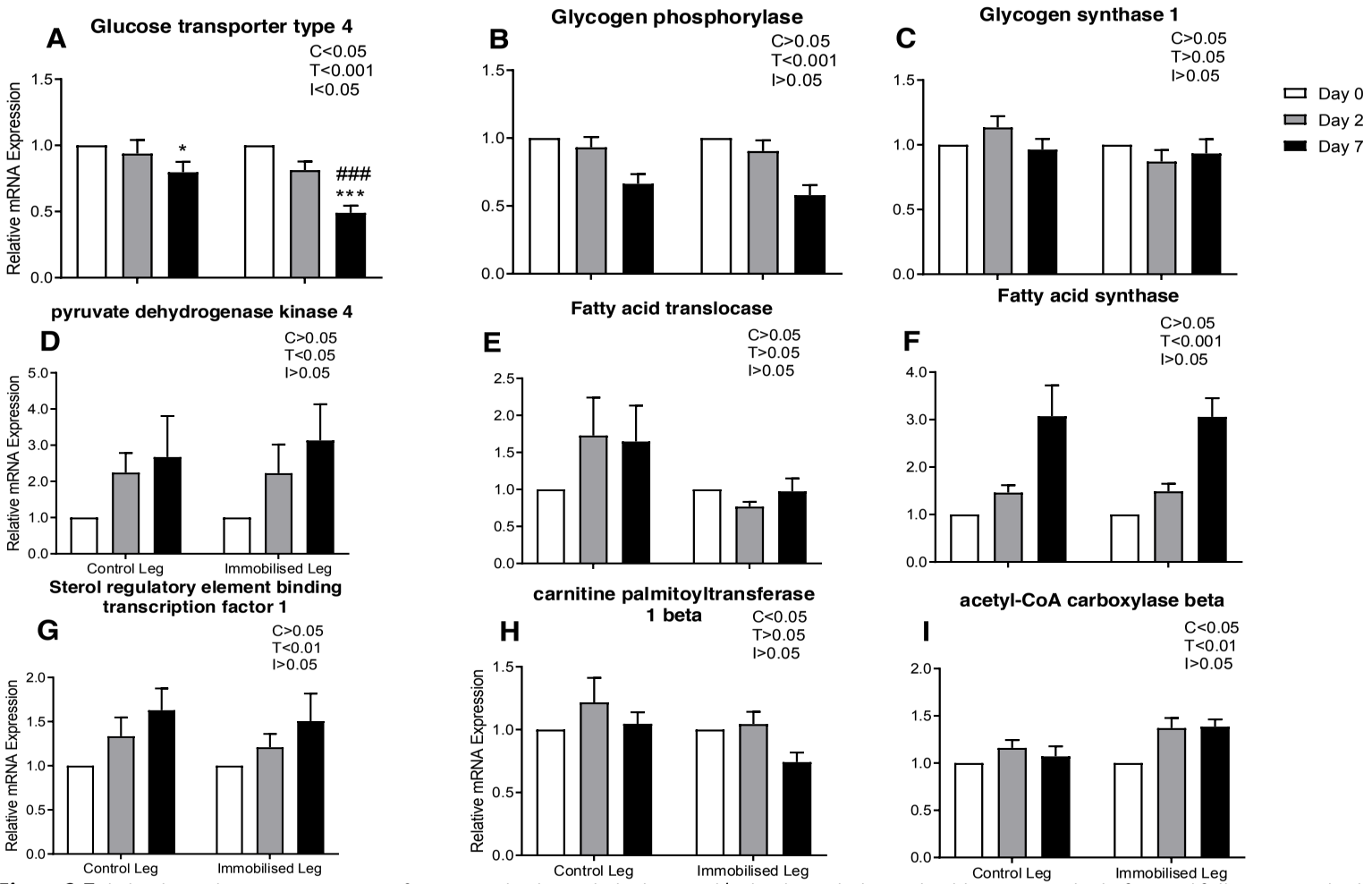


***Figure 3.7*** Skeletal muscle mRNA expression of genes involved in carbohydrate and/or lipid metabolism in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, *, ** and *** denotes *P*<0.05, *P*<0.01 and *P*<0.001 significant differences from day 0 within the same leg, ## and ### denotes *P*<0.01 and *P*<0.001 significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

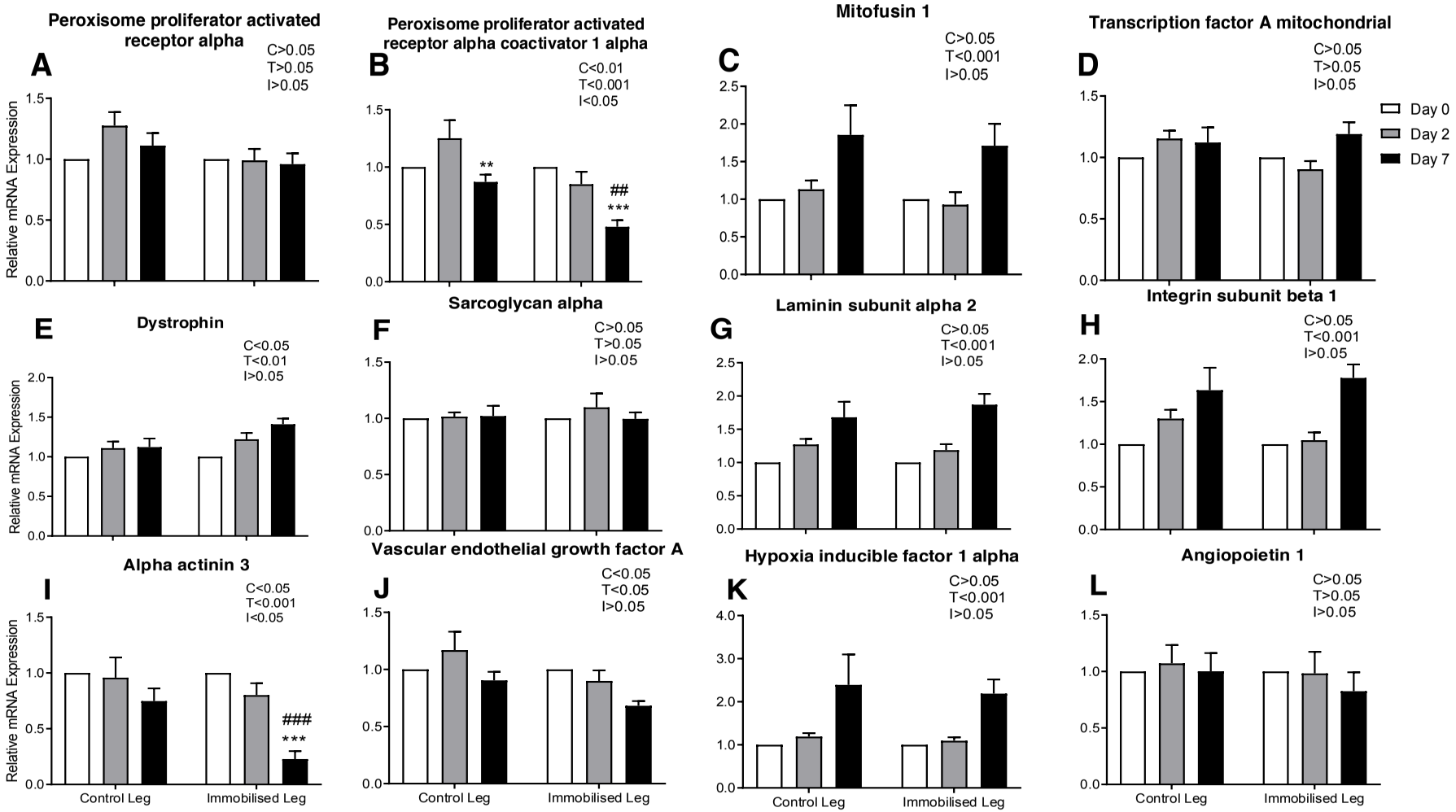


***Figure 3.8*** Skeletal muscle mRNA expression of genes involved in carbohydrate and/or lipid metabolism in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, *, ** and *** denotes *P*<0.05, *P*<0.01 and *P*<0.001 significant differences from day 0 within the same leg, ## and ### denotes *P*<0.01 and *P*<0.001 significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

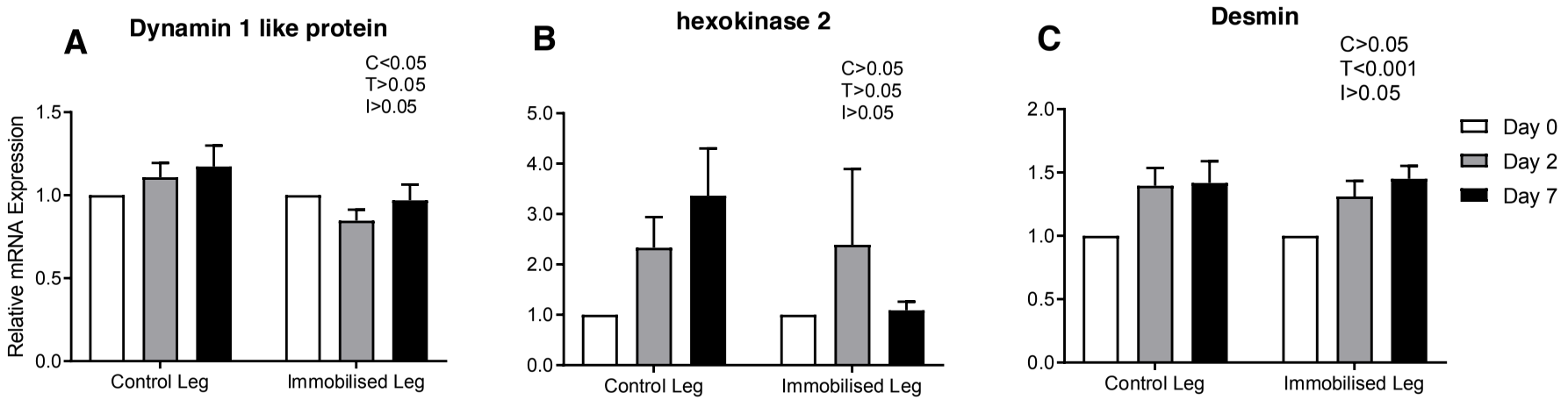


***Figure 3.9*** Skeletal muscle mRNA expression of A) Dynamin like protein 1, B) Desmin, C) Pyruvate dehydrogenase kinase 4 and d) hexokinase 2 in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

## *Discussion*

In the current study we applied a deuterated water approach and performed MRI scans to assess the temporal impact of one week of unilateral leg immobilisation on daily free living myofibrillar protein synthesis rates and quadriceps muscle volume, respectively. We report several novel observations. First, immobilisation lowered daily free living myofibrillar protein synthesis rates by 36% (compared with the control leg) over one week of immobilisation, and the magnitude of this decline was positively correlated with the observed decline in quadriceps volume. Second, this decline in myofibrillar protein synthesis rates occurred rapidly within 2 days, but to a greater extent in the latter part (2-7 days) of the week of immobilisation. Finally, one week, but not two days, of muscle disuse was associated with a coordinated upregulation in the muscle expression of genes involved in the ubiquitin proteasome mediated muscle protein breakdown pathway.

Skeletal muscle loss during a period of disuse has been attributed to a decline in both basal (post-absorptive) (11,22,105) and post-prandial muscle protein synthesis rates (11,45). Currently there are fewer data available on the impact of disuse on muscle protein synthesis rates throughout the day, which includes both basal and post-prandial as well as overnight muscle protein synthesis rates. To gain more insight into the impact of disuse on daily muscle protein synthesis rates over 2, and up to 7 days of immobilisation we applied the use of deuterated water. The ingestion of deuterated water has been applied to assess the muscle protein synthetic rate response to the acute (109) and longer term (70,114) effects of resistance training, as well as in response to various nutritional interventions (114). Recently, other groups have used the provision of deuterated water to measure the effect of disuse on muscle protein synthesis rates (44,72). In line with previous work from our group(s) (109,115) we show that our oral deuterated water regimen resulted in a sustained elevation in body water deuterium (~0.76±0.02%; Figure 2A) and plasma deuterated alanine (~3.1±0.2 MPE; Figure 2A) enrichments throughout the experimental period. With the data from both precursor pools correlating tightly (Figure 2B) and quantitatively relating to one another in agreement to previous work (Figure 2C; (109). Combined with the unilateral immobilisation approach (a within subject comparison of muscle contraction status with identical precursor pool supply) we were able to calculate that disuse reduced free living daily myofibrillar protein synthesis rates by ~36% over a week of immobilisation (Figure 3B). This reduction is quantitatively in line with what would be predicted from previous studies that have assessed the effects of 5 to 14 days of leg immobilisation on myofibrillar protein synthesis rates using both stable isotope labelled amino acid tracers (8,11,22) and deuterium oxide approaches (44). Moreover, we also report that the individual decline in myofibrillar protein synthesis rates was strongly positively correlated with the amount of muscle tissue lost during the week of disuse (Figure 4A). Thus, we provide robust evidence to show that a chronic (i.e. 24 h per day) decline in daily myofibrillar protein synthesis rates plays a major role in driving muscle disuse atrophy *in vivo* in humans. Worthy of note, this decline occurred despite our volunteers maintaining a relatively high dietary protein intake of 1.6 $g \cdot kg \cdot d^{-1}$ (see Table 1), suggesting that the impairments were exclusively a result of the disuse *per se*, and not further augmented by any compensatory decrease in protein (or energy) intake. In addition, the decline in synthetic rate was observed within the myofibrillar fraction of the muscle protein pool, underlining that targeting the maintenance (or stimulation) of myofibrillar protein synthesis rates during short-term disuse should clearly represent a primary strategy to combat the loss of muscle mass and associated declines in function

(44,98,116,117).

A clear picture of how muscle protein synthesis rates change over time during a period of disuse is not yet available, primarily due to the technical difficulties associated with making multiple acute measurements of myofibrillar protein synthesis rates within the same person (118). As a result, it is not clear how quickly myofibrillar protein synthesis rates decline consequent with disuse, or whether the magnitude of decline is dependent on the duration of disuse. Experiments utilizing static molecular markers within muscle tissue have suggested that muscle disuse atrophy may be differentially regulated within the first few days compared with one or more weeks (76,119). In the present work we show that although the decline in MPS did not reach statistical significance over the first 2 days, the decline in myofibrillar protein synthesis rates during this period (8±6% per day) was of roughly similar magnitude as the decline observed over days 2-7 (9±6% per day; Figure 3 D and E). Clearly, the decline in muscle protein synthesis rates (8±6% per day) as a primary factor in driving muscle loss (Figure 3D and E) is a process that occurs rapidly and seems to be a key process in explaining the fast decline in quadriceps volume that we observed during the first 2 days. However, the decline in myofibrillar protein synthesis rates occurred to a greater extent during the latter phase of the disuse period (i.e. 2-7 days; Figure 3D and E). This could suggest that the effect of disuse is cumulative with time, at least for a few days. Alternatively, these data may indicate that an immediate drop in myofibrillar protein synthesis rates occurs, but is variable in the time it takes to manifest across individuals (11/13 subjects decline after 2 days and 13/13 decline after 7 days). Of note, the tight correlation of declining myofibrillar protein synthesis rates with rate of muscle atrophy was less clear when examining the early phase of disuse (Figure 4). It is therefore of value to speculate to what extent declines in myofibrillar protein synthesis rates can quantitatively explain the observed muscle atrophy.

Previous work, using data obtained from acute measurements of hourly muscle protein synthesis rates have estimated that reductions in muscle protein synthesis rates can fully (43) (or even over) explain (8) observed muscle atrophy after 3-6 weeks of leg immobilisation. In contrast, we have previously estimated from our work that reductions in basal and/or post-prandial muscle protein synthesis rates could explain ~80% of the muscle atrophy seen during 5-14 days of disuse (107). However, these estimations rely on a number of assumptions, most importantly that measuring myofibrillar protein synthesis rates after a period of disuse reflect the changes in chronic myofibrillar protein synthesis rates that occur throughout the entire period of disuse. In the present study, where we have captured a cumulative muscle protein synthetic response throughout the entire period of disuse, alongside this we have also calculated quadriceps mass and how this responds over time to disuse. We therefore estimate that from days 0-2 approximately 25%, and from days 0-7 approximately 47%, of the decline in quadriceps mass could be attributed to a decline in daily muscle protein synthesis rates. This leaves room for the possibility of a contribution from increasing muscle protein breakdown rates.

With the difficulty of measuring *in vivo* MPB rates, we applied a custom-designed low-density RT-qPCR microarray gene card to measure the relative expression of 46 targeted genes that encode proteins associated with the regulation of muscle mass, muscle deconditioning/reconditioning and muscle metabolism, with a specific sub-focus on genes involved in MPB. Thirteen genes were unaffected with a further 20 showing an equivalent change in both legs ((potentially due to a repeated biopsy effect (120)). Thirteen of our selected genes changed differentially in the immobilised compared with control leg, and a striking observation is that 7 of those genes are involved in muscle protein breakdown, 4 of which

encode proteins involved in the ubiquitin proteasome system (UPS) (*PSMB1* [Figure 5F], *MAFbx* [Figure 5I], *MuRF1* [Figure 5J]) (121) and associated transcription factors (e.g. *FOXO3* [Figure 5B]). This coordinated change in gene expression of components of the UPS typically manifested as an increase after 7 but not 2 days of immobilisation. In contrast, genes involved in other muscle protein breakdown pathways, for example the calcium dependent calpain system (122), were either unaffected (*CLPN1* [Figure 5]) or tended to be reduced after 7 but not 2 days of immobilisation (*CLPN3* [Figure 5]). These findings are line with and extend on our (11) and other research groups' (123) previous findings that genes specifically involved in the ubiquitination and degradation of myofibrillar proteins show a transcriptional rise consequent with disuse. Previous work has suggested that such a rise in gene expression is rapid (2-7 days) and transient (≤one week), and possibly indicative of an increased rate of muscle deconditioning during this early phase (76). It is therefore perhaps surprising that we observed this transcriptional response at 7 rather than 2 days of disuse. We therefore speculate that, rather than being indicative of changes in muscle protein breakdown or deconditioning *per se*, this coordinated change in gene expression may reflect an 'echo' of the metabolic process (i.e. muscle deconditioning) that preceded and therefore drove the transcriptional response. In support of this thesis, the expression of various genes involved in insulin mediated glucose uptake (e.g. *IRS1* and *GLUT4*) also declined after 7 but not 2 days of immobilisation (i.e. at time points known to succeed the manifestation of insulin resistance; (9,124,125)). Besides the loss of muscle mass and volume, changes in muscle structure and function are evident due to muscle deconditioning which is also evident from our gene array data.

In conclusion, muscle disuse induces a rapid decline in muscle volume (within 2 days) that is further increased with prolonged disuse (up to 7 days). The decline in muscle volume is accompanied by a ~36% decline in daily myofibrillar protein synthesis rates in healthy young men over one week of disuse. These data highlight the key responsible role that declining myofibrillar protein synthesis rates play in the development of muscle disuse atrophy *in vivo* in humans.

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# Chapter 4

Mycoprotein represents a bioavailable and insulinotropic non-animal derived dietary protein source: *a dose-response study*

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## *Abstract*

Background The anabolic potential of a dietary protein is determined by its ability to elicit postprandial rises in circulating essential amino acids (leucine in particular) and insulin. Minimal data exist regarding the bioavailability and insulinotropic effects of non-animal derived protein sources. Mycoprotein is a sustainable and rich source of non-animal derived dietary protein.

Objective To investigate the impact of mycoprotein ingestion in a dose-response manner on acute postprandial hyperaminoacidaemia and hyperinsulinaemia.

Methods Twelve healthy young (28±2 y) men completed five experimental trials in a randomised, single-blind, crossover design. During each trial, volunteers consumed a test drink containing either 20 g milk protein (MLK20) or a mass matched (not protein matched due to the high fibre content of mycoprotein) bolus of mycoprotein (20 g; MYC20), a protein matched bolus of mycoprotein (40 g; MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein. Arterialized venous blood samples were collected in the fasted state and at regular intervals throughout a 4 h postprandial period to assess circulating amino acid, insulin and uric acid concentrations, and clinical chemistry profiles.

Results Mycoprotein ingestion resulted in slower but more sustained hyperinsulinaemia and hyperaminoacidaemia compared with milk when protein matched (MLK20 vs MYC40), with overall bioavailability equivalent between conditions ($P$>0.05). Increasing the dose of mycoprotein amplified these effects, with some evidence of a plateau at 80 g. Peak postprandial leucine concentrations were 201±24 (30 min), 118±10 (90 min), 150±14 (90 min), 173±23 (45 min) and 201±21 (90 min) $\mu mol \cdot L^{-1}$ for MLK20, MYC20, MYC40, MYC60 and MYC80, respectively.

Conclusions Mycoprotein ingestion represents a bioavailable and insulinotropic dietary protein source. Consequently, mycoprotein likely represents a useful source of dietary protein to stimulate muscle protein synthesis rates.

## *Introduction*

A growing body of research suggests that increasing dietary protein consumption beyond currently recommended amounts (i.e. 0.75-0.8 $g \cdot kg^{-1}$ body $weight \cdot day^{-1}$ in the UK/USA; 1, 2) can aid in the maintenance/gain of skeletal muscle mass, optimise tissue reconditioning in response to exercise, and/or promote cardio-metabolic health and weight management (3-6). Consequently, recommendations to increase dietary protein consumption in various populations, such as older adults, athletes, and those at risk of metabolic disease, are beginning to emerge (3-6). However, this trend is occurring in the face of mounting challenges associated with the sustainability of increased production of animal proteins (7). As a result, nutritional research is beginning to address the efficacy of alternative, plant-based protein sources (8, 9).

Mycoprotein, a food source produced by continuous fermentation of the filamentous fungus *Fusarium venenatum*, represents an alternative dietary protein source which, compared with animal derived sources, imposes a significantly lower environmental burden (10-13). Interestingly, previous work has shown benefits of mycoprotein consumption on blood cholesterol and lipid profiles, satiety and glycaemic control in both healthy and metabolically compromised individuals (11, 14-18). However, the potential for mycoprotein to support muscle mass maintenance and/or reconditioning remains to be investigated.

Physiological regulation of skeletal muscle mass is controlled in large part by dietary protein intake (19). Dietary protein ingestion increases muscle protein synthesis rates and, to a lesser extent, inhibits muscle protein breakdown rates, thereby allowing net muscle protein accretion (the 'anabolic response'). The postprandial elevation in muscle protein synthesis rates is driven by the rise in plasma essential amino acids (20) and leucine in particular (21, 22), whereas the inhibition of protein breakdown is mainly attributed to hyperinsulinaemia (23, 24). These postprandial periods offset the net loss of muscle protein which occurs during fasting periods. Accordingly, the potential utility (and amount required) of a specific dietary protein to support the maintenance, gain or reconditioning of muscle tissue is contingent on its ability to mount a sufficient anabolic response. The anabolic response, in turn, is dependent upon the dietary proteins bioavailability and insulinotropic properties. To date, data concerning these postprandial plasma profiles following mycoprotein ingestion are not available.

Mycoprotein is also a source of dietary purines, primarily present as nucleic acids. It has been proposed that dietary nucleic acid consumption should be limited due to concerns that excessive consumption can result in elevated serum uric acid concentrations (25-27), the latter representing an independent risk factor for the development of gout and an indicator of type-2 diabetes (28). However, these recommendations are based on studies that have fed large quantities of isolated, or yeast derived, nucleic acids above levels likely to be found under normal nutritional conditions (29, 30). Such findings require corroboration following the ingestion of nucleic acid containing whole food sources.

The aim of the present study was to provide a detailed acute postprandial plasma hyperaminoacidaemic and hyperinsulinaemic profile in response to the ingestion of graded quantities of mycoprotein compared with a reference, animal derived protein source (milk protein). Due to mycoprotein naturally possessing a large fibre content, we chose to compare to milk protein on a gram for gram total food (i.e. mass matched) and gram for gram total protein (i.e. protein matched) basis. We hypothesised that hyperaminoacidaemia and hyperinsulinaemia would be more rapid with milk protein, though bioavailability of amino acids would be similar between protein sources, and increase in accordance with

dose. As a secondary aim, we investigated the acute circulating serum uric acid and plasma clinical chemistry responses to the ingestion of increasing doses of mycoprotein.

## *Materials and methods*

### *Subjects*

Twelve healthy young men (age: 28±2 y; BMI: 26±1 $kg \cdot m^{-2}$) were included in the present study. Subjects' characteristics and habitual diet are presented in **Table 4.1**. Prior to inclusion in the study, subjects completed a routine medical screening to ensure suitability for acceptance onto the study. This screening involved the determination of height, weight, BMI, resting blood pressure and body composition. Body fat and lean mass were determined by Air Displacement Plethysmography (Bodpod; Life Measurement, Inc., Concord, CA, USA). During the screening, subjects also completed a general health questionnaire. Exclusion criteria were a BMI below 18.5 or above 30 $kg \cdot m^{-2}$, regular smoker, type-2 diabetes mellitus or cardiovascular disease/complications. Subjects were also instructed to cease taking any nutritional supplements for two weeks prior to the study and until all study visits were completed. During the screening, subjects were provided with a 3-day food diary and were instructed by a nutritionist in how to complete the diary in as much detail as possible. Food and drink intake was recorded for three consecutive days including two week days and a weekend day. The habitual energy and macro/micro-nutrient intake of the habitual diet was subsequently calculated using dedicated nutritional software (Nutritics Professional Nutritional Analysis Software, Swords, Dublin, Ireland). All subjects were informed on the nature and risks of the experiment before written informed consent was obtained. The study was approved by the Department of Sport and Health Sciences, University of Exeter's Ethical Committee and conducted in accordance with the Declaration of Helsinki.

***Table 4.1*** *Subjects' characteristics* and habitual diet

| **Characteristics** | |
|---|---|
| Age (y) | 28±2 |
| Body mass (kg) | 80±3 |
| Height (cm) | 177±2 |
| BMI ($kg \cdot m^2$) | 26±1 |
| Lean mass (kg) | 67±2 |
| Body fat (%) | 17±2 |
| Fasting plasma glucose ($mmol \cdot L^{-1}$) | 5.5±0.1 |
| Fasting serum insulin ($mU \cdot L^{-1}$) | 9.2±0.6 |
| Systolic blood pressure (mmHg) | 127±2 |
| Diastolic blood pressure (mmHg) | 78±2 |
| Mean arterial pressure (mmHg) | 93±2 |
| Resting metabolic rate ($kcal \cdot d^{-1}$) | 2278±48 |
| **Nutritional parameters** | |
| Energy intake ($kcal \cdot d^{-1}$) | 2257±138 |
| Protein intake ($g \cdot d^{-1}$) | 107±10 |
| Protein intake ($g \cdot kg\ bw^{-1} \cdot d^{-1}$) | 1.3±0.2 |
| Habitual fat intake ($g \cdot d^{-1}$) | 91±5 |
| Carbohydrate intake ($g \cdot d^{-1}$) | 259±28 |
| Alcohol intake ($g \cdot d^{-1}$) | 4±3 |
| Protein intake (En%) | 19±2 |
| Fat intake (En%) | 37±2 |
| Carbohydrate intake (En%) | 43±3 |
| Alcohol intake (En%) | 1±1 |
| Caffeine intake ($mg \cdot d^{-1}$) | 85±22 |

*Experimental overview and design*

In a randomized, single blind, crossover design, subjects participated in five laboratory test days. During each visit subjects ingested a test drink containing 20 g milk protein (MLK20), a 'mass matched' bolus of mycoprotein (20 g; MYC20), a protein matched bolus of mycoprotein (40 g; MYC40), or 60 (MYC60) or 80 (MYC80) g boluses of mycoprotein. Arterialized venous blood samples were collected in the fasted state and at regular intervals throughout a 4 h postprandial period to assess circulating amino acid, insulin and uric acid concentrations, and detail the plasma clinical chemistry profile. Indirect calorimetry and visual analogue scales (VAS) were used at regular intervals to determine whole body energy expenditure and subjective appetite scores, respectively.

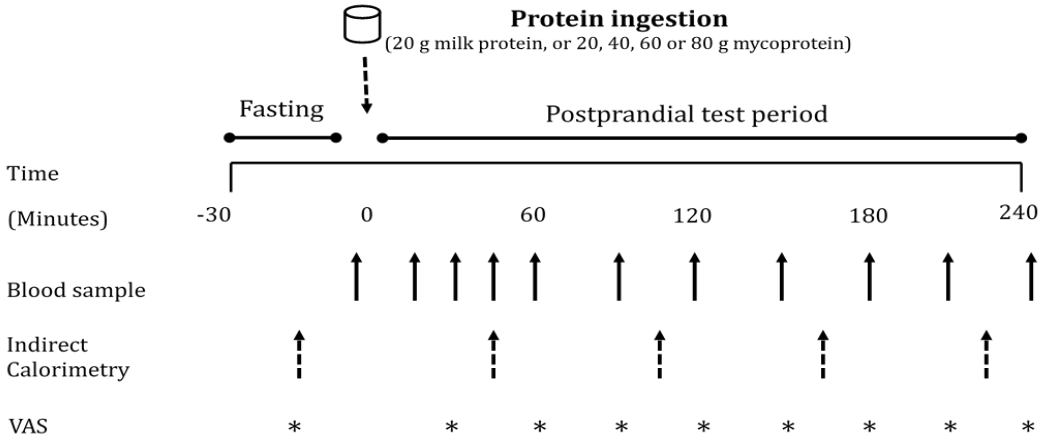


***Figure 4.1*** Overview of the experimental protocol. VAS, Visual Analogue Scale.

*Experimental visits*

An overview of the experimental setup for each test day is illustrated in **Figure 4.1**. Following inclusion into the study, volunteers attended the laboratory on five separate occasions, with each visit being separated by at least three days to ensure complete digestion, absorption and metabolism of the test meal. Volunteers were asked to abstain from strenuous physical activity and alcohol consumption for at least 48 h prior to each visit. For each test day, volunteers arrived at the laboratory at 08.30 h in the fasted state, voided their bladder and rested semi-supine on a hospital bed for 30 min. Thereafter, resting whole body metabolic rate was determined using expired gas collections for indirect calorimetry via a mixing chamber which obtained 20 sec averages (Cortex Metalyzer 2R gas analyser, Cortex, Leipzig, Germany). Expired gases were collected and recorded for a 15 min period, the last 5 min of which were used to obtain average $\dot{V}O_2$ and $\dot{V}CO_2$ values to determine substrate oxidation rates according to the non-protein stoichiometric equations detailed by Frayn (31). Total energy expenditure during this period was calculated as the sum of energy production from fat and carbohydrate, assuming that the oxidation of 1 g of triacylglycerol (862 $g \cdot mol^{-1}$) liberates 39.4 kJ and 1 g of glucose (180 $g \cdot mol^{-1}$) liberates 15.6 kJ of energy. This was then used to calculate resting 24 h energy expenditure at this given time point. Following this, volunteers completed a subjective appetite VAS (32). These 100 mm paper based scales detailed

questions regarding fullness, hunger, satisfaction, prospective food consumption and satiety which were anchored by diametrically opposed feelings of extremity. Volunteers reported on each scale their perceived feelings in the same order each time. Ratings were subsequently measured by the same researcher each time to minimize discrepancies and used to calculate an appetite score as reported previously (32). A cannula was inserted retrogradely into a superficial vein on the dorsal surface of the hand. This hand was kept in a hand-warming unit (air temperature 50-60$^{o}$C) for 15 min to arterialize the venous drainage of the hand (33) after which a fasting (8 mL) blood sample was collected and a 2 mL flush of saline was used to keep the cannula patent for further blood sampling. Thereafter, volunteers ingested one of the test drinks (in a randomized, counterbalanced for order and single blind [the volunteer] fashion) containing either 20 g milk protein (MLK20), 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein, within a total fluid volume of 650 mL. Volunteers were instructed to consume the test drink within 5 min, with the first visit providing the precise time to be repeated on subsequent visits to minimize any effect that speed of consumption may have on observed plasma amino acid kinetics. Completion of drink consumption signified the beginning of a 4 h postprandial testing period. Further indirect calorimetry measurements were taken for the final 15 min of each hour (with the final 5 min used to determine energy expenditure as described above), and further VAS scales were completed every 30 min throughout the postprandial period. Additional arterialized venous blood samples were collected from the hand cannula at 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min into the postprandial period, with additional 2 mL saline flushes used after each. Following the final blood collection the cannula was removed, volunteers were provided with a meal, and were then free to leave the laboratory.

*Test drink preparation and consumption*

Isolated milk was obtained from a commercial supplier (Mega milk protein 85, Hench Nutrition Ltd. Norfolk, UK) and freeze dried mycoprotein was obtained for drink preparation from Marlow Foods Ltd., North Yorkshire, UK. Test drinks were prepared by adding the requisite type/amount of protein to 300 ml water and 75 ml non-caloric artificial coconut or caramel flavouring (depending on volunteer preference but maintained the same for all visits within an individual; Jordan's sugar free skinny syrups, The Protein Pick and Mix Ltd., East Sussex, UK) and mixing thoroughly using a food blender. Water was then added to make up a total volume of 600 mL and mixed again. Following drink consumption by the volunteer an additional 50 mL of water was then added to 'wash' the bottle and ensure all protein was consumed, making a total volume of 650 mL consumed by a volunteer with each test drink. All drinks were well tolerated, consumed within the allotted time (i.e. 5 min) and no adverse effects were reported during or after the test day. Following ingestion of each drink, volunteers were asked to identify which condition they thought they had received which was noted down without feedback. The overall success rate for volunteers correctly identifying the condition was 69%. Individual condition success rates were as follows: MLK20, 78%; MYC20, 67%; MYC40, 78%; MYC60, 67%; MYC80, 56%. The nutritional content and amino acid composition of the different drinks are presented in **Table 4.2.**

***Table 4.2*** *Nutritional content of the drinks*

| **Macronutrient composition** | | | | | |
|---|---|---|---|---|---|
| | MLK20 | MYC20 | MYC40 | MYC60 | MYC80 |
| Energy | 69 | 68 | 136 | 204 | 272 |
| Protein | 16 | 9 | 18 | 27 | 36 |
| Fat | 0.2 | 3 | 5 | 8 | 10 |
| Carbohydrate | 0.7 | 2 | 4 | 6 | 8 |
| Fibre | 0.7 | 5 | 10 | 15 | 20 |
| **Amino acid content** | | | | | |
| | MLK20 | MYC20 | MYC40 | MYC60 | MYC80 |
| Aspartic acid | 1.2 | 1.0 | 1.9 | 2.9 | 3.8 |
| Serine | 0.8 | 0.4 | 0.9 | 1.3 | 1.8 |
| Glutamic Acid | 3.2 | 1.1 | 2.2 | 3.4 | 4.5 |
| Glycine | 0.3 | 0.4 | 0.9 | 1.3 | 1.7 |
| Histidine | 0.4 | 0.2 | 0.4 | 0.7 | 0.9 |
| Arginine | 0.5 | 0.6 | 1.3 | 1.9 | 2.5 |
| Threonine | 0.7 | 0.5 | 1.0 | 1.5 | 2.0 |
| Alanine | 0.5 | 0.6 | 1.2 | 1.7 | 2.3 |
| Proline | 1.5 | 0.4 | 0.9 | 1.3 | 1.8 |
| Cysteine | 0.6 | 0.6 | 1.2 | 1.7 | 2.3 |
| Tyrosine | 0.8 | 0.4 | 0.7 | 1.1 | 1.4 |
| Valine | 1.0 | 0.5 | 1.1 | 1.6 | 2.2 |
| Methionine | 0.3 | 0.6 | 1.2 | 1.7 | 2.3 |
| Lysine | 1.3 | 0.7 | 1.5 | 2.2 | 2.9 |
| Isoleucine | 0.8 | 0.4 | 0.9 | 1.3 | 1.8 |
| Leucine | 1.5 | 0.7 | 1.4 | 2.1 | 2.9 |
| Phenylalanine | 0.7 | 0.4 | 0.8 | 1.3 | 1.7 |
| Tryptophan | 0.2 | 0.6 | 1.2 | 0.6 | 0.6 |
| | | | | | |
| *EAA* | **6.9** | 4.7 | 9.4 | 13.0 | 17.1 |
| *NEAA* | **9.4** | 5.5 | 11.1 | 16.6 | 22.1 |
| *BCAA* | **3.3** | 1.7 | 3.4 | 5.1 | 6.8 |

## *Blood sample collection and analyses*

Four mL of each blood sample was collected into EDTA containing tubes (BD vacutainer LH) and centrifuged immediately at 3000 *g* at 4°C for 10 min. Blood plasma was obtained, then aliquoted and frozen at –80°C for subsequent analyses. The remaining 4 mL of each blood sample was collected into additional vacutainers (BD vacutainers SST II) which were left to clot at room temperature for at least 60 min and then centrifuged at 3000 *g* and 21°C for 15 min to obtain blood serum. Serum was then removed and aliquoted before freezing at –80°C for subsequent analyses.

Plasma amino acid profiles were determined via LC-MS. Briefly, proteins were precipitated out from the samples. The supernatant was then filtered and derivitized with the addition of stable isotope-labelled amino acid internal standards ((L-amino acid mix; Sigma-Aldrich Co., St Louis, MO, USA). Thereafter, HPLC-ESI-MS/MS quantitative analysis of amino acids in plasma was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) hyphenated to a 1200 series Rapid Resolution HPLC system (Agilent Technologies, Palo Alto, USA). Data analysis was undertaken using Agilent Mass Hunter Quantitative analysis software for QQQ (Version B.07.01). Accurate quantification used the

stable isotope labelled internal standards added during sample extraction.
Serum insulin concentrations were determined in duplicate using a commercially available ELISA assay kit (Oxford Biosystems Ltd., Oxford, UK) with a within-batch coefficient of variation of 3.2%. Serum uric acid concentrations were determined enzymatically via colorimetry (Cobas 8000 automated analyser, Roche Diagnostics, USA) as described previously (34) with a within-batch coefficient of variation of 2.6%. Plasma glucose, urea, creatinine and additional electrolyte/clinical chemistry profiles were determined using an automated analyser (Stat profile pHOx Ultra Analyzer, Nova Biomedical, UK).

### *Statistical analyses and data presentation*

All data are expressed as means±SEM. Fasting and postprandial kinetic responses for each of the variables are displayed in two separate graphs to avoid congestion, and present a clear comparison in one graph of MLK20 vs its mass (MYC20) and protein (MYC40) matched conditions, and a second graph allowing the mycoprotein dose-response relationship to be visualized (i.e. comparing MYC20, MYC40, MYC60 and MYC80). However, for all parameters, all five conditions were compared within the same statistical test, and analyzed with a two-way ANOVA with repeated measures (with condition and time as factors). In the event of a significant main effect, Bonferroni post-hoc tests were applied to locate individual differences, with each postprandial value being compared to the corresponding fasting value so the impact of protein ingestion within each condition could be evaluated. Where area under the curves (AUCs) were calculated, a one-way ANOVA was performed to detect any significant effect of treatment. If a significant main effect was detected, multiple *t*-tests were used to compare each condition with each other. Plasma biochemistry profiles, indirect calorimetry and VAS data were averaged as fasting, early postprandial (average of all data collected within the first 2 h post protein consumption) and late postprandial (average of all data collected 2-4 h post protein consumption) responses and analyzed with a two-way ANOVA and Bonferonni post-hoc tests as described above. Statistical significance was set at $P<0.05$. All calculations were performed by using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

## *Results*

### *Plasma amino acid concentrations*

Fasting and postprandial plasma amino acid concentrations are shown in **Figures 4.2 and 4.3**. From similar fasting levels, plasma total amino acid concentrations (Figure 4.2A and B) increased with protein ingestion (time effect: $P<0.0001$) and by differing degrees depending upon condition (time *x* treatment interaction effect; $P<0.001$). MLK20 exhibited the most rapid peak concentration (30 min) and remained elevated relatively briefly for only 45 min. Conversely, mycoprotein conditions generally showed a more delayed rise to peak concentrations, with a more sustained availability: MYC40, MYC60 and MYC80 peaked between 45 and 120 min, but stayed elevated above fasting levels for between 120 and 240 min. MYC20 did not show a significant rise in postprandial total amino acid concentrations. When expressed as an area under the curve (AUC; Figure 4.2C), a significant treatment effect on 4 h postprandial total amino acid availability was detected ($P<0.05$) indicating a dose-response effect, with individual responses showing MYC60 and MYC80 were greater when compared with MYC20 (both $P<0.05$), and MYC80 greater when compared with MYC40 ($P<0.05$). Fasting levels of essential amino acids (Figure 2D and E) were equivalent between conditions. Protein ingestion led to a rise in plasma essential amino acid concentrations (time effect; $P<0.0001$) which differed across conditions (time *x* treatment interaction; $P<0.05$). Specifically, plasma essential amino acid concentrations increased briefly from 30 to 45 min in MLK20 ($P<0.05$), were unaffected in MYC20 ($P>0.05$) and increased in a more sustained manner (but less rapidly compared with MLK20) in MYC40 (from 60-120 min; $P<0.05$), MYC60 (from 45-180 min; $P<0.05$) and MYC80 (from 45-240 min; $P<0.05$). The AUC of the essential amino acid response (Figure 2F) to protein ingestion showed an effect of treatment ($P<0.05$) indicating a dose-response relationship, with MYC60 and MYC80 conditions being significantly greater compared with MYC20 (both $P<0.05$). From equivalent fasting values, plasma non-essential amino acid concentrations (Figure 2G and H) increased with protein ingestion (time effect; $P<0.0001$) in a varying manner depending upon condition (time *x* treatment interaction; $P<0.01$). Specifically, MLK20 showed a sharp, transient rise between 15 and 45 min ($P<0.01$) into the postprandial period, MYC20 did not change, MYC40 displayed a relatively slower increase (90-120 min), whereas MYC60 and MYC80 exhibited more sustained elevations (from 15-120 and 45-240 min, respectively [both $P<0.05$]). Non-essential amino acid AUC (Figure 2I) also showed a significant dose-response relationship ($P<0.05$), with MYC60 showing a greater response compared with MYC20 ($P<0.05$) and MYC80 being higher than both MYC20 ($P<0.05$) and MYC40 ($P<0.05$).

Plasma total branched chain amino acids (Figure 3A and B), from similar fasting values (MLK20: 488±31 $\mu mol \cdot L^{-1}$, MYC20: 442±51 $\mu mol \cdot L^{-1}$, MYC40: 459±41 $\mu mol \cdot L^{-1}$, MYC60: 497±57 $\mu mol \cdot L^{-1}$, MYC80: 526±52 $\mu mol \cdot L^{-1}$), showed a significant effect of time ($P<0.0001$) and a time *x* treatment interaction ($P<0.0001$). Postprandial branched chain amino acid concentrations in MLK20 were elevated above fasting levels from 15-90 min ($P<0.05$) and peaked at 45 min (848±102 $\mu mol \cdot L^{-1}$), did not change in MYC20 (though numerically peaking at 90 min; 581±54 $\mu mol \cdot L^{-1}$), were raised in MYC40 from 45-150 min ($P<0.05$) and peaking at 120 min (752±80 $\mu mol \cdot L^{-1}$), and were elevated in MYC60 and MYC80 from 45-240 min ($P<0.05$), both peaking at 120 min (831±109 and 943±102, $\mu mol \cdot L^{-1}$, respectively). Plasma postprandial branched chain amino acid concentration AUCs (Figure 3C) showed a clear dose-response relationship, with MYC20

displaying lower concentrations than MLK20 (P<0.05) and MYC40, MYC60 and MYC80 all showing greater responses compared with MYC20 (all *P*<0.05), and MYC80 also significantly higher than MLK20 (*P*<0.05).

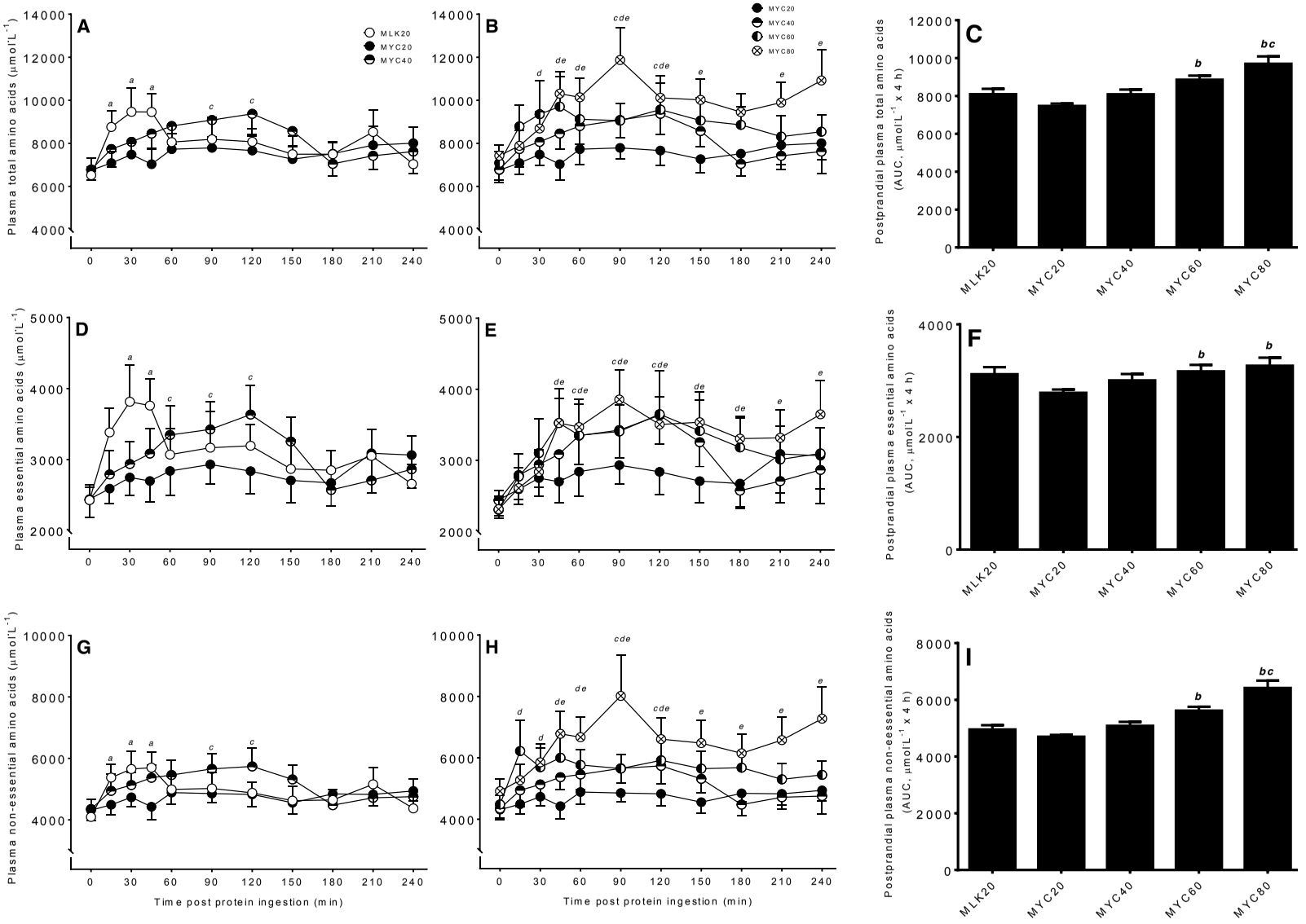


***Figure 4.2*** Mean (±SEM) plasma total (**A-B**), total essential (**D-E**) and total non-essential (**G-H**) amino acid concentrations in the fasting state (*t*=0) and at regular intervals during a 4 h postprandial period following the ingestion of 20 g milk protein (MLK20), or 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein in healthy, young men (*n*=12). For each variable, data are separated into two graphs for clear comparison of relevant conditions, but all conditions were statistically analysed together with two-way repeated measures ANOVAs and Bonferroni post hoc tests applied to locate individual differences: *a*, *b*, *c*, *d*, and *e* indicates value different to corresponding fasting value for MLK20, MYC20, MYC40, MYC60 and MYC80 conditions, respectively. Data are also expressed as area under the curves (AUC) for the total 4 h postprandial responses for total (**C**), essential (**F**) and non-essential (**I**) amino acids. Data were analysed for a main effect with a one-way ANOVA and individual *t*-tests were applied to locate individual differences: *a*, *b*, *c*, and *d* indicates value different to MLK20, MYC20, MYC40, and MYC60 conditions, respectively.

When examining the branched chain amino acids individually, plasma leucine concentrations (Figure 3D and E) were influenced by protein ingestion (time effect: *P*<0.0001) and condition (treatment effect: *P*<0.05), and a significant time *x* condition interaction (*P*<0.0001) was also detected. Specifically, from similar fasting levels (~90 μmol·L$^{-1}$), peak postprandial leucine concentrations increased to 201±24 (at 30 min), 118±10 (at 90 min), 150±14 (at 90 min), 173±23 (at 45 min) and 201±21 (at 90 min) μmol·L$^{-1}$ for MLK20, MYC20, MYC40, MYC60 and MYC80, respectively. Individual responses of leucine were similar (but more pronounced) to those observed for the above detailed sub-groups of amino acids. Specifically, MYC20 did not show any individual changes. MLK20 resulted in more rapid, but less sustained leucinaemia (from 15-90 min; *P*<0.05) compared with all mycoprotein conditions >20 g: MYC40, 45-150 min (*P*<0.05); MYC60, 30-180 min (*P*<0.05); and MYC80, 45-240 min (*P*<0.05). Postprandial leucine AUC (Figure 4.3F) also showed a clear dose-response relationship (*P*<0.05). Specifically, the postprandial leucine response in MYC20 was lower compared with MLK20 (*P*<0.05), and responses in MYC40, MYC60 and MYC80 were all greater than MYC20 (all *P*<0.05), and MYC80 also showing a higher level compared with MLK20 (*P*<0.05) and MYC40 (*P*<0.05). Plasma isoleucine (time effect; *P*<0.0001, treatment; *P*<0.05), interaction *P*<0.0001) and valine (time effect; *P*<0.0001, treatment; *P*=0.015, interaction *P*<0.01) showed similar main effects to leucine, with individual differences, peaks, concentrations and AUCs illustrated in Figures 3G-L.

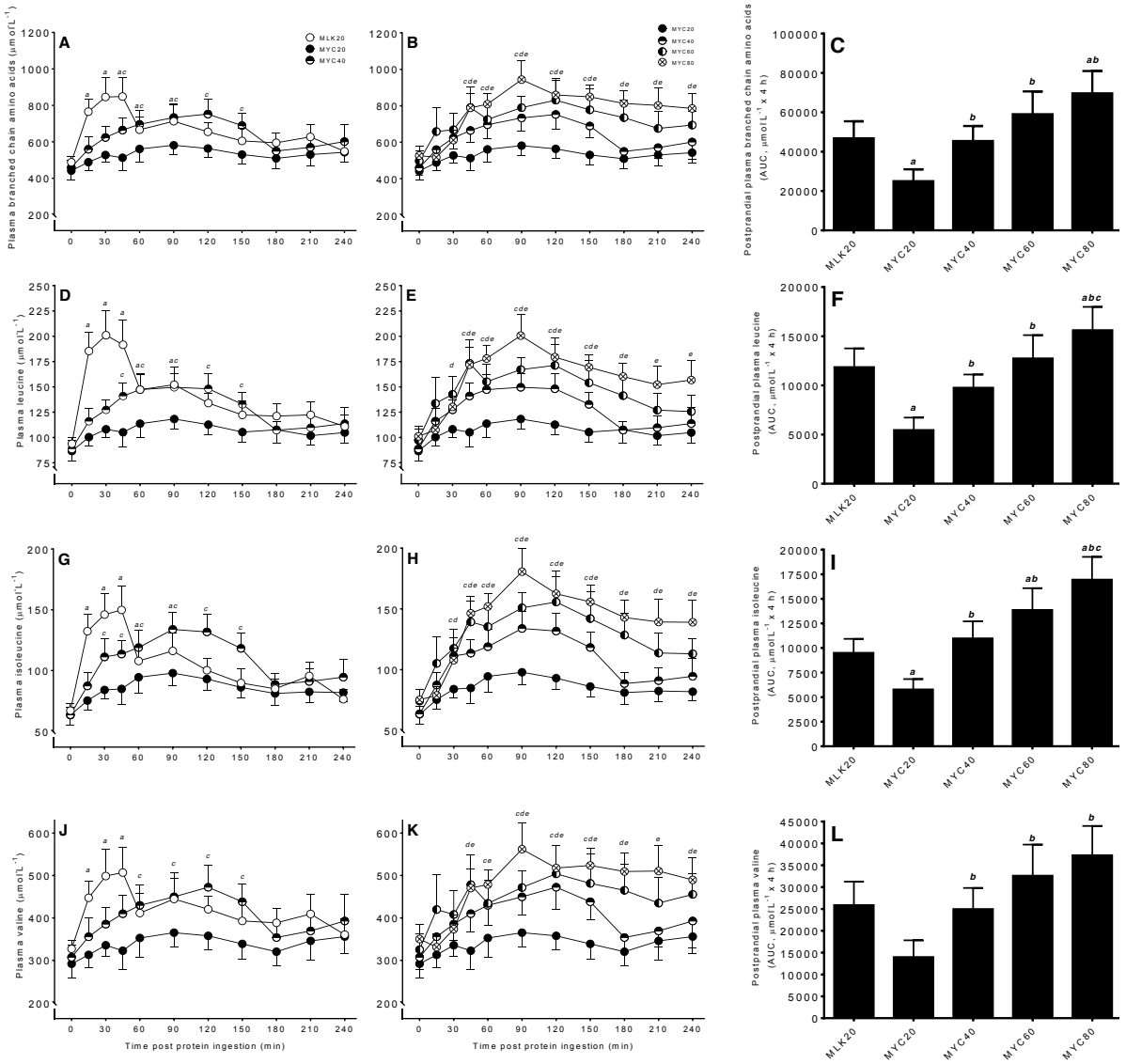


***Figure 4.3*** Mean (±SEM) plasma total branched chain amino acid (**A-B**), leucine (**D-E**), isoleucine (**G-H**) and valine (**J-K**) concentrations in the fasting state (*t*=0) and at regular intervals during a 4 h postprandial period following the ingestion of 20 g milk protein (MLK20), or 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein in healthy, young men (*n*=12). For each variable, data are separated into two graphs for clear comparison of relevant conditions, but all conditions were statistically analysed together with two-way repeated measures ANOVAs and Bonferroni post hoc tests applied to locate individual differences: *a*, *b*, *c*, *d*, and *e* indicates value different to corresponding fasting value for MLK20, MYC20, MYC40, MYC60 and MYC80 conditions, respectively. Data are also expressed as area under the curves (AUC) for the total 4 h postprandial responses for total branched chain amino acids (**C**), leucine (**F**), isoleucine (**I**) and valine (**L**). Data were analysed for a main effect with a one-way ANOVA and individual *t*-tests were applied to locate individual differences: *a*, *b*, *c*, and *d* indicates value different to MLK20, MYC20, MYC40, and MYC60 conditions, respectively.

*Serum insulin concentrations*

Fasting and postprandial serum insulin concentrations are depicted in **Figure 4**. Fasting levels did not differ between conditions (MLK, 10.4±1.9 $mU \cdot L^{-1}$; MYC20, 8.7±1.2 $mU \cdot L^{-1}$; MYC40, 8.9±1.6 $mU \cdot L^{-1}$; MYC60, 8.9±1.5 $mU \cdot L^{-1}$; MYC80, 9.0±1.4 $mU \cdot L^{-1}$; $P>0.05$). Significant effects of time ($P<0.0001$) and a treatment *x* time interaction ($P<0.0001$) were observed. MLK20 resulted in a rapid (at 15 min) but brief (until 30 min; $P<0.001$) rise in circulating insulin levels, which returned to fasting levels after 45 min. MYC20 did not alter serum insulin concentrations, though MYC40, MYC60 and MYC80 all increased circulating insulin concentrations at 15 min (all $P<0.05$) and remained elevated until 60 (MYC40; $P<0.01$) to 90 (MYC60 and MYC80; $P<0.05$) min. Peak insulin concentrations were observed at 15 min for the MLK20 (21.8±4.1 $mU \cdot L^{-1}$), MYC20 (10.6±1.6 $mU \cdot L^{-1}$) and MYC40 (16.2±2.9 $mU \cdot L^{-1}$) conditions, and after 45 min in MYC60 (19.3±2.8 $mU \cdot L^{-1}$) and MYC80 (22.9±3.2 $mU \cdot L^{-1}$), though the magnitudes of the peaks did not differ ($P>0.05$). When comparing the overall postprandial insulin response between conditions as an AUC (peaks above baseline) (Figure 4.4C) there was no difference between MLK20, MYC20 and MYC40 conditions, though MYC80 showed a greater response compared with MLK20 ($P<0.05$), and MYC60 and MYC80 showed a greater response compared with MYC20 ($P<0.01$ and $P<0.001$, respectively). MYC40, MYC60 and MYC80 AUCs did not differ from one another.

*Serum uric acid concentrations*

Serum uric acid concentrations in the fasting state and over a 4 h postprandial period following protein ingestion are depicted in **Figure 4.5**. Fasting plasma uric acid concentrations were similar in all conditions (MLK20, 338±21 $\mu mol \cdot L^{-1}$; MYC20, 362±17 $\mu mol \cdot L^{-1}$; MYC40, 354±18 $\mu mol \cdot L^{-1}$; MYC60, 365±20 $\mu mol \cdot L^{-1}$; MYC80, 350±12 $\mu mol \cdot L^{-1}$; $P>0.05$). There were significant time ($P<0.0001$), treatment ($P<0.01$) and time *x* treatment interaction ($P<0.0001$) effects detected. In MLK20 and MYC40 fasting plasma uric acid concentrations remained unaltered across the postprandial period. MYC20 had minimal impact on serum uric acid concentrations, though there was a significant decrease at 150 min ($P<0.05$) only. With MYC60 uric acid concentrations increased modestly by 30 min ($P<0.05$), and remained elevated until 150 min before returning to fasting values, with the peak concentration of 387±20 $\mu mol \cdot L^{-1}$ occurring 60 min after protein ingestion ($P<0.0001$). Similarly, MYC80 resulted in an increase in serum uric acid concentrations by 30 min ($P<0.01$) with levels again peaking at 60 min (at 378±13 $\mu mol \cdot L^{-1}$; $P<0.0001$) and, despite then decreasing back towards fasting levels, remaining elevated throughout the entire postprandial period ($P<0.05$).

*Whole body energy expenditure*

Resting 24 h energy expenditure in the fasting state and during the early (0-2 h) and late (2-4 h) postprandial period following the ingestion of dietary protein is displayed in **Figure 4.6**. Fasting 24 h energy expenditure (in Kcals) was equivalent between visits (MLK20, 2362±95; MYC20, 2247±97; MYC40, 2331±124; MYC60, 2210±72; MYC80, 2170±108; $P>0.05$). Time ($P<0.05$) and time *x* treatment interaction ($P<0.001$) effects were observed such that protein ingestion resulted in a significant decrease in energy expenditure in MLK20 during the late phase ($P<0.05$), and an increase in energy expenditure in the MYC60 condition during the early postprandial phase ($P<0.0001$) and in the MYC80 condition during both the early ($P<0.0001$) and late ($P<0.001$) postprandial phases.

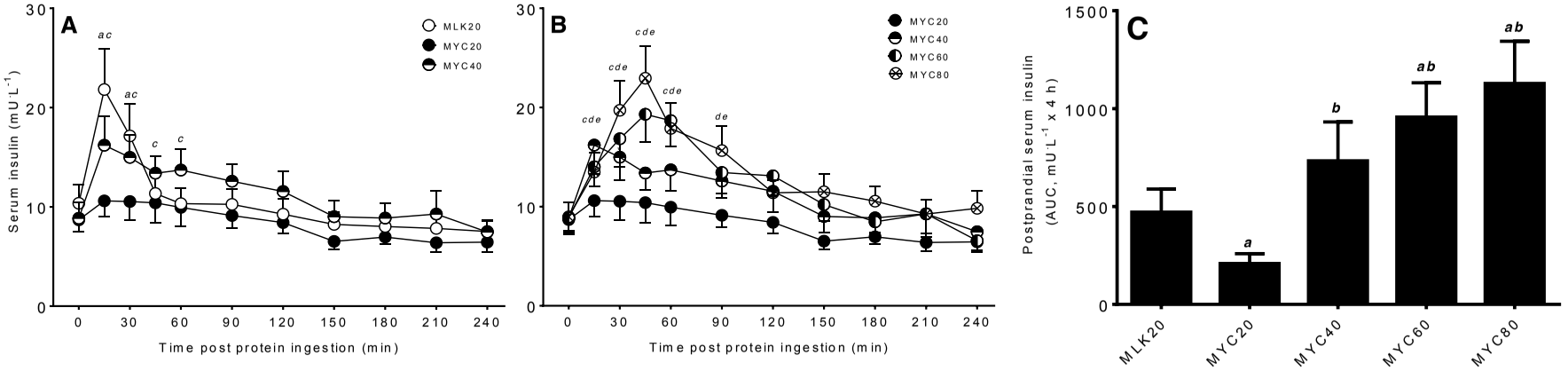


***Figure 4.4*** Mean (±SEM) serum insulin (**A-B**) concentrations in the fasting state (*t*=0) and at regular intervals during a 4 h postprandial period following the ingestion of 20 g milk protein (MLK20), or 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein in healthy, young men (*n*=12). Data are separated into two graphs for clear comparison of relevant conditions, but all conditions were statistically analysed together with a two-way repeated measures ANOVA and Bonferroni post hoc tests applied to locate individual differences: *a*, *b*, *c*, *d*, and *e* indicates value different to corresponding fasting value for MLK20, MYC20, MYC40, MYC60 and MYC80 conditions, respectively. Data are also expressed as area under the curve (AUC) for the total 4 h postprandial response (**C**). Data were analysed for a main effect with a one-way ANOVA and individual *t*-tests were applied to locate individual differences: *a*, *b*, *c*, *d* and *e* indicates value different to MLK20, MYC20, MYC40, MYC60 and MYC80 conditions, respectively.

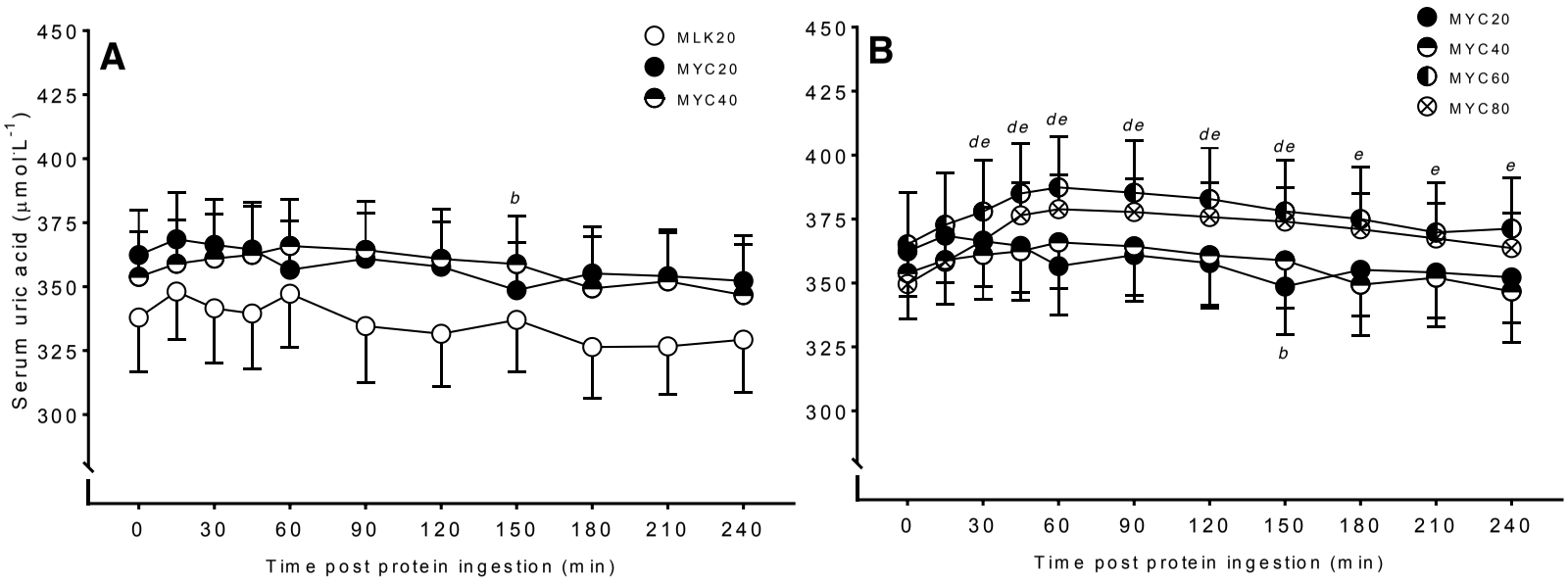


***Figure 4.5*** Mean (±SEM) serum uric acid (**A-B**) concentrations in the fasting state (*t*=0) and at regular intervals during a 4 h postprandial period following the ingestion of 20 g milk protein (MLK20), or 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein in healthy, young men (*n*=12). Data are separated into two graphs for clear comparison of relevant conditions, but all conditions were statistically analysed together with a two-way repeated measures ANOVA and Bonferroni post hoc tests applied to locate individual differences: *a*, *b*, *c*, *d*, and *e* indicates value different to corresponding fasting value for MLK20, MYC20, MYC40, MYC60 and MYC80 conditions, respectively. Data are also expressed as area under the curve (AUC) for the total 4 h postprandial response (**C**). Data were analysed for a main effect with a one-way ANOVA and individual *t*-tests were applied to locate individual differences: *a*, *b*, *c*, *d* and *e* indicates value different to MLK20, MYC20, MYC40, MYC60 and MYC80 conditions, respectively.

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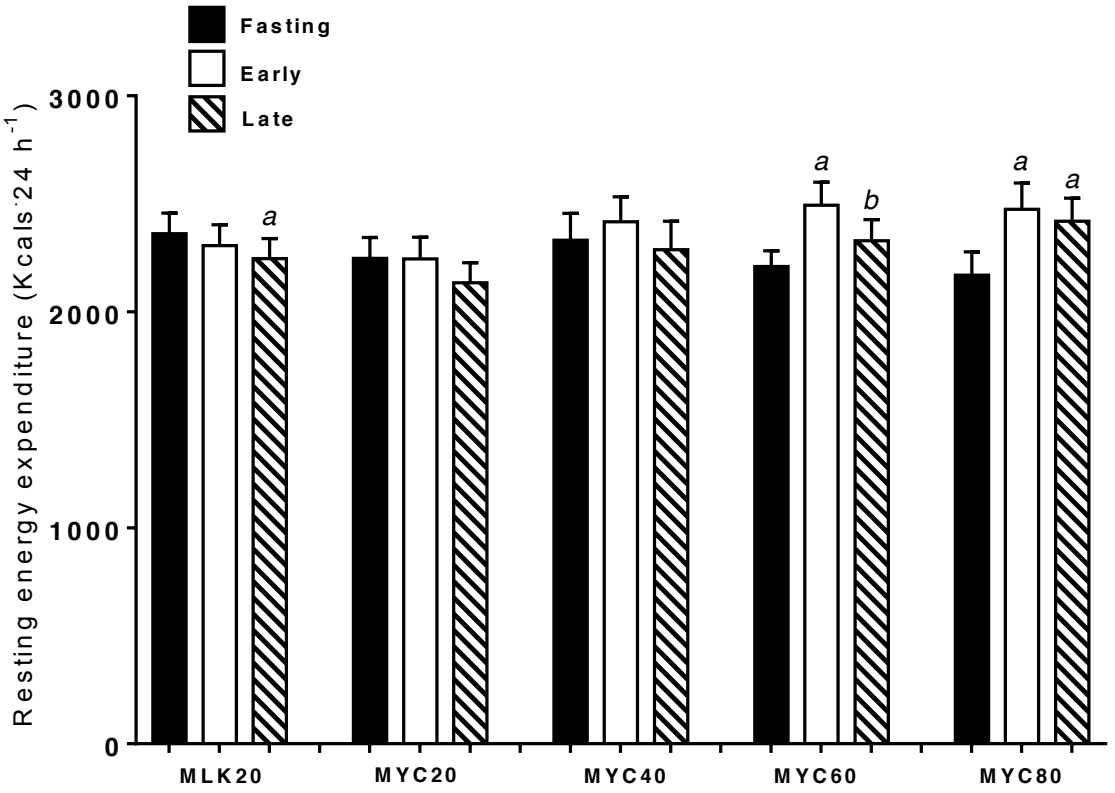


***Figure 4.6*** Mean (±SEM) resting energy expenditure in the fasting state and during the early (i.e. 0.2 h) and late (i.e. 2-4 h) phases of a 4 h postprandial period following the ingestion of 20 g milk protein (MLK20), or 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein in healthy, young men (*n*=12). Data were analysed with a two-way repeated measures ANOVA and Bonferroni post hoc tests applied to locate individual differences: *a* and *b* indicates value different to corresponding fasting or early postprandial value, respectively.

### *Appetite responses*

In the fasting state, appetite scores were similar between all conditions at ~62 ($P$>0.05). Appetite score was affected by protein ingestion (time effect; $P$<0.0001) and condition (treatment effect; $P$<0.05), though only a trend for an interaction between time and treatment was observed (interaction effect; $P$=0.097). Specifically, all conditions showed a reduced appetite score 30 min following protein ingestion (all $P$<0.01) which persisted to 60 min in MLK20 ($P$<0.05) and MYC20 ($P$<0.01) conditions, and until 120 min in the MYC80 condition ($P$<0.001) before returning towards fasting levels. Compared with fasting values, all conditions showed a significantly greater appetite score for the final 60 (MLK20 and MYC20 conditions; $P$<0.01) or 90 min (MYC40 and MYC60 conditions; $P$<0.05) of the postprandial period, with the exception of the MYC80 condition where this increase in appetite was not evident. **Figure 4.7** depicts these data as fasting, mean early (30-120 min) and mean late (150-240 min) appetite responses.

### *Plasma biochemistry profile*

Plasma clinical biochemistry profiles are reported in Table 4.3.

| Parameter | Fasting | | | | | Early postprandial | | | | | Late postprandial | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **MLK20** | **MYC20** | **MYC40** | **MYC60** | **MYC80** | **MLK20** | **MYC20** | **MYC40** | **MYC60** | **MYC80** | **MLK20** | **MYC20** | **MYC40** | **MYC60** | **MYC80** |
| pH | 7.56±0.01 | 7.56±0.01 | 7.56±0.01 | 7.57±0.01 | 7.57±0.01 | 7.55±0.01 | 7.56±0.01 | 7.56±0.01 | 7.55±0.01 | 7.55±0.01* | 7.56±0.01 | 7.55±0.01 | 7.54±0.01 | 7.54±0.01* | 7.57±0.01 |
| $Na^{+}$ ($mmol{\cdot}L^{-1}$) | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 | 137±1 |
| $K^{+}$ ($mmol{\cdot}L^{-1}$) | 4.3±0.1 | 4.3±0.1 | 4.2±0.1 | 4.1±0.1 | 4.2±0.1 | 4.2±0.1 | 4.2±0.1 | 4.2±0.1 | 4.1±0.1 | 4.2±0.1 | 4.0±0.1* | 4.1±0.1* | 4.1±0.1 | 4.1±0.1 | 4.1±0.1 |
| $Cl^{-}$ ($mEq{\cdot}L^{-1}$) | 108.4±0.5 | 108.3±0.4 | 108.0±0.3 | 108.0±0.3 | 107.9±0.3 | 107.2±0.3* | 107.7±0.3 | 107.6±0.3 | 107.4±0.5 | 107.4±0.3 | 107.5±0.2* | 107.9±0.3 | 107.5±0.2 | 107.6±0.3 | 108±0.3 |
| $Ca^{++}$ ($mmol{\cdot}L^{-1}$) | 1.17±0.01 | 1.16±0.01 | 1.17±0.01 | 1.17±0.01 | 1.17±0.01 | 1.18±0.01* | 1.17±0.01 | 1.17±0.01 | 1.17±0.01 | 1.18±0.01* | 1.19±0.01* | 1.17±0.01 | 1.17±0.01 | 1.17±0.01 | 1.17±0.01† |
| $Mg^{++}$ ($mmol{\cdot}L^{-1}$) | 0.52±0.01 | 0.53±0.01 | 0.54±0.01 | 0.52±0.01 | 0.53±0.01 | 0.53±0.01 | 0.54±0.01 | 0.54±0.01 | 0.54±0.01* | 0.55±0.01* | 0.53±0.01 | 0.54±0.01* | 0.55±0.01 | 0.55±0.01* | 0.54±0.01 |
| Glucose ($mmol{\cdot}L^{-1}$) | 5.5±0.1 | 5.4±0.2 | 5.5±0.1 | 5.4±0.1 | 5.4±0.1 | 5.3±0.1 | 5.4±0.1 | 5.5±0.1 | 5.5±0.1 | 5.4±0.1 | 5.2±0.1*† | 5.2±0.1*† | 5.3±0.1*† | 5.3±0.1† | 5.4±0.1 |
| Lactate ($mmol{\cdot}L^{-1}$) | 0.69±0.07 | 0.76±0.10 | 0.71±0.05 | 0.68±0.07 | 0.73±0.08 | 0.75±0.08 | 0.61±0.05* | 0.64±0.06 | 0.67±0.06 | 0.72±0.06 | 0.57±0.05† | 0.61±0.04* | 0.57±0.04* | 0.57±0.05 | 0.61±0.05 |
| Urea ($mmol{\cdot}L^{-1}$) | 5.6±0.3 | 5.6±0.4 | 5.7±0.3 | 5.5±0.3 | 5.3±0.3 | 5.7±0.3 | 5.5±0.3 | 5.7±0.3 | 5.6±0.3 | 5.7±0.3* | 5.4±0.3*† | 5.3±0.3*† | 5.7±0.2 | 5.8±0.3 | 6.0±0.3*† |
| Creatinine ($\mu mol{\cdot}L^{-1}$) | 70±3 | 72±3 | 69±3 | 69±2 | 71±3 | 69±3 | 69±2 | 68±3 | 68±2 | 69±3* | 68±2* | 69±2* | 68±2 | 68±1 | 68±3* |
| TCO2 ($mEq{\cdot}L^{-1}$) | 24.1±0.3 | 23.7±0.4 | 23.8±0.3 | 23.6±0.4 | 24.4±0.4 | 24.4±0.3 | 23.7±0.3 | 23.8±0.4 | 23.6±0.4 | 24.4±0.4 | 24.2±0.3 | 23.7±0.4 | 24.0±0.3 | 23.6±0.4 | 23.3±0.4*† |
| Urea:creatinine | 82±6 | 78±6 | 85±7 | 82±6 | 77±6 | 85±6 | 80±5 | 86±7 | 84±5 | 83±7* | 82±6 | 77±4 | 85±7 | 86±5 | 91±6* |
| Osmalality ($mO{\cdot}kg^{-1}$) | 275±1 | 274±1 | 276±1 | 274±1 | 274±1 | 275±1 | 274±1 | 274±1 | 274±1 | 276±1 | 275±1 | 275±1 | 275±1 | 275±1 | 275±1 |

***Table 4.3*** Plasma clinical chemistry profiles **(**mean±SEM) in the fasting state and average values of samples collected during the early (0-2 h) and late (2-4 h) postprandial periods following the ingestion of 20 g milk protein (MLK20), or 20 (MYC20), 40 (MYC40), 60 (MYC60) or 80 (MYC80) g mycoprotein in healthy, young men (*n*=12). Each parameter was analysed with a two-way repeated measures ANOVA with Bonferroni post hoc tests applied to locate individual differences: * indicates significant difference to corresponding fasting value, † indicates significant difference to corresponding early postprandial phase value.

## *Discussion*

In the present study we investigated the postprandial circulating amino acid and insulin response to mycoprotein ingestion. We assessed mycoprotein both in comparison with a more typical animal derived protein (milk protein), and in a dose-response manner, in young healthy men. Mycoprotein ingestion resulted in equivalent 4 h postprandial availability of serum insulin, and plasma total, essential, non-essential, and branched chain amino acids when compared with milk protein. Mycoprotein, however, resulted in slower (and lower) peak plasma postprandial amino acid and insulin concentrations, likely explained by delayed digestion and absorption kinetics as a result of the high fibre content. Increasing the ingested dose of mycoprotein resulted in a corresponding increase in plasma amino acid availability and augmented the serum insulin response, with some evidence to suggest these responses begin to plateau at a dose of 60-80 g.

We first compared the response of a 'mass matched' meal-like bolus of mycoprotein vs milk protein (i.e. MLK20 vs MYC20). Milk protein was selected as a reference protein due to its prevalence as a protein rich food (i.e. not a supplement) within most diets, as well as commonly studied as a near gold standard protein source with respect to muscle protein anabolism (35, 36). Despite the practical relevance of this comparison, due to its high fibre content mycoprotein is <50% protein (Table 4.2). Consequently, the greater plasma amino acid and insulin responses following mass matched milk protein ingestion (Figures 4.2-4.4) were not surprising. When comparing milk and mycoprotein on a protein matched basis (i.e. MLK20 vs MYC40) the bioavailability of all amino acids (Figure 4.2-4.3) and insulin (Figure 4.4) was equivalent between conditions. Worthy of note, the similar branched chain amino acid and leucine load between the two proteins became equivalently available over the 4 h postprandial period. Within the context of previous studies examining the muscle anabolic responsiveness to milk protein (35, 36), our data collectively infer that a 40 g bolus of mycoprotein provides sufficient hyperaminoacidaemia (and insulinaemia) to mount a robust muscle protein synthetic response in healthy individuals.

The speed and magnitude of the postprandial plasma amino acid (and leucine in particular) peak has been highlighted as a key parameter dictating the postprandial muscle protein synthetic response (21, 37, 38). The ingestion of milk protein resulted in a more rapid rise in plasma essential amino acids (30 vs 120 min, respectively) and leucine (30 vs 90 min, respectively) compared with 40 g mycoprotein. Additionally, both of these responses were also of a greater magnitude, suggesting the milk may have the capacity to mount a greater muscle protein synthetic response. The differential digestion and absorption kinetics are likely explained by the additional fibre contained within mycoprotein, delaying gastric emptying (39) and/or intestinal amino acid absorption (40). Indeed, given the almost identical leucine and branched chain amino acid loads of mycoprotein, any superior anabolic potential of milk is likely directly attributable to the fibre induced slowing of the digestion and absorption kinetics. Presumably as a direct result of this, when matched for protein content mycoprotein also led to a more modest insulin response compared with milk protein. It has previously been shown that insulin is permissive, rather than stimulatory, in mediating postprandial muscle protein synthesis rates (23, 41). That is to say, any rise from basal insulin levels in the postprandial state seems sufficient to facilitate the muscle protein synthetic response, at which point amino acids alone provide the key nutritional signal dictating the magnitude of response (23, 41, 42). As such, the insulin response to the ingestion of 40 g mycoprotein would be unlikely to limit postprandial muscle protein synthesis rates. It should be noted, however, that a greater insulin response (for instance

with increasing dose or with carbohydrate co-ingestion) may further inhibit postprandial proteolysis and thus contribute to the net anabolic response (23). Despite the faster digestion absorption kinetics and insulinotropic impact of milk protein, it also remains to be seen whether the more sustained availability of amino acids and insulin following mycoprotein ingestion would confer benefits with respect to muscle anabolism, particularly over a more extended postprandial period.

Coupled with comparing mycoprotein with a reference animal derived protein, we also sought to assess the impact of graded doses of mycoprotein. We observed a clear dose-response relationship, with 60>40>20 g regarding total postprandial amino acid and insulin availability and magnitude of responses. Increasing the dose of mycoprotein up to 60 g did not delay the rise to peak in plasma concentrations, and in the case of total amino acids, non-essential amino acids and leucine, appeared to expedite the responses (30, 15 and 30 min, respectively, compared with 45-90 min for other mycoprotein conditions). Despite a more rapid peak than other mycoprotein conditions, 60 g led to sustained hyperaminoacidaemia for 3-4 h post ingestion, which would provide the relevant signalling amino acids as well as substrate to enable a sustained muscle protein synthetic response. The 60 g bolus also contains close to what is considered the optimal leucine content to facilitate a maximal muscle protein synthetic response (i.e. ≥ 2.5 g) (9, 21, 22), and this became available rapidly and to an overall greater degree during the 4 h postprandial period compared with lower doses of mycoprotein or milk protein. The absence of any concurrent rise in plasma urea concentrations (Table 3) suggests that the body protein pool would primarily be making use of this dietary protein bolus for synthetic processes and not simply excessively oxidising protein. Collectively, these data suggest that the ingestion of 60 g mycoprotein would be ample for the optimal stimulation of muscle protein synthesis rates in healthy young men.

Increasing the dose of mycoprotein ingestion from 60 to 80 g did not substantially alter postprandial amino acid availability. Indeed, no sub-group or specific amino acid showed a significantly greater AUC in the 80 compared with 60 g condition, and various amino acid peaks exhibiting delayed kinetics in the 80 g condition. Moreover, plasma urea concentrations rose sharply in both the early and late postprandial periods following the ingestion of 80 g mycoprotein (Table 3), implying this large amount of protein was an ‘overload’ and required a sharp rise in oxidation to restore blood homeostasis. Coupled with this, ordinary blood biochemistry was altered substantially (and differentially compared with other conditions) only in MYC80, also implying this was an excessive dose to support normal metabolism. It should be noted, however, that most postprandial plasma amino acid parameters (with the exception of total essential amino acids) and insulin levels were numerically higher when compared with the 60 g mycoprotein condition, and remained consistently elevated for the entire 4 h postprandial period in the 80 g mycoprotein condition only. As such, it may be possible an 80 g dose of mycoprotein may be beneficial in augmenting the muscle protein synthetic response under situations where 60 g is insufficient; for example with ageing (43) or disuse (44) induced anabolic resistance.

Previous work has shown the ingestion of large quantities of nucleotides results in acute hyperuricaemia above clinically accepted levels (29, 30, 45), which has raised concerns over the chronic consumption of nucleotide containing protein sources (46, 47). Mycoprotein (and other plant-based protein sources) generally contain lower amounts of nucleotides compared with meat/fish derived protein sources (48). In keeping with this, we report that the ingestion of moderate doses of mycoprotein (≤40 g) does not modulate serum uric acid concentrations (Figure 4.5) and, in the case of the 20 g dose, actually had a modest and transient lowering effect. The ingestion of 60 and 80 g resulted in elevated serum uric acid

concentrations and, for the latter, this persisted for the 4 h postprandial period. These levels increased from ~350-370 to ~380-390 $\mu mol \cdot L^{-1}$ and thus did not approach a clinically significant concentration (i.e. >420 $\mu mol \cdot L^{-1}$) (28, 45). It should also be noted that even with serum uric acid levels >420 $\mu mol \cdot L^{-1}$, this is only a recognised predictor of gout and/or metabolic complications when existing chronically and in the fasting state, rather than during daily oscillations in response to nutrition (28). Indeed, while circulating uric acid levels are elevated with gout, gout *per se* does not necessarily lead to elevated serum uric acid (28). As such, it remains to be established if hyperuriceamia is a cause or consequence of the clinical condition(s) that it predicts.

Research has highlighted the key role that dietary protein plays in weight management and the promotion of cardio-metabolic health (49). Central to this role is the thermogenic and satiating properties that dietary protein possesses (49). In line, we report that mycoprotein also mounts a robust thermogenic effect, which we specifically detected at the 60 and 80 g doses (Figure 4.6), and a satiating effect under all conditions, comparable to milk protein and sustained over the entire 4 h postprandial period only in the 80 g condition (Figure 4.7). Collectively, this implies that the selection of mycoprotein within the diet would not compromise these important metabolic effects of dietary protein.

To summarise our present data into practical relevance, based on the observed bioavailability, we speculate that the ingestion of 40 g mycoprotein (i.e. 18 g total protein) would be sufficient to mount a robust muscle protein synthetic response, with the ingestion of 60 g mycoprotein (i.e. 27 g total protein) likely necessary to provide an optimal anabolic response. It is unlikely that consuming in excess of 60 g would confer any further benefits in healthy individuals. We conclude that mycoprotein represents a bioavailable and insulinotropic non-animal derived dietary protein. Consumed in sufficient quantities, mycoprotein would be expected to support skeletal muscle anabolism and reconditioning and therefore have clear utility to muscle health in a variety of populations.

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# Chapter 5

## Dietary protein intake does not modulate daily myofibrillar protein synthesis rates or loss of muscle mass and function during short-term immobilisation in young men: a randomized controlled trial

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## *Abstract*

Background Short-term (<1 week) muscle disuse lowers daily myofibrillar protein synthesis (MyoPS) rates resulting in muscle mass loss. The understanding of how daily dietary protein intake influences such muscle deconditioning requires further investigation.

Objective To assess the influence of graded dietary protein intakes on daily MyoPS rates and the loss of muscle mass during 3 days of disuse.

Methods 33 healthy young men (age, 22±1 y; BMI, 23±1 $kg \cdot m^{-2}$) first consumed the same standardized diet for 5 days providing 1.6 g protein·kg $bm \cdot d^{-1}$. Thereafter, participants underwent a 3 day period of unilateral leg immobilisation during which they were randomized into one of three eucaloric diets containing a relatively high, low, or no protein at all (HIGH: 1.6, LOW: 0.5, NO: 0.1 g·kg $bm \cdot d^{-1}$; *n*=11 per group). One day prior to immobilisation participants ingested 400 mL deuterated water ($D_2O$) with 50 mL doses consumed daily thereafter. Prior to and immediately after immobilisation upper leg bilateral MRI scans and *M. vastus lateralis* biopsies were performed for the measurements of *M. quadriceps* volume and daily MyoPS rates, respectively.

Results *M. quadriceps* volume of the control legs remained unchanged throughout the experiment (*P*>0.05). Immobilisation led to 2.3±0.4, 2.7±0.2 and 2.0±0.4% decreases in *M. quadriceps* volume (*P*<0.05) of the immobilised leg in the HIGH, LOW and NO groups (*P*<0.05), respectively, with no significant differences between groups (*P*>0.05). $D_2O$ ingestion resulted in comparable plasma free $^2$H-alanine enrichments during immobilisation (~2.5 MPE) across groups (*P*>0.05). Daily MyoPS rates during immobilisation were 30±2 (HIGH), 26±3 (LOW) and 27±2 (NO) % lower in the immobilised compared with control leg, with no significant differences between groups (*P*>0.05).

Conclusions Three days of muscle disuse induces considerable declines in muscle mass and daily MyoPS rates. However, daily protein intake does not modulate any of these muscle deconditioning responses.

## *Introduction*

Recovery from illness or injury often requires a period of muscle disuse, which typically occurs in the form of bed rest or limb immobilisation. Recent research has focussed on short term periods of muscle disuse (≤ one week) which are common in clinical settings. We and others have shown that merely 2-5 days of disuse already results in substantial loss of muscle mass (3, 4, 5), with associated declines in strength (4, 5). As a result, there is an eagerness of researchers to develop effective (nutritional) countermeasures (e.g. 6, 3, 7).

Skeletal muscle mass loss must ultimately be underpinned by a chronic imbalance between muscle protein synthesis (MPS) and breakdown (MPB) rates. We have previously shown that postabsorptive and postprandial MPS rates decline within a few days of disuse (11). This translates to chronically lower free-living daily myofibrillar protein synthesis (MyoPS) rates during disuse, an effect that manifests within just two days and can explain a large part of muscle atrophy (4). Dietary protein ingestion stimulates MPS rates and inhibits MPB rates which, under normal conditions, allows for postprandial net protein accretion within muscle tissue (46). As a consequence it has been speculated that increasing dietary protein consumption during a period of disuse may alleviate the loss of muscle mass (10). However, we recently showed substantial declines in daily MyoPS rates and muscle disuse atrophy despite participants reporting relatively high habitual dietary protein intakes (1.6 g·kg bm·d$^{-1}$) (11). Though this would theoretically have provided sufficient dietary protein to stimulate MyoPS rates throughout the day (13) and limit muscle atrophy (14, 43), observed rates of muscle loss were in line with the literature (16).

Studies where essential/branched chain amino acid (EAA/BCAA) or protein supplementation has been applied during more prolonged disuse report inconsistent findings concerning loss of muscle mass and function. For example, high dose EAA/BCAA supplementation during 6-28 days of bed-rest or immobilisation has been reported to attenuate losses of muscle mass, strength and/or whole body nitrogen (17, 18, 19). However, studies where dietary protein supplementation has been applied during 5-60 days of immobilisation or bed-rest have typically shown no effect on losses of muscle mass or function (20, 29). For the development of effective nutritional countermeasures, it is important to develop a clear picture of how daily dietary protein intake *per se* (rather than supplementation) influences muscle protein metabolism and mass during disuse. To date, no studies have manipulated total habitual dietary protein consumption under controlled dietary conditions during (short-term) disuse to establish the link between dietary protein intake, daily MyoPS rates and muscle atrophy.

In the present work we conducted a dose-response study comparing how high (1.6 g·kg bm·d$^{-1}$), low (0.5 g·kg bm·d$^{-1}$) and negligible (0.15 g·kg bm·d$^{-1}$) daily dietary protein intakes influence daily MyoPS rates determined using the deuterated water approach, and muscle mass loss determined via MRI during a three day period of unilateral leg immobilisation in healthy males. We hypothesised that declining dietary protein intakes would lead to a greater decline in daily MyoPS rates and a consequent increase in the rate of loss of muscle mass and function.

## *Materials and methods*

### *Subjects*

Thirty-three healthy young men (age; 21±1 y, BMI; 23±1 $kg \cdot m^{-2}$) were included in the present study (see Table 1 for participants' characteristics) and participated in this parallel group randomized controlled trial. The trial was conducted between November 2018 and December 2019 within the Nutritional Physiology Unit at the Department of Sport and Health Sciences at the University of Exeter, Exeter, UK (for the consort flow chart please see **Figure 5.1**). Participants were allocated sequential numbers at the time of screening which were then used as the only identifiable characteristic for all documents containing participant information, and were randomised into groups using an online randomiser (http://www.randomization.com/), recruitment and testing was ended once the trial was fully recruited according to the a priori calculation. Participants attended the laboratory for a routine medical screening and completed a general medical questionnaire to assess their eligibility for participation, and to ensure no adverse health conditions were present. Exclusion criteria included; a (family) history of deep vein thrombosis/cardiovascular disease, metabolic disorders (e.g. type 2 diabetes), musculoskeletal/orthopedic disorders, a body mass index of above 28.5 or below 18.5 $kg \cdot m^{-2}$, participation in a structured resistance training program within 6 months prior to the study, any musculoskeletal injury of the legs within 12 months before the study, use of anticoagulants, any contraindications to MRI scanning (e.g. metallic implants), and consumption of any nutritional supplement prior to and during the study. Participants who consumed a habitual dietary protein intake of above 1.8 or below 0.6 $g \cdot kg \cdot d^{-1}$ were also excluded from the study. During the screening participants' height, body mass and blood pressure were measured, body composition was also assessed by air displacement plethysmography (BODPOD; Life Measurement, Inc. CA, USA). The participants also completed the International Physical Activity Questionnaire (IPAQ). This was used to estimate the participants' physical activity level by reporting time spent sedentary, or undertaking light, moderate and vigorous activities, and multiplying this by the metabolic equivalents for these activities (METs) (21). Participants' habitual diets were recorded for 3 days (two week days and one weekend day) prior to the dietary controlled period by a self-reported written diet diary following detailed instructions and advice from a member of the research team. Furthermore, participants visited the laboratory shortly after the screening where they were familiarized to the exercise tests (described below). All participants were informed of the nature and possible risks of the experimental procedures before providing written informed consent. The study was approved by The Sport and Health Science Ethics committee of the University of Exeter (170712/B/01), in accordance with the guidelines set out in the Declaration of Helsinki, and registered as a clinical trial with clinicaltrials.gov (NCT03797781).

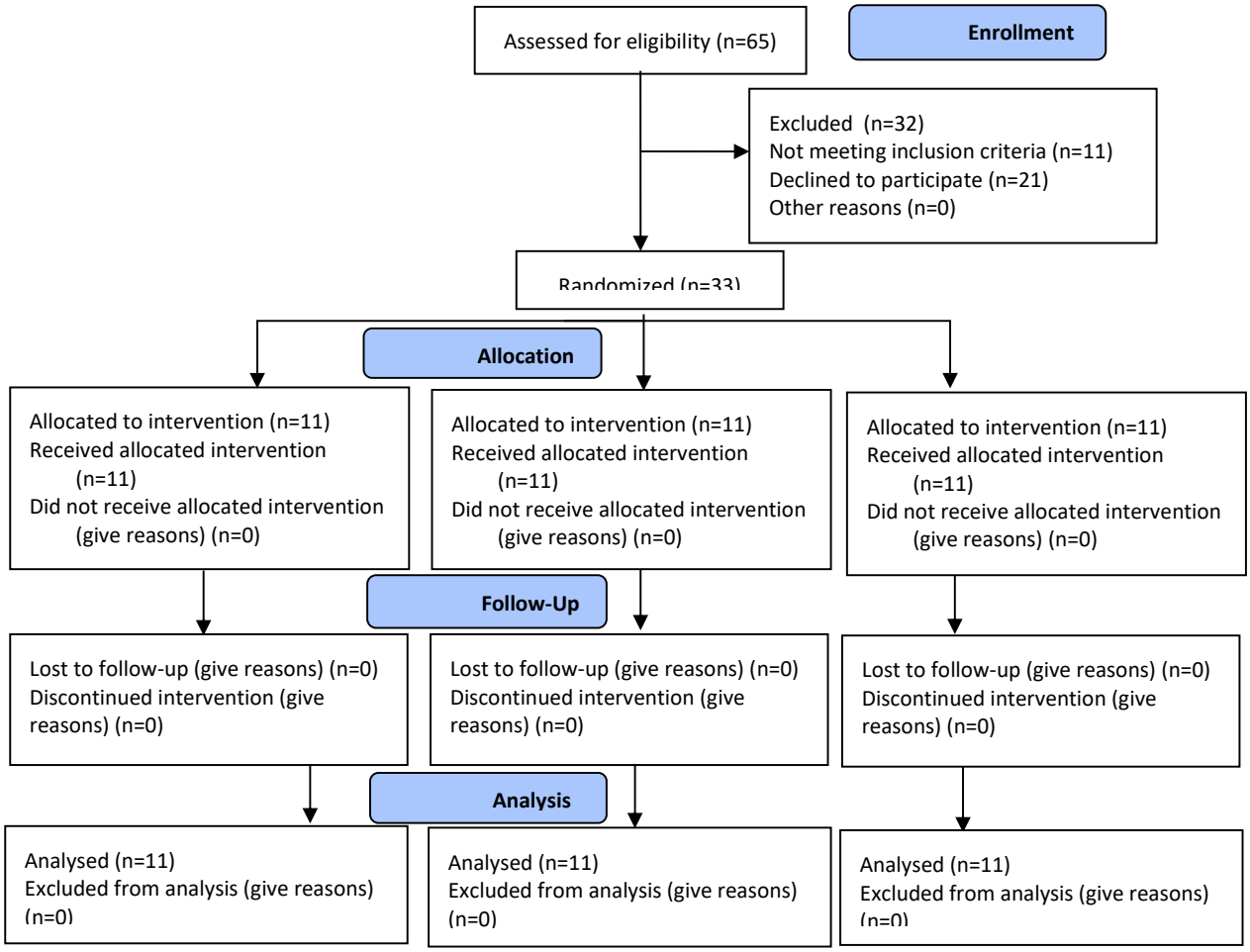


***Figure 5.1*** Consort flow diagram.

### *Experimental protocol*

A graphical representation of the study design is shown in **Figure 5.2**. Following acceptance onto the study participants attended the laboratory in the fasted state for 5 experimental visits across 13 days. This included an 8 day fully controlled dietary intervention period with the final 3 days of the dietary controlled period involving unilateral leg immobilisation using a leg brace and ambulation with crutches. Pre-immobilisation (visit 1) comprehensive unilateral muscle strength and maximal aerobic capacity ($\dot{V}O_2$peak) testing were conducted (protocols described below). Thereafter, five days prior to immobilisation (visit 2) all participants attended the laboratory to collect their first 5 days of food to commence a fully controlled, pre-immobilisation standardized diet with protein intake fixed at 1.6 g·kg bm·d$^{-1}$. To measure daily myofibrillar protein synthesis (MyoPS) rates throughout the immobilisation period participants underwent a deuterium oxide dosing protocol (described below) beginning on visit 3 (i.e. one day prior to commencing immobilisation). This protocol was designed to achieve and maintain 0.8-1.0% body water deuterium enrichment during the measurement periods in line with our previous work (22). The following day (visit 4) participants arrived at the laboratory at ~0800 h and a single *M. vastus lateralis* muscle biopsy was obtained from the (to be) immobilised leg. Following this, participants

were transported to an MRI scanner by wheelchair, avoiding any weight bearing activity and underwent a pre-immobilisation MRI scan of both thigh muscles. Thereafter, participants were randomised, in a single (participant) blind manner into one of three isoenergetic dietary controlled experimental groups (*n*=11 per group) where protein intakes differed between groups; 1.6 (HIGH), 0.5 (LOW) or 0.15 (NO) g·kg bm·d$^{-1}$. Participants were fitted with a leg brace to induce immobilisation of one leg and were given crutches for ambulation, and provided with 3 days of food supply in line with their allotted diet, and this signified the commencement of the 3 day immobilisation period. Following the immobilisation period participants returned to the laboratory for the final visit (visit 5) where *M. vastus lateralis* muscle biopsies were collected from both the immobilised and control legs (the brace was only removed for the biopsy, MRI and exercise testing procedures). Muscle biopsies were all obtained under local anaesthesia, using the percutaneous Bergstrom needle biopsy technique (23) from the *M. vastus lateralis* approx. 15 cm above the patella and approx. ~2 cm below the fascia. Immediately following muscle biopsies, the tissue was quickly assessed and any blood or non-muscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen within one min and stored at -80°C until further analysis. Thereafter, participants were transported via wheel chair to undergo further MRI scans of the thighs of both legs, and finally further unilateral 1RM strength and $\dot{V}O_2$peak testing of both legs separately was performed. This signified the end of the experiment when weight bearing activity of both legs was then permitted.

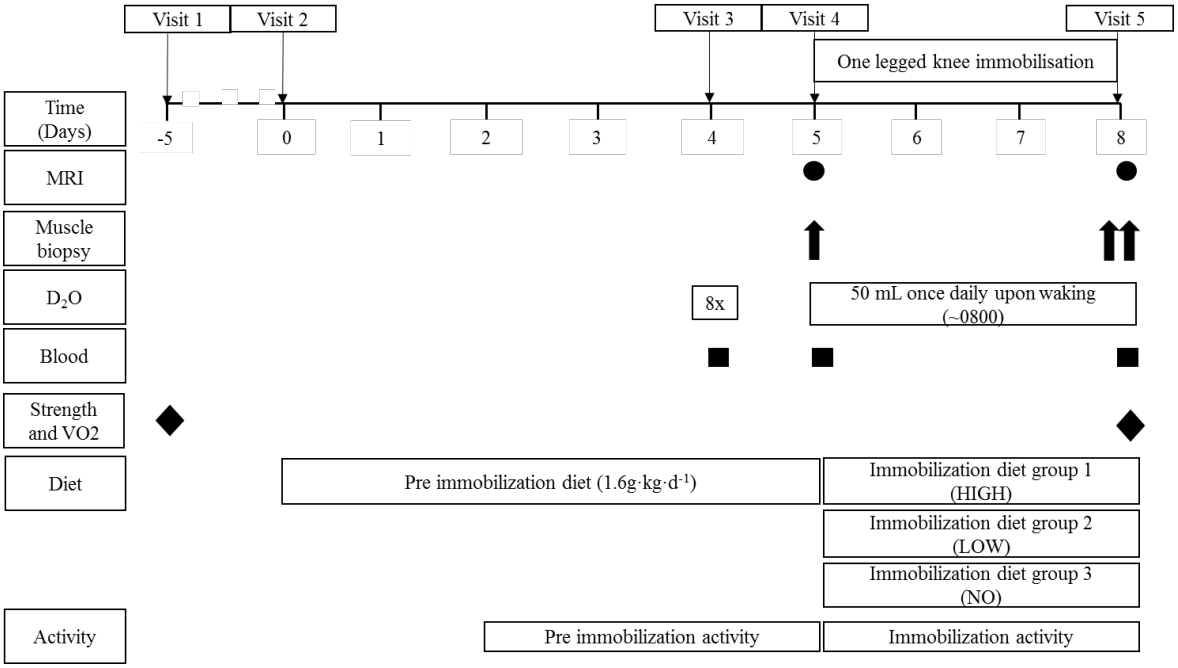


***Figure 5.2*** Study protocol. Thirty-three healthy young males underwent 3 days of unilateral leg immobilization via knee brace. MRI, Magnetic resonance imaging. $D_2O$, deuterated water ingestion. Activity, physical activity measured continuously by GENEactiv wrist watch accelerometry. Diet, all participants underwent 5 days of fully controlled pre-immobilization diet relatively high in dietary protein (1.6 g·kg bm·d$^{-1}$) before being randomized into three groups of varying protein intake HIGH (1.6 g·kg bm·d$^{-1}$), LOW (0.5 g·kg bm·d$^{-1}$) and NO (0.15 g·kg bm·d$^{-1}$) for the 3 day immobilization period. Blood, venous blood sample collection. Arrows represent *M. vastus lateralis* muscle biopsies, (i.e. taken from the immobilized leg only at pre-immobilization and both control and immobilized legs post-immobilization). Strength, unilateral maximal isometric, concentric and eccentric contractions of both the *M. quadriceps* and *M. hamstrings* measured by isokinetic dynamometry. $\dot{V}O_2$peak, single leg peak oxygen uptake was measured using a ramp cycling test to exhaustion. Control and immobilized legs completed all strength and aerobic capacity tests separately.

### *Physical activity and habitual dietary intake*

For 3 days prior to immobilisation (days 2-5 of the pre-immobilisation diet) and for the entirety of the immobilisation period participants' physical activity was measured using an accelerometer (GENEactiv, Activinsights, Cambridgeshire, UK) worn on the non-dominant wrist. Participants were instructed to wear the accelerometer continuously with data being collected at a 60 Hz sampling frequency. Participants' were instructed to refrain from vigorous physical activity during immobilisation but to attempt to maintain their habitual activity levels despite using crutches for ambulation (to avoid whole body sedentariness during immobilisation). Physical activity data from the GENEActiv accelerometers were converted into 60 s epochs and used to estimate time spent performing light, moderate and vigorous physical activity using standard cut-off points (11). Participants were asked to refrain from alcohol intake for one week before and throughout the 8 day dietary control period. Dietary analyses for the calculation of habitual energy and macronutrient intakes were completed using specialized nutrition software (Nutritics Professional Nutritional Analysis Software; Swords, Co. Dublin).

### *Magnetic resonance imaging for determination of M. quadriceps volume*

Prior to and post immobilisation muscle volume of the *M. quadriceps* of both legs was determined via MRI. We described the MRI methodology for the determination of *M. quadriceps* volume in detail previously (11). In brief, a 1.5 tesla (T) MRI scanner was used to make axial plane images over the full length of the femur. A T1-weighted 3D turbo spin echo sequence was used (field of view 500 x 500 mm, reconstructed matrix 512 x 512 mm, echo time 15 ms, repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the supine position, and a 4-element sense body radiofrequency coil was wrapped around both thighs. On average ~45 images were acquired along the length of the femur, with the bottom 25% (from the lateral femoral condyle working proximally) and top 25% (from the greater trochanter working distally) excluded (7, 18). All other images in the axial plane in the middle 50% area of the *M. quadriceps* were analysed via manual segmentation using Philips on-line MRI software. The same experimenter (SPK) performed all manual segmentation of the images. We (11) and others (24) have shown that this region of the *M. quadriceps* muscle undergoes rapid atrophy during disuse and accounts for the vast majority of total *M. quadriceps* volume loss. *M. quadriceps* volume was calculated using a previously published method (25) where the total CSA for all images was calculated and multiplied by the slice gap plus the distance between slices (linear interpolation) (in this case a total 2 cm, comprised of a 5 mm slice thickness and a 15 mm slice gap), summarized by the following equation:

$$muscle\ volume = \sum_{aCSA} \cdot\ (slice\ thickness + slice\ gap)$$

### *Deuterated water protocol*

The deuterated water dosing protocol was based on our previous work (4, 22). Day 1 of the experimental protocol acted as a $D_2O$ loading day where participants consumed 400 mL 70% $D_2O$ separated over the

day as 8 x 50 mL boluses (CK Isotopes Ltd, Leicestershire, UK). Upon arrival at the laboratory (0730 h) background blood and saliva samples were collected before the first bolus of $D_2O$ was ingested. The first dose of $D_2O$ was consumed at ~0800 h with the remaining doses being consumed every 1.5 h thereafter. Participants stayed at the university until 4 out of the 8 loading day $D_2O$ doses had been consumed, with the remaining $D_2O$ doses being consumed at home under instruction of timings (i.e. leaving 1.5 h between each). Every day following the loading day participants consumed a maintenance dose of $D_2O$ (50 mL) upon waking (~0800 h). Blood samples were collected during the test days (i.e. day 5 [pre], 7 [after 2 days of immobilisation] and 12 [post]). Venous blood samples were collected from the antecubital vein via venepuncture and collected into EDTA-containing vacutainers which were centrifuged at 2,500 *g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C until further analysis took place. To ensure uniformity and compliance with the $D_2O$ protocol participants were provided with a log to record the times they consumed the $D_2O$ and were provided with enough doses to last until their next study visit, at which point containers were returned, counted and subsequent doses were provided.

### *Muscle strength and single leg cycling $\dot{V}O_2$peak testing*

Unilateral knee extension and flexion contractions were performed using isokinetic dynamometry (Biodex System 3, Shirley, NY, USA). Isometric, isokinetic concentric and isokinetic eccentric strength for both knee extension (i.e. *M. quadriceps* strength) and flexion (i.e. *M. hamstrings* strength) were all determined in the stated order. After warm-up repetitions at 50, 75 and 85 % of self-determined one repetition maximum (1-RM) participants performed 3 x 3 s maximal isometric repetitions of knee extension followed by knee flexion. Knee angle was fixed at 60° of flexion (0° being full extension) and repetitions were separated by a 2 minute rest and the 2 exercise modalities by a 5 minute rest. Subsequently participants performed 5 repetitions of maximal knee extension isokinetic concentric exercises, and this was repeated for knee extension isokinetic eccentric exercises. Repetitions were sequential with a 2 minute break between the two contraction types, contraction speed was $60°\cdot s^{-1}$ over the central 80° range of motion (verified by goniometry) out of each participant's full range of motion (e.g. from full extension to full flexion). Then following a 5 minute break the same isokinetic concentric and eccentric contractions were repeated for knee flexion.

Unilateral leg peak maximal oxygen uptake ($\dot{V}O_2$peak) was assessed using a previously validated single leg ramp exercise test to exhaustion (28). In brief, a custom designed counterweight pedal (11.4 kg) was fitted to the crank of an electronically braked cycle ergometer (Lode Corival, Groningen, The Netherlands). Participants cycled with one leg, with the non-exercising leg resting on a stationary stool. The counterweight assisted with the upstroke of the cycling phase and eliminated the need to pull up on the pedal. Whole body expired gases were collected via a facemask and oxygen consumption was measured using an online gas analyser (Cortex Metalyzer 3B gas analyser, Cortex, Germany). For all exercises the (to be) immobilised leg was always performed first followed by the control leg.

### *Dietary control*

Nutritional information for the pre-immobilisation (5 days immediately before immobilisation) and immobilisation (3 day immobilisation period) diets is provided in Table 2. Basal metabolic rate (BMR) was

estimated using the Henry equations based on age, gender, and weight (28). Individual energy requirements were then calculated by multiplying the participants' BMR and PAL (calculated from the IPAQ as described above). Thereafter, an individual 8 day meal plan was designed for each participant with all food prepared, weighed and packaged in-house in the Nutritional Physiology Unit's research kitchen facility. Throughout the study all ingredients and instructions/information for preparation were provided to the participants which they prepared at home, and a log was provided to record the times of consumption of each meal. For the first 5 days of the 8 day dietary control (i.e. pre-immobilisation period) all participants consumed a diet containing 1.6 g·kg bm·d$^{-1}$, with ~30% of their energy being provided by fat and the remainder from carbohydrates (~50–55%; variation due to different energy requirements in parallel with clamped protein intake). Alcohol consumption and any other food or drinks (except water, but including tea and coffee) other than that provided were prohibited during the study. Dietary protein intake was equally distributed across four meals (~27±1, 28±1, 28±1 and 28±1 g at breakfast, lunch, dinner and a pre-sleep whey protein beverage) and participants were instructed to consume their meals approximately 4-5 h apart, throughout the day to optimise 24 h muscle protein synthesis rates (30, 31). At each experimental visit participants' body mass was measured (seca 703 column scale, seca GmbH & Co. KG, Hamburg, Germany) wearing light clothing and the researchers discussed with the participants any questions or issues that may have arisen with the diet, and in the event of any substantial weight change (>0.5 kg, with the same upward or downward trend on two consecutive visits) energy content of the next two days was adjusted (via the reduction/increase in carbohydrate). Following the 5 day pre immobilisation period, volunteers commenced with the 3 day immobilisation period during which volunteers were randomised into either the HIGH (1.6 g·kg bm·d$^{-1}$; *n*=11) LOW (0.5 g·kg bm·d$^{-1}$; *n*=11) or NO (0.15 g·kg bm·d$^{-1}$; *n*=11) protein groups. The HIGH group therefore maintained the pre-immobilisation diet precisely, whereas the LOW group had ~68% (~77 g) of their protein (and ~7% [~7 g] of fat) replaced by ~25% (~85 g) more carbohydrate, and the NO protein group had ~91% (~106 g) of their protein (and ~13% [~12 g] of fat) replaced by ~45% (~164 g) of carbohydrate. The amounts of dietary protein were selected to represent a wide spectrum to allow a true dose-response to be investigated. 1.6 g·kg bm·d$^{-1}$ was selected as 'high' based on being double the UK RDA (31), being in line with current recommendations for restricting muscle loss during disuse (14, 33) and also consistent with habitual protein intakes reported in our previous work investigating daily MPS rates and muscle disuse atrophy in young, healthy men (73) The LOW group was selected as 0.5 g·kg bm ·d$^{-1}$ since this is considerably below (38%) the current RDA and also representative of dietary protein intakes that might be expected in patients undergoing a period of disuse in a hospital setting (33). The NO group was designed to remove dietary protein as a stimulus for MPS rates as far as possible while being practically achievable during a diet maintaining energy balance (i.e. 0.15 g·kg bm·d$^{-1}$). All food items in the 1.6 and 0.5 g·kg bm·d$^{-1}$ protein groups were purchased from commercial retailers. To reduce protein intake to 0.15 g·kg bm·d$^{-1}$ certain food products given to this group were purchased from a company that produces specialized low/zero protein food (Promin Metabolics, Stockport, UK). Example meals on each diet consisted of the following: breakfast; scrambled eggs and beans on toast (HIGH), jam on toast (LOW), low protein oatmeal (NO); lunches; a chicken sandwich with snacks (e.g. biscuits, fruit) (HIGH), ham sandwich with fruit (LOW) and vegetable soup with low protein bread rolls (NO); dinner; chicken tikka masala curry with rice and vegetables (onion, green beans, tomatoes) (HIGH), vegetarian stir fry (vegetables, stir fry sauce and rice noodles) (LOW), vegetarian pizza (low protein pizza base, vegetables [sweetcorn, mushrooms, tomatoes, onion, pepper], tomato puree)

(NO).

### *Immobilisation protocol*

We have previously used the knee brace approach to achieve unilateral leg immobilisation and consequent declines in daily MPS rates and muscle mass over 2 and 7 days (11). Briefly, the brace (X-ACT Donjoy brace, DJO global, Vista, CA, USA) was applied and the participant can then ambulate on crutches (after receiving instructions) throughout the immobilisation period. The immobilised leg was randomized and counterbalanced for leg dominance with the non-immobilised leg acting as a within-subject control (for both MPS rates and muscle mass measurements). Using the hinge of the brace the knee was fixed at an angle of 40° flexion (full knee extension = 0°) to ensure no weight bearing occurred. Participants were instructed that all ground contact, and muscle contraction (except for ankle rotation exercises twice per day to activate the venous muscle pump), in the immobilised leg were forbidden. Adhesive tape with the experimenter's signature inscribed was placed around the straps of the brace. Breaking of the tape would indicate that the brace had been altered and resulted in exclusion from the study (11, 35), though it was not necessary to exclude any participants based on this in the present study. A plastic shower cover was provided to the participants to wear over the brace when showering. Daily contact was maintained with the subject throughout the study to ensure proper compliance.

### *Plasma free [$^2$H]alanine enrichments*

Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 5975C MSD & 7890A GC, Wilmington, USA). First the plasma samples were deproteinized using dry 5-sulfosalicylic acid. Subsequently free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, mesh size 100-200 µm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA). The purified amino acids were converted to their *tert*-butyldimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA before analysis via GC-MS. The plasma free alanine mass isotopomers (M and M+1) were measured using selective ion monitoring at *m/z* 232 and 233. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation.

### *Myofibrillar bound $^2$H alanine enrichments*

Myofibrillar protein-enriched fraction was extracted from ~50 mg of wet weight muscle tissue by hand-homogenization on ice using a pestle in a standard extraction buffer (670 µL 1M Sucrose, 500 µL 1M Tris/HCl, 500 µL 1M KCl, 100 µL 1M EDTA and dd$H_2O$ was added until a total volume of 10 ml was achieved [10 µL/mg]). The samples were centrifuged at 2,500 *g* and 4°C for 5 min and the pellet was then washed with 500 µL of ddH2O and again centrifuged at 2,500 *g* and 4°C for 10 min. The myofibrillar protein was solubilized by adding 1 mL of 0.3 M NaOH and heating for 30 min at 50°C with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 9,500 *g* and 4°C, the supernatant containing the myofibrillar protein was kept and the collagen protein pellet was discarded. The myofibrillar proteins

were precipitated by the addition of 1 mL of 1 M PCA and spun at 700 *g* and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolyzed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated at 120°C. The free amino acids were subsequently dissolved in 25% acetic acid solution and passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA) and eluted with 2 M $NH_4OH$. Following this the eluted amino acids were dried and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (138). The derivatized amino acids were measured using a gas-chromatograph-isotope ratio mass spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60m DB-17MS column (no. 122-4762; Agilent, Wilmington, USA) and a 5 m precolumn. Ion masses 2 and 3 were analyzed to determine the $^2H/^1H$ ratios of muscle protein-bound alanine. A series of known standards was used to assess the linearity of the mass spectrometer and to control for the loss of tracer.

### *Calculations*

Myofibrillar protein fractional synthesis rates (FSR) were calculated based on the incorporation of [$^2$H] alanine into myofibrillar protein and the mean free plasma [$^2$H] alanine enrichment throughout the immobilisation period as a precursor. FSR was calculated using the standard precursor-product method expressed as daily rates as follows:

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

where $E_{m1}$ and $E_{m2}$ are the myofibrillar muscle protein-bound enrichments on day 0 and 3. $E_{precursor}$ represents mean plasma free [$^2$H] alanine enrichment (mean enrichment between day 0-3). *t* represents the time between biopsies (day 0-3). FSRs were calculated in both legs separately using the biopsy collected from the immobilised leg as baseline for both legs.

### *Statistics*

All data are presented as means±SEM and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). The study sample size was based on a previously reported one week muscle disuse dietary controlled intervention in healthy young males (32). A sample size of 33 (11 per group) was anticipated to detect a 0.3% difference between HIGH, LOW and NO protein intakes groups on *M. quadriceps* muscle mass (SD=0.3, 80% power, α=0.05). A two-way repeated measures ANOVA with leg (control vs immobilised; treated as the repeated factor) and group (HIGH, LOW and NO) as factors were used to compare MyoPS rates. Three-way repeated measures ANOVAs (time [pre vs post], leg [control vs immobilised] and group [HIGH, LOW and NO] as factors, with time and leg considered as repeated factors) were used to compare *M. quadriceps* volume and isometric, concentric and eccentric leg strength and unilateral leg $\dot{V}O_2$peak data. A two-way repeated measures ANOVA with group (HIGH, LOW and NO) and time (habitual vs pre vs during immobilisation) as factors was used to

assess for differences in dietary intake parameters. Two-way repeated measures (time) ANOVAs were used to assess how physical activity and plasma $^{2}$H-alanine enrichments differed between groups from pre to during immobilisation. For all ANOVAs, data were checked and no ANOVA model assumptions were violated, when a significant interaction was found Bonferroni post-hoc tests were applied to locate individual differences. Statistical significance was set at $P<0.05$.

## *Results*

### *Physical activity and diet*

Table 1 displays participants' characteristics and habitual dietary intakes, and Table 2 depicts dietary intake parameters and physical activity during the dietary controlled period for 5 days preceding (pre) and during the 3 day immobilisation period. There were no significant differences in habitual energy or macronutrient intake or habitual physical activity levels between the groups ($P$>0.05). Light, vigorous and total physical activity significantly reduced from pre to during immobilisation (time effects; all $P$<0.05) and to a similar extent (time x group interaction effects; all $P$>0.05) across groups. Moderate physical activity significantly declined in the HIGH and NO protein group only ($P$<0.05). As expected, all dietary parameters were identical across groups during the pre-immobilisation dietary controlled period. By design, energy intake was equivalent across groups during immobilisation, but dietary protein intake differed (group, time and group x time effects; all $P$<0.001) such that HIGH was greater than LOW and NO (both; $P$<0.001) and LOW was greater that NO ($P$<0.001). This resulted in differences in CHO intake across groups (group, time and group x time effects; all $P$<0.001) where HIGH was lower than NO ($P$<0.001) and LOW ($P$<0.05), with the NO group also having higher CHO intake than LOW ($P$<0.001). There were no significant differences between groups for fat intake during immobilisation (group and the group x time effects; both $P$>0.05, time effect; $P$<0.001). (Fat intake [En%] was significantly reduced from pre to during immobilisation in the NO group [$P$<0.05]).

***Table 2.1*** *Dietary intake and physical activity levels during dietary controlled periods pre and during a 3 day period of unilateral knee immobilisation.*

| | HIGH | | LOW | | NO | |
|---|---|---|---|---|---|---|
| | Pre | During | Pre | During | Pre | During |
| Energy intake (MJ·d$^{-1}$) (Kcal·d$^{-1}$) | 11.6±0.3 (2777±64) | 11.7±0.3 (2788±62) | 11.5±0.4 (2741±104) | 11.5±0.5 (2747±114) | 11.7±0.3 (2791±74) | 11.8±0.3 (2801±80) |
| Protein intake (g·d$^{-1}$) | 116±4 | 116±4 | 113±7 | 36±2*$^{a}$ | 116±4 | 10±0.4*$^{a, b}$ |
| Protein intake ( g·kg bm·d$^{-1}$) | 1.6±0.1 | 1.6±0.1 | 1.6±0.1 | 0.51±0.1*$^{a}$ | 1.6±0.1 | 0.14±0.1*$^{a, b}$ |
| Protein intake (En%) | 18±1 | 18±1 | 16±0.4 | 5±0.1*$^{a}$ | 17±0.2 | 1.4±0.03*$^{a, b}$ |
| Protein per meal (g) | 28±1 | 28±1 | 27±2 | 10±1*$^{a}$ | 28±1 | 3±0.1*$^{a, b}$ |
| CHO intake (g·d$^{-1}$) | 362±12 | 368±13 | 341±11 | 426±19*$^{a}$ | 361±11 | 525±13*$^{a, b}$ |
| Carbohydrate intake (En%) | 52±1 | 53±1 | 50±1 | 62±1*$^{a}$ | 52±1 | 71±2*$^{a, b}$ |
| Fat intake (g·d$^{-1}$) | 88±4 | 85±4 | 98±4 | 91±5 | 90±4 | 78±4 |
| Fat intake (En%) | 29±1 | 28±2 | 32±1 | 30±1 | 29±1 | 23±1*$^{a, b}$ |
| Light physical activity (h·d$^{-1}$) | 1.0±0.1 | 0.7±0.1* | 1.2±0.1 | 0.8±0.1* | 1.3±0.1 | 0.8±0.1* |
| Moderate physical activity (h·d$^{-1}$) | 2.1±0.2 | 1.6±0.2* | 2.2±0.3 | 1.7±0.2 | 3.0±0.5 | 1.5±0.2* |
| Vigorous physical activity (h·d$^{-1}$) | 0.3±0.1 | 0.1±0.1* | 0.3±0.1 | 0.1±0.02* | 0.3±0.1 | 0.1±0.1* |
| Total physical activity (h·d$^{-1}$) | 3.4±0.3 | 2.3±0.2* | 3.7±0.4 | 2.6±0.2* | 4.6±0.6 | 2.4±0.4* |

*Values represent means±SEM, n=11 per group. Data were analyzed by using a two way repeated measures ANOVA (with time and group as factors).* $^{*}$*=significant difference from pre immobilisation value, P<0.001,* $^{a}$ *= significant difference from HIGH group during immobilisation P<0.05,* $^{b}$ *= significant difference from LOW group during immobilisation P<0.001. En% = % of total energy intake. Pre denotes the 5 day period of controlled diet before immobilisation. During denotes the 3 day immobilisation period.*

*M. quadriceps muscle volume*

*M. quadriceps* muscle volumes calculated from MRI are displayed in **Figure 5.3**. There were no significant differences in *M. quadriceps* muscle volume between legs or between groups pre immobilisation (group x leg interaction effect, *P*>0.05) (control leg; HIGH = 1412±85 $cm^3$, LOW = 1448±106 $cm^3$, NO = 1495±71 $cm^3$, immobilised leg; HIGH = 1430±82 $cm^3$, LOW = 1439±105 $cm^3$, NO = 1499±83 $cm^3$). The *M. quadriceps* muscle volume of the control leg was unaffected by immobilisation (*P*>0.05). *M. quadriceps* volume of the immobilised leg reduced significantly during immobilisation (leg x time; *P*<0.001) by 2.3±0.4, 2.7±0.2 and 2.0±0.4 % in the HIGH (pre = 1430±82 to post immobilisation = 1396±81 $cm^3$, *P*<0.001), LOW (pre = 1439±105 to post immobilisation = 1400±101 $cm^3$, *P*<0.001) and NO (pre = 1499±83 to post immobilisation = 1469±81 $cm^3$, *P*<0.05) groups, respectively (Figure 5.3B); however, these changes did not differ across groups (Figure 5.3B) (group x time and group x leg x time interactions; *P*>0.05).

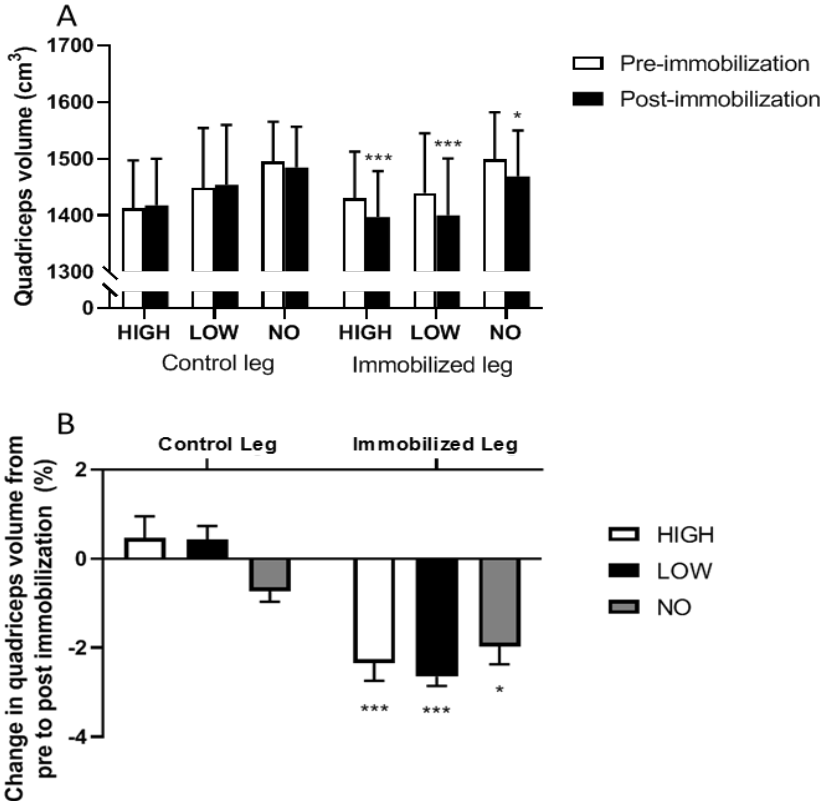


***Figure 5.3*** *M. quadriceps* muscle volume of the control and immobilized legs pre and post 3 days of unilateral leg immobilization where participants consumed a fully controlled energy balanced diet containing a HIGH (*n*=11; 1.6 g·kg bm·$d^{-1}$), LOW (*n*=11; 0.5 g·kg bm·$d^{-1}$) or NO (*n*=11; 0.15 g·kg bm·$d^{-1}$) dietary protein content (A). A three way ANOVA (leg x time x group) was conducted to assess for statistical differences. Main effect of leg *P*>0.05, group *P*>0.05 and time *P*<0.001. Leg x group interaction *P*>0.05, leg x time interaction *P*<0.001, group x time interaction *P*>0.05, leg x group x time interaction *P*>0.05. Bonferroni post tests were conducted to locate individual differences; *** and * denotes a significant difference from pre immobilization within the same group at *P*<0.001 and *P*<0.05, respectively. B expresses the relative difference in *M. quadriceps* volume between the control and immobilized legs for HIGH, LOW and NO groups. Data were analyzed by a two way ANOVA with leg (control vs immobilized) and group (HIGH vs LOW vs NO) as factors. Main effect of leg *P*<0.001, group *P*>0.05, leg x group interaction was *P*<0.05. Bonferroni post tests were conducted to locate individual differences; * and *** denote a significant difference from pre immobilization within the same group at *P*<0.05 and *P*<0.001, respectively. Data presented are means±SEM.

*Muscle strength and single leg cycling $\dot{V}O_2$peak*

*M. quadriceps* and *M. hamstrings* muscle strength data are displayed in **Figures 5.4 and 5.5**, respectively. There were no significant differences in any contraction type for the *M. quadriceps* or *M. hamstrings* muscle strength between legs or between groups pre immobilisation (group x leg interaction effect, *P*>0.05). No parameter of strength was altered throughout the experiment in the control leg for either the *M. quadriceps* or *M. hamstrings* muscles (*P*>0.05) and there were no significant differences between groups (*P*>0.05). Immobilisation decreased *M. quadriceps* maximal isometric (HIGH = by 24±8%, LOW = by 24±6%, NO = by 26±5%; time x leg effect; *P*<0.001), concentric (HIGH = by 23±8%, LOW = by 22±6%, NO = by 25±4%; time x leg effect; *P*<0.001) and eccentric (HIGH = by 16±7 %, LOW = by 18±8 %, NO = by 23±6 %; time x leg effect; *P*<0.001) strength to a similar extent across groups (group x leg interaction; *P*>0.05 for all 3 contraction types). In contrast, immobilisation only reduced *M. hamstrings* maximal concentric strength (HIGH = by 7±2 %, LOW = by 8±6 %, NO = by 5±5 %) (time x leg; *P*<0.05), again with no significant differences between groups (group x leg interaction; *P*>0.05), whereas *M. hamstrings* maximal isometric and eccentric strength were unaffected by immobilisation (all main and interaction effects; *P*>0.05, except hamstring isometric time effect; *P*<0.01). Unilateral $\dot{V}O_2$peak (**Figure 5.6**) was not affected by immobilisation (*P*>0.05), group (*P*>0.05) in either leg (interactions all; *P*>0.05).

## *Plasma free $^2H$ alanine precursor pool enrichment*

Plasma free [$^2$H] alanine enrichments (**Figure 5.7**) were 2.4±0.1, 2.4±0.1 and 2.3±0.1 mole percent excess (MPE) at the start of immobilisation in the HIGH, LOW and NO groups, respectively, and tended (time effect; *P*=0.06) to increase post immobilisation. However, there were no significant differences in plasma free [$^2$H] alanine enrichments between groups (*P*>0.05).

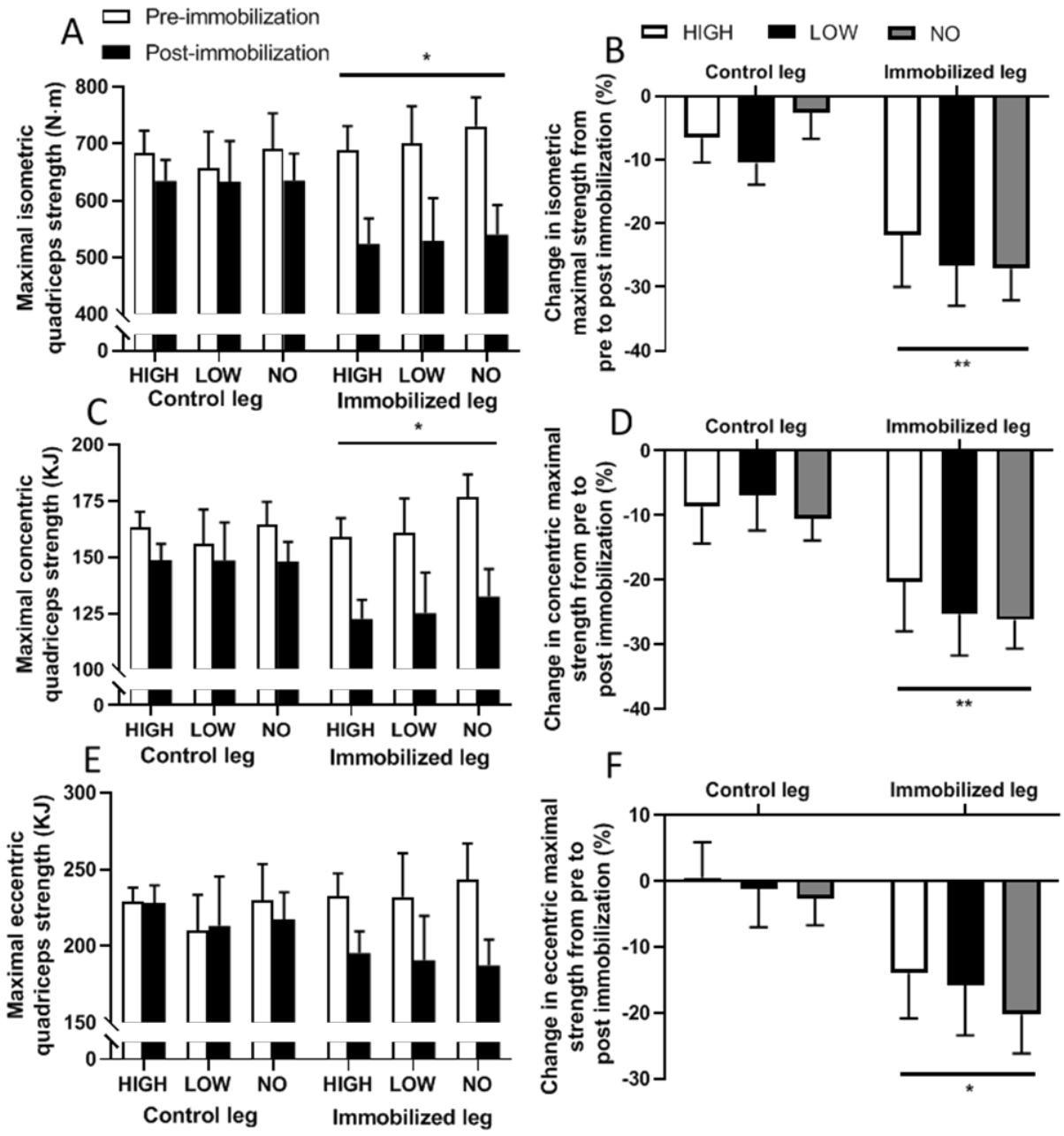


***Figure 5.4*** *M. quadriceps* muscle strength assessed by isokinetic dynamometry. Maximal isometric (3-repetition average) (A), isokinetic concentric (C) and isokinetic eccentric (E) (both 5-repetition average) *M. quadriceps* strength for the control and immobilized legs pre- and post- immobilization for the three groups HIGH ($n$=11; 1.6 g·kg bm·d$^{-1}$), LOW ($n$=11; 0.5 g·kg bm·d$^{-1}$) and NO ($n$=11; 0.15 g·kg bm·d$^{-1}$). Data were analyzed by three way ANOVA with leg x group x time as factors. A, C, and E all showed no main effect of group ($P$>0.05), but for isometric and concentric *M. quadriceps* strength a main effect of leg was detected $P$<0.05, but for eccentric the main effect of leg was $P$>0.05. For all three exercises (graphs A, C and E) the main effect of time was $P$<0.001. The group x leg and group x time interactions were $P$>0.05 for 3 graphs (A, C and E), the leg x time interactions were $P$<0.001 for all 3 graphs (A, C and E). The group x leg x time interactions were $P$>0.05 for all three graphs. Graphs B, D and F show the relative change in maximal isometric, concentric and eccentric *M. quadriceps* strength respectively, for the control and immobilized leg and for the HIGH, LOW and NO groups. Data were analyzed by two way ANOVA with leg and group as factors. The main effect of group was $P$>0.05 for all 3 graphs (B, D, F), the main effect of leg was $P$<0.05 for isometric (B) and eccentric (E) exercises and $P$<0.01 for concentric (D). * and ** denote the main effect of leg at $P$<0.05 and $P$<0.01 level respectively. The group x leg interaction effects were $P$>0.05 for all 3 exercises (graph B, D, F). Data are means±SEM.

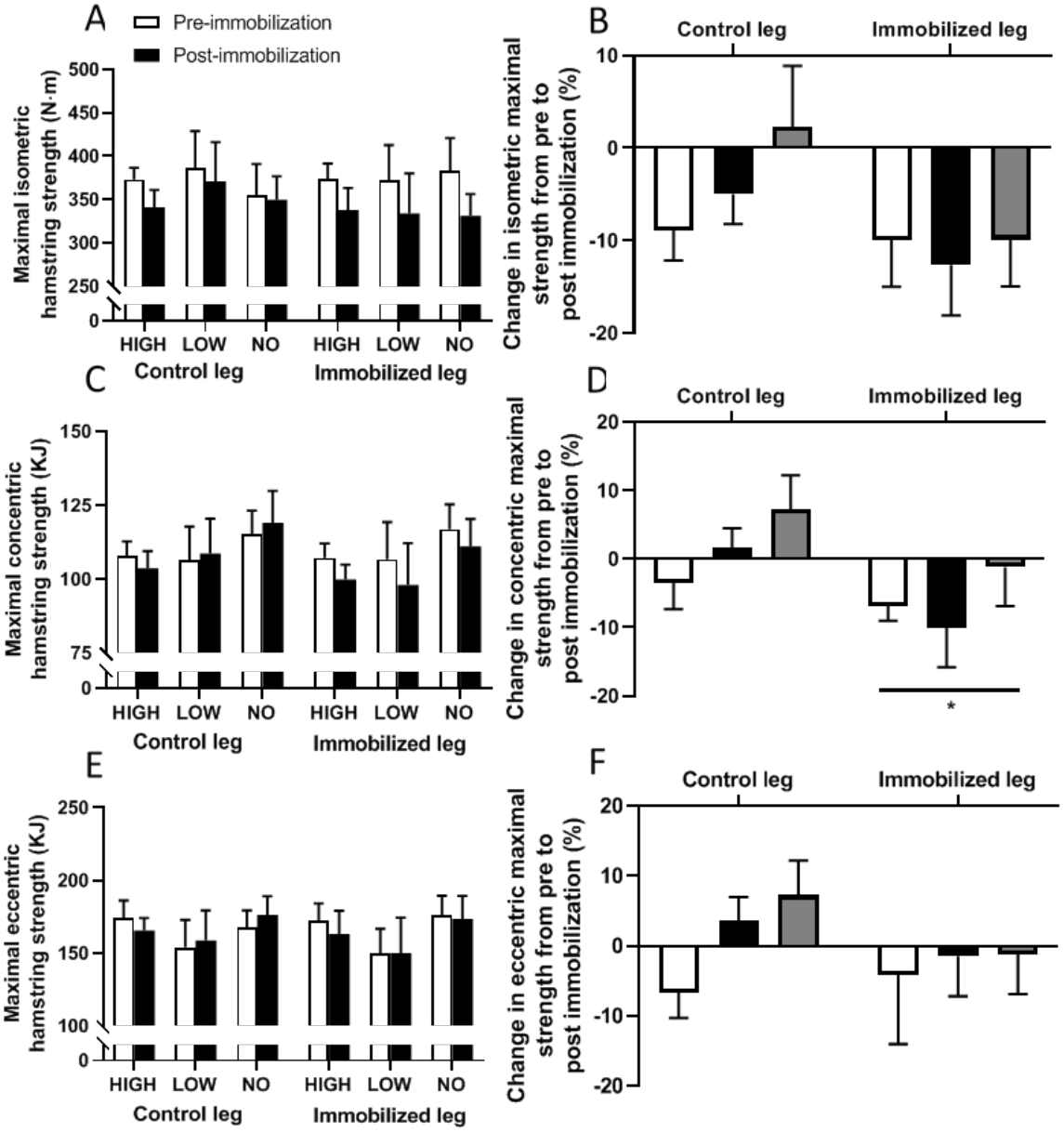


***Figure 5.5*** *M. hamstrings* muscle strength assessed by isokinetic dynamometry. Maximal isometric (3-repetition average) (A), isokinetic concentric (C) and isokinetic eccentric (E) (both 5-repetition average) *M. hamstrings* strength, for the control and immobilized legs pre- and post- immobilization for three groups HIGH ($n$=11; 1.6 g·kg bm·d$^{-1}$), LOW ($n$=11; 0.5 g·kg bm·d$^{-1}$) and NO ($n$=11; 0.15 g·kg bm·d$^{-1}$). Data were analyzed by three way ANOVA with leg x group x time as factors. A, C and E all showed no main effect of leg $P$>0.05, main effect of time was $P$>0.05 for concentric (C) and eccentric (E), but P<0.01 for isometric (A). Group x leg and group x time interactions were $P$>0.05 for 3 graphs (A, C and E), the leg x time interactions were $P$>0.05 for isometric (A) and eccentric (E), but $P$<0.05 for concentric (C). The group x leg x time interactions were $P$>0.05 for all three graphs. Graphs B, D and F show the relative change in maximal isometric, concentric and eccentric *M. hamstrings* strength respectively, for the control and immobilized leg and for the HIGH, LOW and NO groups. Data were analyzed by two way ANOVA with leg and group as factors. The main effect of group was $P$>0.05 for all 3 graphs (B, D, F), the main effect of leg was $P$<0.05 for concentric (C) and $P$>0.05 for eccentric (E) exercises and concentric (D). * denotes the main effect of leg $P$<0.05. The group x leg interaction effects were $P$>0.05 for all 3 exercises (graph B, D, F). Data are means±SEM.

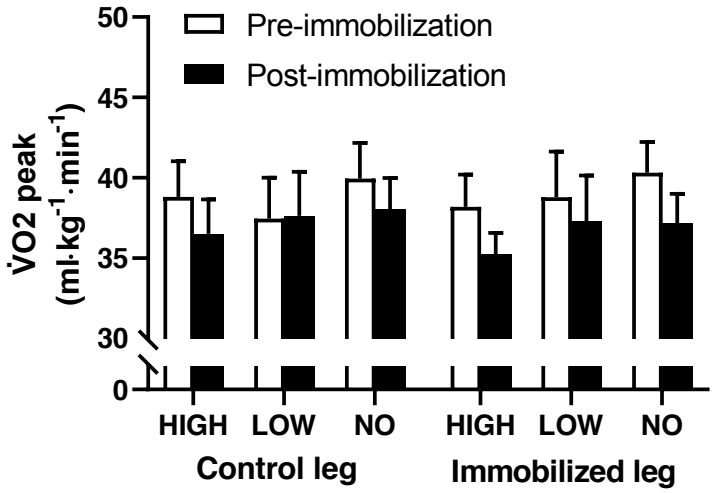


***Figure 5.6*** $\dot{V}O_2$peak achieved during a unilateral cycling ramp test. The graph expresses data for both the control and immobilized leg and for both pre- and post- immobilization. Data were analysed by three way ANOVA with leg x group x time as factors. Main effect of both group and leg were P>0.05, but time was P<0.05. The group x time, group x leg and leg x time interactions were all P>0.05, the group x time x leg interaction was P>0.05. Data were analyzed by two way ANOVA with leg and group as factors. Main effects of group, leg were both P>0.05, the group x time interaction was P>0.05. Data are means±SEM, n=11 per group.

### *Daily myofibrillar protein synthesis rates*

Myofibrillar protein bound [$^2$H] alanine enrichments increased pre- to post- immobilisation (main effect of time; *P*<0.001), and showed differences between legs (main effect of group; *P*<0.001) with the control leg increasing more than the immobilised leg (group x time interaction; *P*<0.001). After the 3 day immobilisation period myofibrillar protein bound [$^2$H] alanine enrichments increased by 44±4, 35±4 and 39±5 % more in the control compared with the immobilised leg in the HIGH (control leg to 0.1149±0.0045 MPE; immobilised leg to 0.0797±0.0024 MPE), LOW (control leg to 0.1191±0.0057 MPE; immobilised leg to 0.0885±0.0036 MPE) and NO (control leg to 0.1041±0.0041 MPE; immobilised leg to 0.0755±0.0031 MPE) groups respectively (data not shown). There were no significant differences between groups (*P*>0.05), or any group interactions (all *P*>0.05) such that dietary protein intake had no effect.

Daily myofibrillar protein FSRs (%·d$^{-1}$) during immobilisation, calculated separately in the control and immobilised legs using mean plasma free [$^2$H] alanine enrichments as the precursor pool, are displayed in **Figure 5.8**. Daily myofibrillar protein FSRs were 30±2, 26±3 and 27±2 % lower in the immobilised compared with the control leg in the HIGH (1.55±0.05 vs 1.08±0.04 %·d$^{-1}$), LOW (1.57±0.10 vs 1.16±0.06 %·d$^{-1}$) and NO (1.40±0.08 vs 1.03±0.07 %·d$^{-1}$) groups respectively, with no significant differences between groups (*P*>0.05).

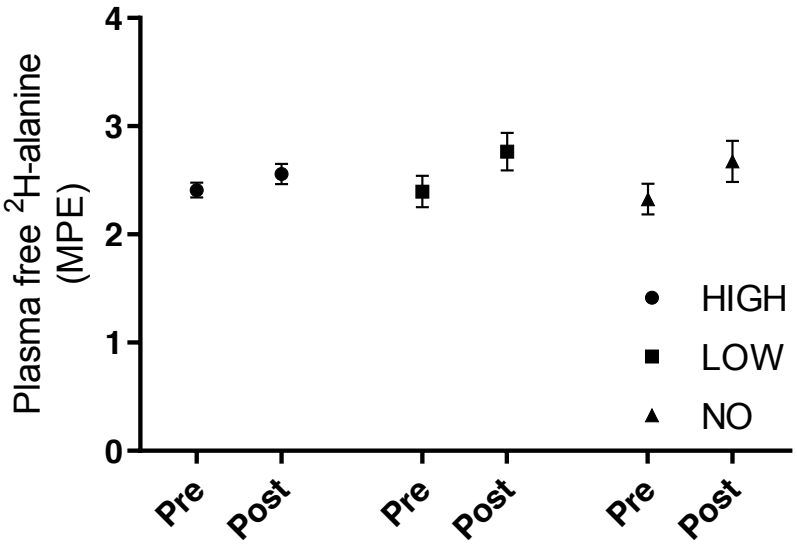


***Figure 5.7*** *Plasma free $^{2}$H-alanine (MPE) enrichments pre and post a 3 day period of unilateral knee immobilization where participants consumed a fully controlled energy balance diet containing a HIGH (n=11; 1.6 g·kg bm·d$^{-1}$), LOW (n=11; 0.5 g·kg bm·d$^{-1}$) and NO (n=11; 0.15 g·kg bm·d$^{-1}$) dietary protein content. Data were analyzed using a two way repeated measures ANOVA with time (pre vs post immobilization) and group (HIGH vs LOW vs NO) as factors. Main effect of group P>0.05, time P>0.05 and group x time interaction P>0.05. Data are means±SEM.*

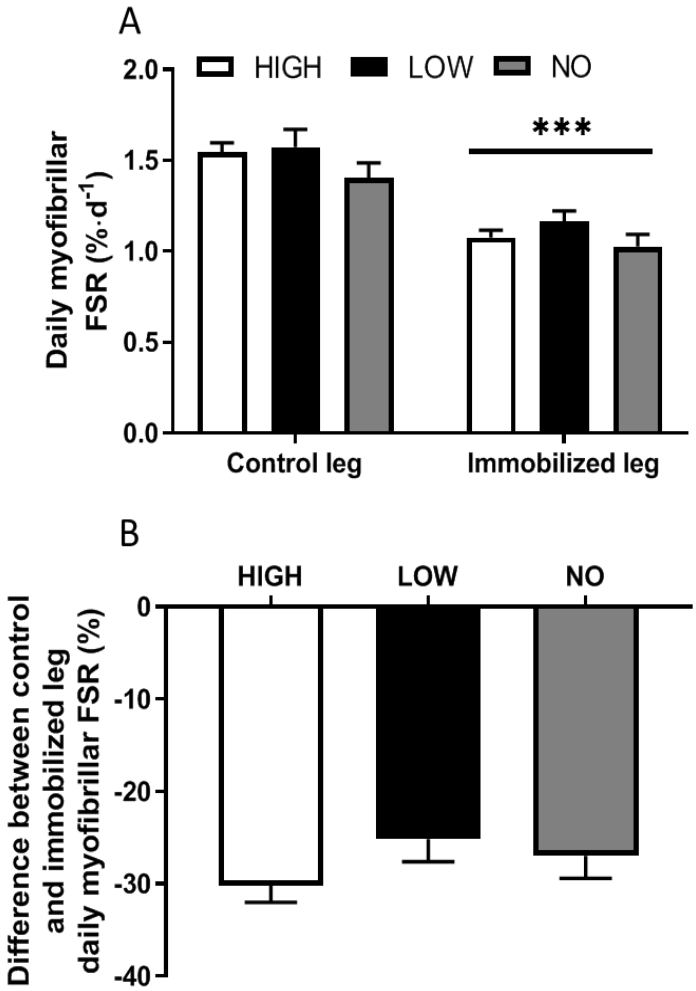


***Figure 5.8*** Daily myofibrillar fractional synthesis rates (FSR; %·d$^{-1}$) over a 3 day immobilization period via unilateral knee immobilization calculated from the plasma $^{2}$H-alanine precursor pool, where participants consumed a fully controlled energy balance diet containing a HIGH (*n*=11; 1.6 g·kg bm·d$^{-1}$), LOW (*n*=11; 0.5 g·kg bm·d$^{-1}$) and NO (*n*=11; 0.15 g·kg bm·d$^{-1}$) dietary protein content. Data were assessed by two way ANOVA with leg (control vs immobilized) and group (HIGH vs LOW vs NO) as factors. Main effect of leg *P*<0.001 (***), group *P*>0.05, leg x group interaction *P*>0.05. B expresses the difference in daily myofibrillar fractional synthesis rates between the control and immobilized leg for the three groups. Data were analyzed by one way ANOVA, *P*>0.05.

## *Discussion*

We assessed the impact of graded intakes of daily dietary protein during a short-term (3 day) period of muscle disuse (knee immobilisation) in healthy young men on daily myofibrillar protein synthesis (MyoPS) rates, muscle mass and function. We report that 3 days of immobilisation resulted in a considerable decline in daily MyoPS rates and loss of *M. quadriceps* volume and leg muscle strength. However, none of these muscle deconditioning responses to immobilisation were modulated by daily dietary protein intake, despite our design spanning a virtual absence of protein through to relatively high intakes.

Dietary protein ingestion transiently stimulates MyoPS rates for 2-5 h (37). As such, the repeated postprandial stimulation at each meal contributes considerably to daily, 24 h MyoPS rates and, thus, muscle mass maintenance. We recently demonstrated that a major physiological driver of muscle loss during short-term disuse is a considerable decline in daily, free-living MyoPS rates (4). Importantly, in that work we reported that disuse lowered daily MyoPS rates and consequently induced muscle atrophy despite participants (self) reporting habitual protein intakes double that of the daily UK RDA (31) (i.e. 1.6 g·kg bm·$d^{-1}$) and in line with current recommendations to limit muscle disuse atrophy (14, 33). However, this study was not conducted under dietary controlled conditions, nor did we compare groups according to protein intakes, and thus in the present work we aimed to establish the role of daily dietary protein intake on daily MyoPS rates and muscle loss during disuse. We recreated the relatively high habitual protein intake previously observed (which was also in line with the habitual protein intakes of the current participants; see Table 2) and habituated all participants to this diet for five days prior to immobilisation. This allowed the application of a dose-response approach during the immobilisation period only, where a control group maintained the same intake, one group consumed a diet virtually devoid of protein, and another group a sub-optimal amount indicative of consumption levels during hospitalization (33). In line with our previous work (4), immobilisation lowered MyoPS rates by ~28% (or ~9%·$d^{-1}$) compared with the control legs. However, contrary to our hypothesis, this decline was comparable across the groups, with the higher, low and negligible protein intakes resulting in 30, 26, and 27% lower MyoPS rates, respectively, in the immobilised compared with control legs. As well as the considerable difference in total protein intakes across groups, these data also occurred in the face of the control group being provided their daily protein intake equally across four meals (breakfast, lunch, dinner, pre-bed; resulting in ~28 g protein per meal) each separated by ~4 h. We had reasoned such an approach would result in sufficient protein per meal (31) and appropriately timed (39, 38) to maximise daily MyoPS rates. It would be remiss of us not to mention that significant differences in daily MyoPS rate across groups also did not occur in the *non*-immobilised leg. It is likely that this represents a type-2 error given that the NO protein group displayed (numerically) lower daily MyoPS rates coupled with a numerical loss (compared with a numerical gain in the other groups) of muscle mass.

In line with the lack of effect of dietary protein intake on daily MyoPS rates, we also observed no impact on muscle mass loss during disuse (Figure 5.3). We have recently shown that leg immobilisation results in substantial atrophy of the *M. quadriceps* within two days (11). In line with those data, and previous reports (5, 15, 40), we observed a ~2.4% (i.e. 0.8%·$d^{-1}$) decline in *M. quadriceps* volume following 3 days of immobilisation. However, atrophy was comparable across the dietary intervention groups with the high, low and zero protein intake groups' *M. quadriceps* volume declining by 2.3 (~0.8%·$d^{-1}$), 2.7 (~0.9%·$d^{-1}$) and 2.0% (~0.7%·$d^{-1}$), respectively. These data were also consistent with the lack of effect of dietary protein

intake on the decline in a wide array of muscle function tests following immobilisation (Figures 5.5 and 5.6). Indeed, with the exception of concentric contractions (2%·d$^{-1}$ decline), *M. hamstrings* strength was remarkably resistant to disuse induced declines, whereas *M. quadriceps* concentric (~8%·d$^{-1}$), eccentric (~6%·d$^{-1}$) and isometric (~8%·d$^{-1}$) muscle strength all declined at rates in line with the literature (39). Though the numerical decrease in single leg V̇O2peak seen with immobilisation was not significant (Figure 5.7), comparable effects were seen across groups. Accordingly, our data conclusively show that the decline in muscle function during short-term disuse is rapid, but not modulated by dietary protein intake. Previous studies have also found that manipulating protein intake during disuse does not modulate the rate of muscle disuse atrophy. For example, protein supplementation studies which have increased protein intakes to; 1.6 (vs 1.1) g·kg bm·d$^{-1}$ during 5 days of immobilisation in older men (52); 1.3 (vs 1.0) g·kg bm·d$^{-1}$ during 2 weeks of immobilisation in young men (3); and to 1.6 (vs 1.0) g·kg bm·d$^{-1}$ during 29 days of bed-rest in young men and women (20), have all shown no protective effect on muscle mass. Taken together, therefore, it would seem that dietary protein consumption within 'normal ranges' (i.e. ≤1.6 g·kg bm·d$^{-1}$) during disuse does not modulate the rate of muscle loss. We have reasoned previously that such findings may be due to control groups also consuming adequate protein (3). However, our data refute this notion with the novel observation that daily MyoPS rates and muscle loss are still not modulated even when relatively high, evenly spaced protein intakes are compared with low or negligible protein diets. Collectively, these studies' findings may be explained by the reduced MPS response to each dietary protein meal that occurs consequent with disuse (i.e. 'anabolic resistance'; (26, 35, 43)). Our data extend on the concept of disuse induced anabolic resistance by implying that this phenomenon manifests virtually immediately (given the short time frame of disuse), is not overcome by modest increases in protein intake, and is not exacerbated by dramatic reductions in protein intake.

Research where specific essential amino acid (EAA) or leucine supplementation has been applied during a period of disuse has generally (18, 37, 44), but not always (6) attenuated muscle loss. It is possible that this apparent discrepancy with protein supplementation studies can be attributed to such approaches translating to a greater amount of total protein provided. That is, extrapolating total daily protein intakes from these supplementation studies suggests the equivalent of ~87-158 (1.2-1.9 g·kg bm·d$^{-1}$) g total protein was consumed in the treatment conditions, and thus generally higher than the present and previous data concerning protein manipulation. Alternatively, the specific provision of high dose EAA and/or leucine may have had the capacity to overcome/compensate for muscle anabolic resistance more effectively than the present work. In support, such supplementation studies have provided daily EAA and leucine intakes of ~43-88 and ~15-19 g per day, respectively (17, 18, 37, 44), compared with ~51 and ~8 g in the present work, or ~19-50 and ~3-10 g in previous protein manipulation studies ineffective at attenuating muscle disuse atrophy (19, 20, 29). This would imply that the availability of amino acids *per se* is not limiting to MPS during disuse, but rather a dramatic increase in the threshold required for EAA/leucine to stimulate intracellular anabolic signalling pathways rates occurs. The inference therefore would be that maximising selective intracellular transport of these key amino acids (rather than raising global amino acid availability) is the prudent goal during disuse. However, such a notion clearly warrants further research, especially given not all leucine supplementation studies have been successful at attenuating muscle disuse atrophy (6). Additionally, the model of disuse (i.e. single limb immobilisation vs whole body bedrest) is a further important consideration. The amount of inactive tissue will undoubtedly affect amino acid availability during disuse, and could therefore conceivably contribute towards

discrepancies across studies or nutritional strategies depending on the nature of disuse. It may be that future nutritional strategies may be more effective by focussing on sensitising the intracellular anabolic signalling pathways (rather than increasing the stimulus), which appears to explain the proposed beneficial effects of prolonged fish oil supplementation on MPS rates and muscle mass maintenance in ageing (46) and disuse (45).

In conclusion, graded dietary protein intakes of 0.15, 0.5 or 1.6 g·kg bm·d$^{-1}$ did not influence the rapid decline in MyoPS, muscle mass or function during 3 days of unilateral leg immobilisation. This study is the first to evaluate the role of dietary protein intake *per se* under controlled dietary conditions on the rate of skeletal muscle deconditioning during short-term muscle disuse.

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# Chapter 6

General Discussion

Short periods of disuse due to illness or injury often result in negative health consequences including the loss of muscle mass and function, even in otherwise healthy individuals. In the present thesis, we studied the physiological mechanisms responsible for muscle atrophy during short term disuse and assessed the role of protein ingestion as a strategy to ameliorate the loss of muscle mass. In this chapter we will cover the implications of the presented findings and discuss them in a broader context. At the end of this final chapter, we have outlined important questions for future research on this topic.

*Individual muscle atrophy over time during short term disuse*

The major muscle groups of the thigh (e.g. quadriceps) experience atrophy after just 3 days of disuse (1) and to a greater degree after 1 week (2–4). This rapid loss of muscle mass during disuse is clinically relevant given that the average length of hospital stay is 5 to 7 days (5) and the recovery from a mild illness at home can also take up to a week of low levels of physical activity. In addition, short term disuse results in muscle atrophy, it also confers other negative effects on muscle including reductions in strength (2–4) and compromised insulin sensitivity (3). However, it still remains unclear as to how individual thigh muscles respond over time during short term leg immobilisation.

Most of the previous work that assessed muscle atrophy during short term disuse has utilised computed tomography (CT) scanning (2,3,6,7). While this allows for the accurate measurement of how the major muscle groups (e.g. quadriceps) atrophy during disuse, it does not provide proper resolution to accurately assess how individual muscles of the thigh, e.g. vastus lateralis, respond. Furthermore, CT scanning typically involves capturing a single cross-sectional image of the quadriceps muscle halfway between the knee and hip to assess muscle atrophy and to measure changes in muscle cross sectional area (2,8). Therefore, it does not allow us to gain much insight on how muscle volume responds during short term disuse and makes estimates of muscle loss during disuse more complex. Magnetic resonance imaging (MRI) provides a powerful tool with better resolution to accurately measure changes in muscle volume and individual muscle cross sectional area. Belavy and colleagues (2009) applied MRI scanning in a long term bed rest study (e.g. ≥1 month) and observed that the vastii muscle group experienced the greatest atrophy of all the individual muscle groups. This finding is also supported by Akima and colleagues (2001) who also reported differential atrophy between synergistic muscles, finding that the vastus lateralis and vastus intermedialis demonstrated greater atrophy than the vastus medialis after long term bed rest. However, it is still unknown, if individual muscle groups atrophy in a similar pattern over a shorter duration (< seven days) of disuse.

In Chapter 2 we used serial MRI scanning of the upper leg over time during one week of unilateral leg immobilisation to provide a temporal picture of atrophy in the major muscle groups of the thigh. We demonstrated that the quadriceps and hamstring muscle atrophied by 1.7 and 1.4 % respectively after just 2 days of disuse. Following 7 days of immobilisation, the quadriceps muscle group had atrophied by 6.7 %, at a rate of approximately 1 % per day. The quadriceps was responsible for the majority (60%) of thigh muscle atrophy, when compared to the hamstring which had atrophied by 3.5 % after 7 days (11% of total thigh atrophy). The discrepancy between muscle groups could be attributed to a number of factors. The quadriceps muscle is composed of a greater percentage of type 2 muscle fibres (9,10) which may be more susceptible to atrophy (11). Additionally, the quadriceps group is more frequently recruited during habitual activities like walking, sitting and standing when compared to the hamstring (12). Thus, the removal of all physical activity via the leg brace may have resulted in a greater removal of stimuli for

the quadriceps compared to the hamstring, which resulted in greater atrophy for the quadriceps. The model of disuse with the knee immobilised at ~30 degrees of flexion may also have contributed to the difference in atrophy, because the quadriceps was immobilised at a longer length than the hamstring. Previous research in a rodent model has shown that the length at which a muscle is immobilised will affect the degree of atrophy experienced (13). When a muscle is immobilised at a shorter length than its normal resting length, muscle atrophy is exacerbated due to a lower neuromuscular activity occurring within the muscle (13).

The use of serial MRI scanning provided the resolution to determine muscle atrophy of the individual muscles that make up the larger muscle groups e.g. the vastus lateralis and the semitendinosus. After one week of unilateral leg immobilisation the vastii muscles atrophied to the greatest extent and significantly more than the adductor group and biceps femoris short head. The gracilis and sartorius exhibited no atrophy. Furthermore, multiple MRI images were taken along the length of the muscle (between origin and insertion points) which made it possible to determine that the mid-belly sections of the quadriceps and hamstring atrophied to a greater extent that the proximal and distal sections. These findings regarding the spatial-temporal changes in atrophy of specific muscles within the thigh provide insights to refine strategies that aim to reduce muscle atrophy during disuse, such as neuromuscular electrical stimulation (NMES). Specifically, the application of NMES should begin as soon as it is safe to do so after the onset of disuse with protocols that allow for longer stimulation periods of the quadriceps, specifically the individual vastii muscles, with less focus on the adductor, gracilis and sartorius muscles. This should allow for a more timely, effective NMES treatment to attenuate muscle mass loss by targeting the specific muscle groups that are at a greater risk of atrophy.

Changes in strength of the different muscle groups following seven days of immobilisation mirrored the differential changes in individual muscle atrophy. Where the quadriceps experienced a significant reduction in strength, in comparison the hamstring muscle group remained unaffected. This information can be used to inform the development of rehabilitation strategies. Specifically, rehabilitation strategies should incorporate more quadriceps compared to hamstring exercises to aid the faster recovery of quadriceps muscle strength.

*The temporal response of muscle protein synthesis rates during short term disuse*

The loss of muscle mass in response to disuse is largely thought to be driven by decrements in both post-absorptive muscle protein synthesis (MPS) rates (2,6,14) and the anabolic response to protein ingestion (2,15), with minimal contribution of an increase in muscle protein breakdown rates (16). These findings hold true following both short term (14,15) and long term periods of disuse (17,18). Metabolic studies to identify the changes in muscle anabolism during disuse have primarily utilised the continuous infusion of stable isotope labelled amino acid tracers (e.g. L-[ring-$^{2}H_{5}$] phenylalanine) over a period of hours before and after the disuse period (2,14,15,17). Although this approach allows for the controlled assessment of muscle protein synthesis rates, using specialist equipment like infusion pumps in a laboratory environment, continuous stable isotope infusion tracers can only be used for ~12 hours before tracer recycling (i.e. protein-bound tracers being released back into precursor pools) becomes a confounding factor (19,20). Consequently, these studies have only captured a snapshot in time that a participant spends in disuse (e.g. 1-2 % at the start and end of a period of disuse). Additionally, stable isotope infusion methodology does not account for different factors that occur outside of a controlled, experimental

condition that may influence MPS rates throughout the period of disuse, such as repeated meal ingestion and varied physical activity levels.

Deuterated oxide ($^2H_2O$) is a stable isotope tracer used for *in vivo* studies of human metabolism (21). Shortly after being consumed, $^2H_2O$ increases body water deuterium enrichment ($^2H$) and is gradually incorporated into numerous *de novo* synthesised molecules including proteins, lipids and metabolites (22,23). In relation to protein metabolism, amino acids will become labelled with deuterium over time via transaminase reactions (24). Notably, alanine becomes highly enriched (~3.7 times greater than body water deuterium) in a short amount of time (i.e. < 2 hours). Because of this metabolic property, alanine is one of the most convenient amino acids to use as a tracer when measuring MPS rates with deuterium oxide. Deuterium has a long half-life within the body water pool (~10 to 14 days), and it is relatively easy to maintain a steady state over a number of weeks (25) by consuming a small, daily bolus of $^2H_2O$. The relative practicality of utilising deuterium in free-living research volunteers undergoing disuse protocols makes it a suitable method to assess MPS rates over sustained periods of time outside of a laboratory environment.

In chapter 3, we implemented deuterated oxide to measure temporal changes in MPS rates during short term unilateral leg immobilisation. We demonstrated that disuse induced a rapid and sustained decline in daily MPS rates in the immobilised leg only, contributing to a 36% reduction in MPS over seven days of immobilisation. This reduction is consistent with previous research that has assessed the changes in either post-absorptive/prandial (2,14,17) or daily MPS rates (26) after short term disuse. More specifically, MPS rates reduced by 16% in the first 2 days (i.e. 8% per day) and by 45% between days 2 and 7 of disuse. This result was remarkably consistent across participants. The daily rate of decline in muscle protein synthesis rates was similar between the first 2 days and last 5 days, 8 and 9 % per day, respectively. Assuming that a linear decrease occurred a greater reduction actually occurred over the last 5 days (i.e. 16 % between days 0 – 2 and 45 % between days 2 and 5). This suggests that there is a stepwise reduction in muscle protein synthesis rates, at least during the first week of disuse. Surprisingly, the decline in MPS rates occurred despite participants reporting the consumption of a high protein diet (1.6 g·kg body weight$^{-1}$·day$^{-1}$), exceeding the RDA of 0.8 g·kg body weight$^{-1}$·day$^{-1}$. Together, these data suggest the decline in muscle protein synthesis rates was due to disuse and not because participants were not consuming enough protein.

Alongside the reduction in MPS rates, we utlilised MRI scanning to measure quadriceps volume. We observed a rapid decline in quadriceps volume of 1.7 % after 2 days and 6.7 % after 7 days (~0.9 % per day), which is in line with other studies (27). To estimate the change in quadriceps muscle mass following 7 days of leg immobilisation, we assumed that the skeletal muscle density did not change (mass = volume x density). After 2 days the quadriceps muscle mass had declined by 42 g, and by 162 g after 7 days. The reductions in quadriceps mass and MPS rates were correlated between days 0 – 2 ($r = 0.48$) and between days 2 and 7 ($r = 0.58$). This suggests that the reduction in muscle protein synthesis rates and muscle atrophy seen during short term disuse are closely linked.

By applying deuterium oxide to measure daily MPS rates and MRI scanning to measure muscle volume, we were able to make more accurate estimations on how reductions in MPS rates contribute to the observed muscle atrophy. To make this calculation previous work had assumed that muscle protein breakdown rates do not change during muscle disuse and that in the control leg daily muscle protein synthesis and breakdown rates are equivalent (16). Based on approach we calculated that the expected

loss of muscle mass as a result of decreased daily MPS rates as follows: net muscle protein loss = daily MPB – daily MPS. We also calculated a net muscle protein loss of 0.21 and 0.45 % per day over the first 2 days and entire week, respectively. Given that we know the muscle mass of the participants' quadriceps at baseline, this equates to an expected muscle protein loss from the immobilised quadriceps that is directly attributable to reduced MPS rates of 11 g and 76 g over 2 and 7 days, respectively. Surprisingly, this accounts for only 25 and 47 % of the 42 and 162 g of estimated (measured) muscle loss. Although we did not directly measure muscle protein breakdown rates during disuse, we did measure the temporal changes of key markers of muscle protein breakdown from multiple proteolytic pathways. Interestingly, the only molecular pathway that showed a consistent response were genes within the ubiquitin proteasome system (e.g. MAFbx and MURF1), which were upregulated after 7 but not 2 days of immobilisation. Although this is not a direct measurement of muscle protein breakdown rates it does lend support for the argument that muscle protein breakdown may have increased during the week of unilateral leg immobilisation and contributed to the observed muscle atrophy.

Taken together, the results presented in chapter 3 imply that muscle protein breakdown rates likely increased during the week of muscle disuse and contributed to the muscle atrophy we report. Our finding that the reductions in MPS rates only accounts for 25 and 47% of the muscle loss, after 2 and 7 days respectively, is far below what previous studies utilising acute measurements of MPS rates over a period of hours after participants finish a short bout of disuse have reported (6,16). Findings from these previous studies led the authors to conclude that reductions in MPS rates after short term disuse could fully explain the muscle atrophy measured (i.e. muscle protein breakdown rates remained unchanged (16), or possibly decreased during disuse (6)). Indeed, previous work from our group estimated that reductions in basal and/or postprandial MPS rates could explain ~80% of the muscle atrophy seen during 5–14 days of disuse (28). However, differences in the methodology between past studies and the present study may explain some of the differences in the findings. Most notably the application of deuterium oxide instead of labelled amino acid stable isotope infusion tracers and MRI scanning instead of CT scanning allowed us to address some of the assumptions made in previous work. Most importantly, we addressed the assumption that measuring myofibrillar protein synthesis rates after a period of disuse reflects the changes in chronic myofibrillar protein synthesis rates that occur throughout the entire period of disuse and usually inferring muscle mass from single slice cross-sectional measurements.

Despite being able to refine calculations of how a decline in MPS contributes to atrophy during disuse, our methods still involve several assumptions. We assumed that muscle density does not differ substantially across volunteers or in response to disuse. It is a common concern that leg immobilisation will result in lower leg oedema, but this was not observed in any of the participants. Considering this and our observation that oedema does not occur in the upper leg, it is unlikely that the one week of leg immobilisation altered intra- or extra-cellular volume of the muscle cells within the vastus lateralis. Therefore, it is unlikely that muscle density changed during short-term immobilisation; although this was not measured in chapter 3, nor has it been measured after a period of muscle disuse in previous research. Secondly, we assumed that vastus lateralis muscle protein turnover rates are analogous to the turnover rates in entire quadriceps muscle. Furthermore, that the incorporation rate of alanine is equivalent to all other amino acids from the intracellular pool into myofibrillar proteins. Lastly, we assumed that myofibrillar protein atrophy is equivalent to total protein loss. These are all assumptions that future studies should address to make more accurate estimations of how much reductions in MPS rates can

explain the atrophy observed during a period of disuse.

It is widely accepted that the synthetic response of certain protein sub-fractions, including the myofibrillar fraction, are diminished during short term disuse. However, there are hundreds of individual proteins within each sub-cellular protein fraction within skeletal muscle. By only assessing how the major sub-cellular fractions respond to disuse, we lack global insight as to how the individual proteins within each protein fraction respond. It is possible that more abundant proteins within each sub-fraction skew the overall response of the subfraction, or that certain individual proteins are particularly affected by muscle disuse. Modern high throughput mass spectrometry techniques (e.g. LC-MS/MS) in combination with deuterated oxide methodology allow for the identification and detection of the peptides and how their deuterium enrichment changes over time, which can be used to estimate the fractional synthesis rates of the individual proteins. This methodology has recently been applied in human skeletal muscle tissue to measure the proteome synthetic response to dietary and exercise interventions (29). Implementing a proteomic based approach may lead to the development of new strategies that aim to reduce muscle atrophy during short term disuse.

*The role of protein intake in modulating muscle mass and muscle protein synthesis rates during short term disuse.*

The recommended dietary protein intake for healthy adults is 0.8 g·kg body weight$^{-1}$·day$^{-1}$ (30). For an individual who weighs 80 kg, this would equate to a recommendation of ~65 g of dietary protein per day. However, during periods of disuse food intake and protein intake are often reduced (31), typically due to a reduction in appetite (32) and / or a decline in energy requirements primarily due to reduced physical activity (33,34). The reduced protein intake during a period of disuse may contribute to greater muscle atrophy during disuse, due to a reduction in protein-related MPS stimulation and MPB inhibition (35). Therefore, it has been suggested that providing individuals with greater amounts of dietary protein intake to overcome disuse-induced anabolic resistance (2,15) and stimulate muscle protein synthetic rates during disuse could reduce muscle atrophy (36,37). Research in this area is lacking but some long term bed rest studies have observed that supplementation of crystalline amino acids attenuates the loss of muscle mass during the disuse period (18). This is also supported by more recent research that shows that consumption of an essential amino acid mixture also reduced the amount of muscle mass lost during short term leg immobilisation (38). However, research in this area is inconclusive as several other studies have shown that supplementing with additional protein or essential amino acids during short term leg immobilisation did not reduce muscle atrophy observed (39,40). These discrepant research findings could be explained by the protein content of the diet provided during the disuse phase of these studies and how protein or essential amino acids were supplemented alongside their habitual diet. For example, Paddon-Jones and colleagues (2004) provided a daily protein intake of ~0.8 g·kg body weight$^{-1}$·day$^{-1}$, which was then supplemented with 3 x 16.5 g of crystalline essential amino acids daily, which conferred a protective effect on leg lean muscle mass after 28 days of bed rest. In contrast, Dirks and colleagues (2014) saw no effect of 21 g of whey protein supplementation twice daily, when the participants' habitual dietary protein intake was 1.14 g·kg body weight$^{-1}$·day$^{-1}$.

In addition to the amount (41) and the type (42,43) of dietary protein, the distribution of when protein is consumed throughout the day may also influence muscle protein synthesis rates (44). In a traditional Western diet, most of the protein ingested throughout the day is skewed towards dinner, where more

protein rich foods (e.g. meat) are more likely to be consumed (45,46). This unbalanced consumption of protein throughout the day (e.g. ~10 g at breakfast and ~40 g at dinner) results in a lower muscle protein synthetic response to protein ingestion over a full day when compared to more evenly distributed protein ingestion (e.g. ~25 g per meal) throughout the day (44).

In Chapter 5 we assessed the effect of graded dietary protein intakes on muscle atrophy and the change in daily muscle protein synthesis rates during 3 days of unilateral leg immobilisation. To assess how protein intake may influence muscle atrophy during disuse we used a parallel groups design. One group consumed an optimally designed diet consisting of 1.6 g·kg body weight$^{-1}$·day$^{-1}$ (HIGH) protein that was equally distributed throughout the day (~28 g per meal) which included a pre-bed protein meal that was aimed at stimulating overnight muscle protein synthesis rates during disuse (47,48). A second group consumed 0.5 g·kg body weight$^{-1}$·day$^{-1}$ (LOW) protein and a third group consumed 0.15 g·kg body weight$^{-1}$·day$^{-1}$ (NO) protein. After 3 days of disuse, we observed a significant reduction in daily myofibrillar protein synthesis rates, muscle strength and quadriceps volume in all three groups, HIGH, LOW and NO protein. Surprisingly, we observed no differences between the different protein intake groups in the response of myofibrillar protein synthesis rates (which declined by ~28 % on average in the three groups) and muscle atrophy (which declined by ~2.3 % on average in the three groups). It is likely that the three days of muscle disuse induced some anabolic resistance to protein ingestion that could not be overcome by the ~28 g of protein ingested per meal. This may indicate that disuse-induced anabolic resistance occurred earlier than previous studies have reported (i.e. < 5 days) (2,15). Conversely, if muscle protein breakdown rates increased during disuse this could help explain why the high protein intake per meal in the HIGH protein group (e.g. ~28 g) did not alleviate the reduction in muscle mass or muscle protein synthesis rates during disuse. An increased muscle protein breakdown would lead the endogenous liberation of amino acids in the disused muscle that could then be reconstituted into muscle proteins. This would mean that the extra protein consumed in the HIGH protein group would not be required as a means to provide the substrate (i.e. amino acids) required to synthesise muscle proteins, nor did it stimulate intracellular signaling cascades (e.g. mTOR pathway) that increase muscle protein synthesis rates.

In support of our findings in chapter 5, most of the previous research that has assessed the effect of differing protein intakes during disuse have found no beneficial effect of higher protein intakes during disuse (40,49). A primary difference between the work presented in Chapter 5 and previous work is the large differences in the total amount of protein provided per group. We provided participants with 1.6, 0.5 and 0.15 g·kg body weight$^{-1}$·day$^{-1}$ of dietary protein, in the HIGH, LOW and NO groups, respectively. Other groups investigating the role of protein intake during disuse provided participants in the control group with ~1.0-1.1 g·kg body weight$^{-1}$·day$^{-1}$, while participants in the supplemented group received ~1.4-1.6 g·kg body weight$^{-1}$·day$^{-1}$ (40,49,50). Therefore, the null effect of protein supplementation on alleviating muscle atrophy during disuse may be attributed to the control group receiving a dietary protein intake above the recommended daily amount (i.e. 0.8 g·kg body weight$^{-1}$·day$^{-1}$). In contrast, studies where participants are supplemented with a mixture of amino acids or leucine (18,38) have generally reported a beneficial effect on reducing muscle atrophy during disuse. The discrepancy in the effectiveness of these studies could be due to the EAA/leucine supplementation studies providing a greater source of EAA (~43-88 g) and leucine (~8-15 g) (18,38), compared to protein supplementation studies (EAA provided = ~19-50 g, leucine provided = ~3-10 g) (40,49). It could be that muscle disuse substantially increases the threshold for leucine or EAA to stimulate muscle protein synthesis rates. Hence the studies that have

supplemented EAA/leucine may have prevented muscle atrophy in contrast to those who have supplemented intact protein sources (i.e. all amino acids). However, there is evidence from other research projects that show that leucine supplementation had no effect at preventing muscle atrophy during short term disuse (39).

Future work should investigate the optimal mix of amino acids that should be provided per meal during disuse to best maintain muscle protein synthesis rates. For example, disuse may increase the competition between amino acids for intracellular transport into the muscle. Thus, future work may aim to control the provision of certain amino acids, so the amino acids shown to increase muscle protein synthesis rates e.g. leucine have a greater chance at being transported into the muscle cell. Conversely, protein or amino acid supplementation may be more effective when combined with other supplements that may either increase blood flow and hence delivery of amino acids to the muscle, e.g. nitrate (51), or sensitise the muscle to the amino acids e.g. omega 3 supplementation (52) or combined electrical stimulation and protein ingestion (53).

*Future research*

Despite the novel findings presented in this thesis there are many unanswered questions that future research should address. All participants in the studies presented in this thesis were young, healthy males. There is still very little known about how females respond to disuse, thus future research should aim to unveil potential sex-specific differences to short term disuse. Given that males have higher habitual muscle mass than females, this may predispose them to greater disuse induced muscle atrophy, as there is a correlation between these two factors. Furthermore, the hormonal differences between sexes may also result in differential atrophy during disuse. Where males have higher testosterone levels, which has an anabolic effect on muscle in habitual conditions, females have elevated levels of estrogen which seems to have limited anabolic properties (54).

Not only is our understanding of sex-specific differences in response to muscle disuse lacking, how middle-aged adults (ages 40 – 65 y) respond to disuse is also lacking. The majority of muscle disuse research has focused on young healthy individuals, aged 18-30 y, and to a lesser extent on older individuals (aged over 65 y). However, there is very little research on how middle aged individuals respond to disuse. This age group is important as the gradual loss of muscle mass seems0 to begin at around aged 35 y and is further exacerbated after the age of 50 y. It has been hypothesised that repeated bouts of short term muscle disuse accelerates muscle loss with aging. Thus, to better preserve muscle mass across the lifespan it is vital to further our understanding on how muscle disuse results in muscle atrophy in middle aged subjects.

On a more mechanistic level, the work put forth in this thesis has greatly refined our understanding of how the reduction in muscle protein synthesis rates contributes to muscle atrophy during short term disuse. Unlike previous research studies, we concluded that reductions in muscle protein synthesis rates could not fully explain the muscle atrophy observed. Although we did not measure muscle protein breakdown rates in chapter 3, to explain the quantity of muscle atrophy observed muscle protein breakdown rates more than likely must have been increased. Despite technical challenges future work should aim to better understand the contribution of muscle protein breakdown to muscle atrophy during muscle disuse. This could be achieved by applying arteriovenous blood sampling in combination with infusion of stable isotope labeled amino acids, typically phenylalanine to achieve a steady state and measure unlabeled phenylalanine efflux from muscle to estimate muscle protein breakdown rates using

a two or three pool model (55). This technique could be applied before and after a short period of disuse to measure how disuse affects muscle protein breakdown rates. If stronger evidence supporting an increase in muscle protein breakdown is established this knowledge could be used to better direct nutritional or pharmacological interventions that are aimed at reducing any increase in muscle protein breakdown rates during disuse and reduce muscle atrophy.

To further improve our understanding of how changes in muscle protein synthesis and breakdown rates contribute to muscle atrophy, future work should focus on the most precise measurements of muscle mass. Our findings in chapter 2 could be extended by measuring individual muscle volume, particularly vastus lateralis muscle volume after disuse as this is the most common muscle where muscle protein synthesis and breakdown rates are measured. Despite multiple studies now showing that short term muscle disuse reduces muscle protein synthesis rates, our understanding of the molecular regulation of these changes could be improved. The majority of studies have focused on measuring the abundance or phosphorylation status of individual proteins. Future research could employ more global approaches such as transcriptomics or proteomics that may provide more insight as to how the muscle responds to disuse. This may provide the identification of novel molecular regulators of muscle disuse that could prove to be viable targets of new pharmacological interventions.

Nutritionally it is essential that future investigations elucidate whether the anabolic resistance to protein ingestion seen after short term muscle disuse can be overcome by the ingestion of greater protein intakes. Does a muscle protein synthetic dose-response to protein ingestion also exist after short term disuse that is akin to what occurs in the aging process? This will help inform us on how much protein should be ingested per meal during disuse with the aim of overcoming this anabolic resistance and stimulating muscle protein synthesis rates during disuse. This will also help with the designing of optimal diets that contain the preferred amount of protein per meal that may be able to reduce muscle atrophy during short term disuse. If anabolic resistance after short term disuse can be overcome with higher protein intakes, future work should also assess if the same is possible with more sustainable protein sources for example plant based proteins, further supported by the use of novel supplements e.g. HMB (56) or ursolic acid (57).

*Conclusions*

The studies described in this thesis further extend our understanding of how muscle disuse induces muscle mass and strength loss. We employed novel techniques such as the use of deuterium oxide to measure daily muscle protein synthesis rates following disuse applied over time in a free-living setting. We observed a rapid and sustained decline in daily muscle protein synthesis rates that strongly contributed to the observed muscle loss. By deduction, we also conclude that increases in muscle protein breakdown must also contribute to the loss of muscle mass during short term disuse. We utilised MRI scanning to show how individual muscles of the thigh atrophy during a period of disuse. Nutritionally we investigated the role of graded dietary protein intakes and how these impact muscle protein synthesis rates and subsequent muscle atrophy during disuse. The levels of protein intake had no effect on daily muscle protein synthesis rates and/or the amount of muscle that was lost. These findings will be applied to develop future studies and to design alternative nutritional strategies to attenuate muscle atrophy during short term disuse.

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# Chapter 7

Summary

Impact

Acknowledgements

Curriculum Vitae

## *Summary*

Previous research has shown that short periods of both bed rest and leg immobilisation result in a reduction in muscle size and strength, particularly of the quadriceps and hamstring muscles. Furthermore, longer term bed rest studies lasting months have shown in more detail how individual muscles of the thigh atrophy over a longer time period. However, there were no data available on how individual muscles of the thigh atrophy during short term disuse (i.e. up to one week). Therefore, in Chapter 2 we assessed how the individual muscles of the thigh respond to one week of unilateral leg immobilisation. We report in this study that thigh muscle atrophy occurs rapidly (within just 2 days) and at a sustained rate (by approximately 0.8% per day) during one week of immobilisation. The atrophy is mainly attributed to the loss of *M. quadriceps* tissue mass. Furthermore, the constituent muscles of the thigh atrophy during immobilisation at markedly differing rates (~0.4 - 1.0% per day) with the *M. vastus lateralis* experiencing the most and *M. gracilis* the least atrophy. The preponderance towards *M. quadriceps* rather than hamstrings atrophy during immobilisation is accompanied by functional declines manifesting distinctly within leg extension movements.

Extensive research has shown that muscle disuse lowers both post-absorptive and post-prandial muscle protein synthesis rates over a short term period (i.e. one week) and after long term disuse (i.e. more than 1 month), however these measurements have only been made using stable isotope labelled amino acid tracers which only allow for the investigation of muscle protein synthesis after a period of disuse. Thus there were no data on how muscle protein synthesis rates changes over time throughout the entire period of muscle disuse. Recently, the application of deuterated water ($^2H_2O$) has re-emerged in the field as an approach to assess muscle protein synthesis rates over multiple days or weeks *in vivo* in humans. In chapter 3, 13 healthy young males underwent one week of unilateral leg immobilisation. Muscle disuse induced a rapid decline in muscle volume (within 2 days) that was further increased with prolonged disuse (up to 7 days). The decline in muscle volume was accompanied by a ~36% decline in daily myofibrillar protein synthesis rates in healthy young men over one week of disuse. These data highlight the key role that declining myofibrillar protein synthesis rates play in the development of skeletal muscle disuse atrophy *in vivo* in humans.

A small but growing body of research has highlighted how higher protein/amino acid intakes may act to reduce muscle atrophy during a period of disuse. These research studies generally assessed the impact of consuming animal based protein sources. In Chapter 4 we assessed the bioavailability of mycoprotein in an attempt to gain more insight into the potential anabolic properties of more sustainable, non-animal protein sources. Mycoprotein is a sustainable non-animal derived protein source that is produced by the continuous fermentation of the filamentous fungus *Fusarium venenatum.* Based on the observed bioavailability, we speculate that the ingestion of 40 g mycoprotein (i.e. 18 g total protein) would be sufficient to mount a robust muscle protein synthetic response, with the ingestion of 60 g mycoprotein (i.e. 27 g total protein) likely necessary to provide an optimal anabolic response. It is unlikely that consuming an excess of 60 g would confer any further benefits in healthy individuals. We conclude that mycoprotein represents a bioavailable and insulinotropic, non-animal derived dietary protein source. Consumed in sufficient quantities, mycoprotein would be expected to support skeletal muscle anabolism and reconditioning.

In Chapters 2, 3 and 4 we identified that skeletal muscle atrophy occurs rapidly in just 2 days of disuse, which is attributed, at least partly, to a rapid reduction in muscle protein synthesis rates. Although some research studies investigated the effects of increased amino acid or protein intake on muscle atrophy during disuse, there were no studies that assessed this while consuming different amounts of protein. Thus, in Chapter 5 we investigated how graded protein intakes of 0.15, 0.5 and 1.6 g·kg bm·d$^{-1}$ may modulate muscle atrophy and muscle protein synthesis rates during a short period of disuse. We found that graded dietary protein intakes of 0.15, 0.5 or 1.6 g·kg bm·d$^{-1}$ did not attenuate the rapid decline in myofibrillar protein synthesis rates, muscle mass, or function during 3 days of unilateral leg immobilisation. This study is the first to evaluate the role of dietary protein intake *per se* under controlled dietary conditions on the rate of skeletal muscle deconditioning during short-term muscle disuse.

## *Impact*

*What is the main objective of the thesis, and what are the most important results and conclusions?*

Skeletal muscle plays an important role in human health, providing the capacity for ambulation and postural control, as well as the regulation of whole body protein metabolism. Muscle mass and strength are both positively correlated with quality of life and functional capacity, while low muscle mass and strength are associated with a reduced physical function and mortality. Periods of physical activity are commonplace particularly due to illness, hospitalisation or injury and this is associated with high health care costs. Recent research shows that the average length of stay in hospital is 7 days. Furthermore, it has also been shown that during short term hospitalisation patients spend 83% of their entire time in bed, further highlighting the prevalence of short periods of disuse. During these periods of physical inactivity a loss of muscle function and a loss of muscle mass will occur. Recent research has shown that just 3 days of muscle disuse will cause a reduction in muscle mass and strength, highlighting the fast rate at which muscle deconditioning occurs. These short periods of physical inactivity can contribute to age related diseases including sarcopenia and dynapenia as well as a decline in the metabolic condition of the muscle.

The main objectives of this thesis included; firstly to investigate the effect of short term unilateral leg immobilisation on temporal muscle atrophy of the major muscle groups of the thigh as well as the constituent individual muscles. Secondly, we aimed to investigate how skeletal muscle protein synthesis rates are affected by short term leg immobilisation, how the rate may change over time and how any possible reduction in muscle protein synthesis rates may contribute to muscle atrophy. We also aimed to assess how different dietary protein intakes ranging from a relatively high protein intake to a diet containing very low protein may affect muscle atrophy, function and protein synthesis rates during short term disuse. Lastly, we aimed to understand the plasma amino acid bioavailability as well as the postprandial insulinemic profile after the consumption of the sustainable protein - mycoprotein when compared to animal (milk) protein.

In **Chapter 2**, we assessed thigh muscle size multiple times over 1 week of unilateral leg immobilisation, to understand how short term disuse affected upper leg skeletal muscle mass. This disuse model can be used to mimic physical inactivity that may occur in a hospitalisation setting without the disease burden. We showed that muscle atrophy can occur within just 2 days of disuse, with reductions in muscle strength occurring after 7 days. The thigh muscle atrophy was mainly due to the more habitually utilised muscle groups, where the quadriceps atrophied to a greater extent than the hamstring. This demonstrates the need for future research to assess the effect of a variety of different interventions aimed at reducing muscle atrophy during short term disuse. Alongside investigating muscle atrophy of the major muscle groups of the thigh, in **Chapter 3** we employed the use of the stable isotope tracer deuterium oxide to measure daily muscle protein synthesis rates during short term disuse. This allowed us to better understand the mechanistic cause of skeletal muscle atrophy. We demonstrated that muscle protein synthesis rates rapidly decrease during one week of leg immobilisation. However, we calculated that the decline in muscle protein synthetic rates could not fully explain the muscle atrophy we observed. Thus it is likely that muscle protein breakdown rates also increased during disuse. This finding opens up a line of investigation for future research to try to find interventions that can prevent this reduction in muscle protein synthesis and, therefore, attenuate muscle atrophy.

To understand the role of graded protein ingestion on the response of muscle mass and protein synthesis rates during disuse, in **Chapter 5** we provided subjects who were undergoing short term leg immobilisation with differing amounts of protein intake, from high protein (1.6 g g·kg bm·$d^{-1}$), to low protein (0.5 g·kg bm·$d^{-1}$), to minimal protein (0.15 g·kg bm·$d^{-1}$). We demonstrated that there was no difference in the ability of a high, low or minimal protein intake during disuse at preventing muscle atrophy or reductions in muscle function during short term leg immobilisation. Although providing a diet that contained a relatively high amount of animal protein that was evenly well distributed throughout the day, this did not prevent muscle atrophy or reductions in muscle strength during short term immobilisation. In **Chapter 4**, we did establish that the sustainable protein source, mycoprotein, is a viable alternative in terms of amino acid bioavailability when compared to animal based proteins (e.g. milk protein).

*What is the contribution of the research results to science and society?*

The results presented in this thesis contribute to the scientific field of skeletal muscle metabolism and the regulation of skeletal muscle mass. They help to better understand how individual muscles of the thigh respond to short term leg immobilisation. With this information both physiotherapists and clinicians can help target and investigate interventions that may help alleviate reductions in muscle mass and function not only during disuse, but also enhance muscle recovery during rehabilitation. Furthermore, as we have displayed the drastic detrimental effect of physical inactivity on skeletal muscle mass, future work should investigate the effect of pre-habilitation exercise interventions. Specifically, exercise interventions to increase the mass and function of skeletal muscle prior to a known period of physical inactivity in patients – this could include certain cancer patients or patients undergoing joint replacement surgery. If patients enter treatment in an improved physical condition, it may be the case that the treatment may not have such a detrimental effect on their muscle mass, function and overall wellbeing. Moreover, their recovery would hopefully be enhanced leading to improved discharge rates, relieving a burden on the healthcare system. We identified that a diet that contained an optimal amount, distribution and quality of protein intake was not able to prevent reductions in muscle function and mass during short term disuse. Future work should consider combining interventions for example both neuromuscular electrical stimulation and protein/meal ingestion to assess if this helps alleviate the reduction in muscle mass and function compared to either intervention applied in isolation.

*To whom are the research results relevant?*

Besides the relevance for the scientific community, the results of this thesis are of considerable interest to clinicians and physiotherapists. In this thesis we have investigated the effect of short term period of physical activity on muscle mass and function as well as some countermeasures. Both clinicians and physiotherapists in clinical and sporting settings treat patients/athletes who are undergoing a period of physical inactivity. Such a rapid decline in muscle mass should encourage clinicians and physiotherapists to begin rehabilitation as soon as it is safe to do so during and/or after the period of disuse.

Furthermore, the results presented in this thesis are also applicable to health care policy makers. We clearly demonstrated that short term muscle disuse results in a decline in muscle mass and function. Given the importance of skeletal muscle mass and function to quality of life as well particularly in the elderly, it is essential to implement strategies to make sure patients retain as much of their muscle mass and function, before, during and after a period of inactivity. It is also likely to be beneficial for the addition/improvement of education on the importance of skeletal muscle to overall health and the role a lack of skeletal muscle can play in non-communicable disease, to be added to medical education at the university level. Particularly how the quality, mass and function of skeletal muscle can be maintained during disease or increased during rehabilitation, especially by the implementation of different types of exercise training and nutrition.

Lastly, we displayed that mycoprotein, which is a sustainably produced protein source, has an amino acid bioavailability that is in line with animal proteins. Thus, there is an opportunity for companies in the food industry to develop new protein dense/rich products that could be used either in a clinical setting or at home. Particularly, food industry companies that focus on developing sustainable plant-based proteins have an opportunity to develop environmentally friendly protein dense supplements and food products.

Several communication strategies are applied to inform the various relevant stakeholders. The results are or will be published in international, peer-reviewed journals. The studies have been presented at several conferences and symposia, which increases the visibility of the results and contributes to new insights and ideas for future research.

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This thesis is dedicated to John Redding and Bridget Kilroe.

## *Curriculum Vitae*

Sean Kilroe was born on October 25, 1992 in Chelmsford, United Kingdom. He completed Secondary School at Hylands School in Chelmsford, United Kingdom, in 2011. In the same year he started his bachelor programme in Sport and Exercise Science at Nottingham Trent University. In 2014 he obtained a Bachelor of Science (BSc honours) in the Upper Second Class category after writing a thesis titled 'The Effect of Acute Oral Creatine Administration on Knee Extensor Function'. Soon after this Sean started a Master of Science in Integrated Physiology in Health and Disease at Nottingham University in September 2015. Here Sean gained more knowledge with Human physiology and particularly skeletal muscle physiology. Sean obtained his MSc passing with a Merit grade with a thesis entitled 'Effects of Remote Ischemic Preconditioning on Muscle Blood Flow, Exercise and Mental Performance'. After obtaining his MSc in 2015 Sean began a PhD at Maastricht and Exeter University in January 2016 under the supervision of Prof Luc van Loon, Dr Benjamin Wall and Dr Sarah Jackman. In the years that followed Sean performed multiple human interventions studies that assessed the impact of short-term muscle disuse on skeletal muscle function, mass and metabolism. As well as focussing on how graded protein intakes may influence the muscle response to short-term disuse and alongside this investigating the anabolic potential of sustainable non-animal derived protein source. Throughout the years Sean won a student poster award at the first Exeter Sports Medicine conference in May 2017, Sean was also shortlisted for a Young Investigator Award at the European College of Sport Science in July 2019.

Currently, Sean Kilroe works in the department of Biochemistry, Cellular and Molecular Biology at The University of Texas Medical Branch at Galveston as a Postdoctoral researcher under the supervision of Professor Douglas Paddon-Jones and Professor Blake Rasmussen.

## *List of publications*

Dunlop, M. V., **Kilroe, S. P**., Bowtell, J. L., Finnigan, T. J., Salmon, D. L., & Wall, B. T. (2017). Mycoprotein represents a bioavailable and insulinotropic non-animal-derived dietary protein source: a dose–response study. *British Journal of Nutrition*, *118*(9), 673-685.

**Kilroe, S. P**., Fulford, J. F., Jackman S. R., van Loon, L. J. C., & Wall, B. T. (2020). Temporal and muscle specific disuse atrophy during one week of leg immobilisation in healthy young men. *Medicine and Science in Sports and Exercise*, 52(4), 944-954.

**Kilroe, S. P**., Fulford, J. F., Jackman S. R., Holwerda, A. M., Gijsen, A. P., van Loon, L. J. C., & Wall, B. T. (2020). Short-term muscle disuse results in a rapid and sustained decline in daily myofibrillar protein synthesis rates. *American Journal of Physiology – Endocrinology and Metabolism*, 318(2), 117-130.

**Kilroe, S. P**., Fulford, J. F., Jackman S. R., Holwerda, A. M., Gijsen, A. P., van Loon, L. J. C., & Wall, B. T. (2020). Dietary protein intake does not modulate daily myofibrillar protein synthesis rates or loss of muscle mass and function during short-term immobilisation in young men: a randomized controlled trial. *American Journal of Clinical Nutrition*, 11;113(3):548-561.

Davenport, D. A., Jameson, T. S. O., **Kilroe, S. P**., Monteyne, A. J., Pavis, G. F., Wall, B. T., Dirks, M. L., & Stephens, F. B. (2020). A randomised, placebo-controlled, crossover study investigating the optimal timing of a caffeine-containing supplement for exercise performance. *Sports Medicine-Open*. 6, 1-12.

Jameson, T. S. O., **Kilroe, S. P**., Fulford, J., Abdelrahman, D. R., Murton, A. J., Dirks, M. L., Stephens, F. B., Wall, B. T. (2021). Muscle damaging eccentric exercise attenuates disuse-induced declines in daily myofibrillar protein synthesis and transiently prevents muscle atrophy in healthy men. *American Journal of Physiology – Endocrinology and Metabolism*, 321(5):E674-E688.

Arentson-Lantz, E. J., **Kilroe, S. P.** (2021). Practical applications of whey protein in supporting skeletal muscle maintenance, recovery, and reconditioning. *Journal of Animal Science*. 1;99(4):skab060.

## *Oral and Poster Presentations*

- ECSS (European College of Sport Science) 2019: Muscle Disuse Results In A Rapid Decline In Myofibrillar Protein Synthesis Rates.
- Exeter Sports Medicine conference 2017: Temporal and muscle specific disuse atrophy during one week of leg immobilisation in healthy young men.

## *Awards*

- Best poster at the Exeter Sports Medicine conference 2017.