CHARACTERISING HUMAN MUSCLE PROTEIN SYNTHETIC RESPONSES ACROSS THE PHYSICAL ACTIVITY SPECTRUM

by

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A thesis submitted to

the University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

School of Sport, Exercise and Rehabilitation Sciences

University of Birmingham

July 2019

ABSTRACT

Maintenance of muscle mass, strength and quality across the lifespan is of vital importance. Whilst the impact of some forms of physical activity (i.e., resistance exercise) and inactivity (i.e., muscle disuse) on muscle mass regulation have been well-established, far less is known about the impact of other activities that span the physical activity spectrum. Similarly, strategies to optimise muscle mass regulation have begun to be developed but there is still scope for optimisation by considering different resistance exercise variables. Accordingly, the purpose of this thesis was to characterise the extent to which longer-term muscle protein synthesis rates are altered by differing activities across the physical activity spectrum. In the first experimental study of this thesis, a step reduction model was utilised to determine the impact of reduced physical activity and increased sedentary time on daily myofibrillar protein synthesis rates in healthy young men. This study provided novel data showing that one week of reduced physical activity and increased sedentary time led to a substantial (~27%) decline in daily myofibrillar protein synthesis rates. This was associated with increased skeletal muscle mRNA expression of myostatin and muscle atrophy F-box (MAFbx) and decreased mRNA expression of the mechanistic target of rapamycin (mTOR). Considering these findings, and the potency of resistance exercise to preserve muscle mass, the second experimental study of this thesis sought to compare daily myofibrillar protein synthesis rates over a seven-day period of volume-matched, low frequency and high frequency resistance exercise in young untrained men. The results demonstrated that resistance exercise frequency did not modulate daily myofibrillar protein synthesis rates or the phosphorylation status and total protein content of selected proteins implicated in skeletal muscle ribosomal biogenesis. These novel data showing that resistance exercise frequency did not modulate daily myofibrillar protein synthesis rates are in line with longer-term training studies. Collectively,

the work contained within this thesis has successfully provided new knowledge and a clearer understanding of the extent to which longer-term muscle protein synthesis rates are altered by different activities that span the physical activity spectrum.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisors Dr Gareth Wallis and Professor Janice Thompson. Gareth, I have learnt a lot from your meticulous approach to science and I cannot express how appreciative I am for your guidance and supervision over the last four years. Your scientific integrity is something that I admire and respect greatly. Thank you for always being available for advice in times of need and our many discussions at your office door. It has been a pleasure to have you as a mentor. Janice, thank you for always putting a smile on my face come rain or shine! I will always appreciate the time you made for me regardless of your many other duties as Head of School. You have always been incredibly supportive and I will count myself fortunate if I reach the high standards you set one day.

During my PhD, I was incredibly fortunate to visit and collaborate with Professor Luc van Loon at the M3 Research Unit at Maastricht University. Luc, thank you for welcoming me to Maastricht, the hospitality you showed me both times I visited and introducing me to the Frape! It was an honour to visit a world-leading lab in skeletal muscle metabolism and the data presented in this thesis would not have been possible without your collaboration. Dr Andy Holwerda (and Evelien), thank you for welcoming me into your home and making my time in Maastricht so enjoyable. Andy, it was a pleasure working in the lab and discussing science with you; I learnt a lot from our discussions over many Belgian beers!

I have been fortunate to work with a lot of great people over the eight years I have been within SportExR. Thank you to Steve and Dave for keeping things going on a day-to-day basis within the School. Dr Leigh Breen, thank you for getting me started on my research journey during my Masters. Also, thank you to Dr Yasir Elhassan for providing medical oversight for the studies contained within this thesis. Helen and Colleen, thank you for

teaching me several important lab skills when I was first starting my PhD. Gav, Jamo and Nathan it was a pleasure living with you; it made the PhD journey considerably easier coming home to a great group of mates! Benny, cheers for the many beers, laughs and scientific discussions. To the rest of SportExR postgrads, Ben S, Steve, Stu, Simon, Ste, Sophie Edwards, Seabright, Sophie Joanisse, Sarah, Sardar, Fletch, Jess, Dan Lou, Tim, Ollie and Matt, it was a pleasure working and socialising with you all!

Cara, thank you for your support over the last year or so of this PhD journey. You've made my life infinitely better, kept me going when my motivation has waned and helped me to maintain a work-life balance. To my sister Sophie, thank you for always being supportive of me and keeping an eye on things back home!

Finally, I must express my gratitude to my Mum and Dad. You have been fantastic role models; always incredibly supportive and encouraging me to follow my dreams. I would not have been able to complete my Masters without your financial support and I would not be the man I am today without you both. I am forever grateful and I hope I have made you proud.

CONTENTS LISTING

LIST OF PUBLICATIONS, ABSTRACTS AND AWARDS

Published

Shad BJ, Thompson JL, Holwerda AM, Stocks B, Elhassan YS, Philp A, van Loon LJC, Wallis GA. One week of step reduction lowers myofibrillar protein synthesis rates in young men. Medicine & Science in Sports & Exercise, 2019 May, PMID: 31083048

Mckendry J, Breen L, **Shad BJ**, Greig CA. Muscle Morphology and Performance in Master Athletes: A Systematic Review and Meta-analyses. Aging Research Reviews, 2018 April, PMID: 29715523.

Shad BJ, Wallis GA, van Loon LJC, Thompson JL. Exercise prescription for the older population: The interactions between physical activity, sedentary time, and adequate nutrition in maintaining musculoskeletal health. Maturitas, 2016 November, PMID: 27338978.

In preparation

Shad BJ, Thompson JL, Mckendry J, Holwerda AM, Elhassan YS, Breen L, van Loon LJC, Wallis GA. Daily myofibrillar protein synthesis rates in response to low and high frequency resistance exercise in young men. Target journal: Journal of Applied Physiology.

Abstracts

Shad BJ, Holwerda AM, Elhassan YS, van Loon LJC, Thompson JL, Wallis GA. Impact of Short-term Sedentariness on Week-to-Week Myofibrillar Protein Synthesis Rates in Physically Active Young Men. American College of Sports Medicine (ACSM) Annual Meeting 2018, Minneapolis. Thematic poster presentation.

Awards

American College of Sports Medicine (ACSM) International Student Award. ACSM Annual Meeting 2018, Minneapolis. Award amount - $1,000. The International Student Award honours students from countries outside of the United States and helps fund travel expenses for travelling to the ACSM Annual Meeting.

American College of Sports Medicine (ACSM) Basic Science World Congress Student Award. ACSM Annual Meeting 2018, Minneapolis. Award amount - $1,000. The Basic Science World Congress Student Award supports up to four travel awards each year to help defray the costs of travel for the ACSM Annual Meeting.

LIST OF ABBREVIATIONS

1RM	one repetition maximum
^{2}H	deuterium
$^{2}H_2O$	deuterated water
4E-BP1	4E-binding protein 1
AUC	area under the curve
AV	arteriovenous
BMI	body mass index
BSA	bovine serum albumin
CDK4	cyclin-dependent kinase 4
cDNA	complementary DNA
CQ	quantitation cycle
CSA	cross-sectional area
DC	detergent compatible
DXA	dual-energy x-ray absorptiometry
EAA	essential amino acids
ELISA	enzyme-linked immunosorbent assay
FBR	fractional breakdown rate

FFM	fat-free mass
FSR	fractional synthetic rate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-IRMS	gas chromatography-isotope ratio mass spectrometer
GC-C-IRMS	gas chromatography combustion isotope ratio mass spectrometry
HF	high frequency
HMB	β-hydroxy-β-methylbutyrate
HPA	habitual physical activity
LF	low frequency
MAFbx	muscle atrophy F-box
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	the mechanistic target of rapamycin
mTORC1	the mechanistic target of rapamycin complex 1
MuRF1	muscle RING finger 1
MVPA	moderate-to-vigorous intensity physical activity

NBAL	net protein balance
NEFA	non-esterified fatty acid
OGTT	oral glucose tolerance test
p70S6K	ribosomal protein S6 kinase beta-1
PDK4	pyruvate dehydrogenase kinase 4
PGC-1α	peroxisome proliferator activated receptor gamma coactivator 1-alpha
PIC	pre-initiation complex
PVDF	polyvinylidene fluoride
Ra	rate of appearance
Rd	rate of disappearance
rDNA	ribosomal DNA
rpS6	ribosomal protein S6
rRNA	ribosomal RNA
RT-qPCR	real-time quantitative polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SR	step reduction
TBST	tris-buffered saline tween 20

UBF	upstream binding factor
UK	United Kingdom
US	United States

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Human metabolism is regulated by the intricate synchronisation of multiple organs throughout the body. Of these organs, skeletal muscle undoubtedly plays a central role from both a metabolic and functional perspective (Wolfe, 2006). Skeletal muscle typically accounts for over 50% of total body mass and ~85% of whole-body glucose disposal (DeFronzo *et al.*, 1981) supporting athletic performance, activities of daily living and functional independence into old age. Muscular strength is positively associated with insulin sensitivity (Karelis *et al.*, 2007; Srikanthan & Karlamangla, 2011), functional capacity (Brill *et al.*, 2000) and quality of life (Stegenga *et al.*, 2012). In contrast, the loss of and/or low muscle mass and strength as a consequence of advancing age and various lifestyle alterations (e.g., injury, illness and/or inactivity) is closely associated with cardio-metabolic disease (Atkins *et al.*, 2014), insulin resistance (Dirks *et al.*, 2016b), loss of physical function (Janssen *et al.*, 2002) and consequently, premature death (Bunout *et al.*, 2011; Srikanthan & Karlamangla, 2014). Ultimately, the loss of and/or low muscle mass and strength is implicated in the progressive development of chronic disease states and functional impairments and thus the maintenance of muscle mass, strength and quality across the lifespan is of vital importance. A better understanding of the extent to which muscle mass regulation is altered across the physical activity spectrum is needed before combative strategies against metabolic and functional decline can be developed. This is the focus of this thesis herein.

This chapter begins by highlighting the prevalence of sedentariness and physical inactivity and the increasingly recognised role that these behaviours may play in regulating skeletal muscle mass and physical function. This is followed by an overview of the key physiological processes which regulate skeletal muscle mass (i.e., muscle protein turnover). Thereafter,

studies that have used different experimental models (i.e., muscle disuse vs. step reduction) to assess the impact of physical inactivity and time spent sedentary on skeletal muscle mass, muscular strength and muscle protein turnover are contrasted and discussed. The final part of this chapter discusses the research and rationale behind low volume, high frequency resistance exercise and its potential to maximise muscle mass and strength adaptations in physically inactive and sedentary populations.

1.1.1 Sedentariness: definition, prevalence and health implications

Sedentariness is generally defined as 'any waking behaviour characterised by an energy expenditure ≤1.5 metabolic equivalents while in a sitting or reclining posture' (Sedentary Behaviour Research Network, 2012). Until relatively recently, our ancestors lived a highly active hunter-gatherer lifestyle which required foraging for food, shelter and subsistence in order to survive. Naturally, this demanded that very little time be spent sedentary. In contrast, present day technological advances and modern conveniences have engineered our environment to encourage sedentary behaviour at every opportunity. Global estimates of the prevalence of sedentariness vary significantly due to factors such as age, gender, occupation, socioeconomic status and local infrastructure (Althoff *et al.*, 2017). Nevertheless, estimates in highly developed nations suggest that in excess of 60% of waking hours are spent sedentary in young individuals and this rises with advancing age (Loyen *et al.*, 2017). Daily step count can also be used as a proxy for sedentariness, with <5000 steps·d^{-1} generally considered to be indicative of a sedentary lifestyle (Tudor-Locke *et al.*, 2013). Large-scale global data suggest that the average daily step count is <5000 steps·d^{-1} and that approximately 17% of the US population average <2500 steps·d^{-1}(Tudor-Locke *et al.*, 2009; Althoff *et al.*, 2017). Clearly sedentariness is at its highest level in human history and its prevalence is projected to further increase in the coming decades (Ng & Popkin, 2012).

It has been proposed that a highly sedentary lifestyle contributes to the development of chronic diseases such as obesity, diabetes and cardiovascular disease, increasing the risk of premature mortality (Hamilton *et al.*, 2008; Booth *et al.*, 2017). Early evidence to support this thesis originated from a classic study demonstrating that coronary heart disease incidences and mortality rates were significantly lower in bus conductors who were regularly climbing stairs compared to bus drivers who spent most of their day sitting (i.e., sedentary) (Morris *et al.*, 1953). These early findings are supported by more recent data from over one million men and women, indicating that highly sedentary, physically inactive individuals have a 59% greater mortality rate than physically active, minimally sedentary individuals (Ekelund *et al.*, 2016).

More recently, it has been recognised that sedentary behaviour may also contribute to the age-related loss of skeletal muscle mass and strength (Gianoudis *et al.*, 2015; Foong *et al.*, 2016). This appears to be independent of physical activity (Gianoudis *et al.*, 2015), which is well appreciated as an important factor in the maintenance of skeletal muscle mass and function. Thus, physical inactivity and sedentary behaviour may act independently and in combination to regulate skeletal muscle mass. However, the physiological processes which may contribute to the negative consequences of physical inactivity and sedentary behaviour on skeletal muscle mass and function are relatively unknown. This is important to understand in order to facilitate the development of strategies to maintain muscle mass, physical function and quality of life in highly inactive and sedentary populations. This will be the focus of the first study of this thesis **(Chapter 3)**.

1.1.2 Physical activity: definition, guidelines and participation

Whilst physical inactivity and sedentariness are typically associated with negative health consequences, physical activity is widely considered as the cornerstone to optimal health and well-being. Physical activity is broadly defined as 'any bodily movement produced by skeletal muscles that requires energy expenditure' (World Health Organization, 2010) and is typically divided into two forms: aerobic exercise (e.g., brisk walking, cycling or jogging) and resistance exercise (e.g., lifting weights). Physical activity guidelines generally advise that adults complete a minimum of 150 minutes per week of moderate-to-vigorous intensity physical activity (MVPA) accumulated in bouts of at least 10 minutes at a time, with additional muscle-strengthening activities also recommended at least two times per week in order to maintain or improve strength and physical function (World Health Organization, 2010).

Regular physical activity typically leads to improvements in insulin sensitivity (Houmard *et al.*, 2004; Malin *et al.*, 2013), reductions in adiposity (Malin *et al.*, 2013; Verheggen *et al.*, 2016) and overall improvements in physical function and fitness (O'Donovan *et al.*, 2005; Tieland *et al.*, 2012). However, unique and divergent responses are observed based on the mode of physical activity undertaken. For example, regular aerobic exercise training increases mitochondrial biogenesis, content and function, an adaptive response less commonly observed following prolonged resistance exercise (Wilkinson *et al.*, 2008; Perry *et al.*, 2010; Groennebaek & Vissing, 2017) . Conversely, increases in muscle mass are frequently observed following regular resistance exercise (Mitchell *et al.*, 2012; Tieland *et al.*, 2012; Snijders *et al.*, 2015), a response less commonly observed following aerobic exercise. When combined, aerobic and resistance exercise provide an effective means of maintaining or improving multiple aspects of overall health, well-being and physical function.

Despite the well-appreciated benefits of regular physical activity, the vast majority of individuals are physically inactive (i.e., they fail to meet the current physical activity guidelines). A large body of evidence suggests that 40-50% of individuals in countries such as the United Kingdom (UK) and United States (US) are currently physically inactive (British Heart Foundation, 2015; Centers for Disease Control and Prevention, 2015). However, these figures are acquired primarily from self-report questionnaires which are often highly subjective and open to bias. Objective measures of physical activity obtained using accelerometer devices demonstrate that the prevalence of physical inactivity is actually significantly higher than self-reported data would suggest, with a study of adults in four European countries indicating that 70-95% of individuals failed to meet the current guidelines (Loyen *et al.*, 2017). This suggests that current physical activity guidelines may be unattainable for the vast majority of the global population, particularly those living in highly developed countries.

The lack of participation in regular physical activity has led to the emergence of research focused on novel strategies to improve physical activity participation and mitigate the negative consequences of a physically inactive and sedentary lifestyle. The primary focus of research conducted to date has been on the concept of breaking up prolonged bouts of sitting, typically associated with many office and desk-bound jobs. For example, numerous studies have shown that breaking up prolonged bouts of sitting, typically with two-to-three minute bouts of low-intensity aerobic physical activity (e.g., walking) every 20-30 minutes, lowers postprandial glucose and insulin responses (Dunstan *et al.*, 2012; Peddie *et al.*, 2013; Dempsey *et al.*, 2016). Recent evidence also demonstrates that low-load resistance exercise protects against loss of muscle mass during a prolonged period of reduced physical activity in the older population (Devries *et al.*, 2015). The increasing recognition that sedentariness

across the lifespan may accelerate loss of muscle mass and strength, coupled with the high prevalence of sedentary behaviour and physical inactivity, highlights the need to design physical activity-based strategies that are effective, realistic and attainable (e.g., frequent, small bouts of exercise) for our largely inactive and sedentary society. This will be the focus of the second study of this thesis **(Chapter 4)**.

1.2 Muscle protein turnover

Skeletal muscle is comprised of several subcellular protein fractions, broadly categorised into sarcoplasmic, collagen, mitochondrial and myofibrillar proteins, with the metabolic properties, functionality and turnover rate of each varying considerably (Burd *et al.*, 2010b; Holm *et al.*, 2010; Di Donato *et al.*, 2014). The myofibrillar proteins are particularly important from a functional perspective as they comprise ~65% of all skeletal muscle proteins and encompass the contractile apparatus (i.e., actin, myosin and titin) (Haus *et al.*, 2007). As such, any substantial change in myofibrillar protein content will directly influence one's ability to be physically active and functional to a level that supports one to be self-sufficient.

Skeletal muscle mass is controlled by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB), with the net protein balance (NBAL) of these two dynamic processes determining whether muscle proteins are accrued, maintained or lost. In the postabsorptive state, rates of MPB exceed MPS rates, creating a negative NBAL and loss of muscle proteins (Phillips *et al.*, 1997; Phillips *et al.*, 1999). The consumption of a protein-containing meal provides the stimulus and substrate (i.e., essential amino acids (EAA)) required to increase and sustain postprandial MPS rates for a period of hours (Rennie *et al.*, 1982; Bohe *et al.*, 2003; Moore *et al.*, 2009; Symons *et al.*, 2009b). The ingestion of protein

and/or carbohydrate may also cause a modest suppression of MPB, primarily via the anti-proteolytic effect of insulin (Greenhaff *et al.*, 2008; Glynn *et al.*, 2010; Groen *et al.*, 2015). This simultaneous increase in MPS and decrease in MPB shifts the NBAL to positive and thus muscle proteins are accrued. The result of these daily fluctuations between the postabsorptive and postprandial state is an overall neutral NBAL and thus muscle mass is neither gained nor lost in the majority of healthy, non-exercising, young individuals.

Muscle protein turnover is particularly responsive to changes in muscular contractile activity, with any discernible change in muscle mass driven primarily by a chronic increase or decrease in contractile stimuli in healthy, young individuals. For example, a single bout of resistance exercise increases post-exercise MPS rates (Chesley *et al.*, 1992; Kumar *et al.*, 2009; Fry *et al.*, 2011). However, without sufficient substrate from food ingestion, MPB is also increased and the NBAL remains negative (Phillips *et al.*, 1997; Phillips *et al.*, 1999).

It follows then that the ingestion of food (i.e., a protein-rich meal) in conjunction with resistance exercise acts synergistically to further enhance MPS and supress MPB, shifting the NBAL to positive (Tipton *et al.*, 2007; Reidy *et al.*, 2014). In fact, resistance exercise sensitises skeletal muscle to the anabolic properties of protein-rich food for at least 24 hours, allowing more of the dietary protein-derived amino acids to be incorporated into myofibrillar protein (Burd *et al.*, 2011; Holwerda *et al.*, 2016; Wall *et al.*, 2016a). This synergy between resistance exercise and food ingestion allows a greater proportion of the day to be spent in a positive NBAL and this is thought to underpin increases in muscle mass observed following prolonged and regular resistance training in various populations.

In contrast, removal of muscular contractile activity (e.g., bed rest and limb immobilisation) leads to substantial reductions in postabsorptive and postprandial MPS rates in humans and

reduces the ability of skeletal muscle to utilise dietary protein-derived amino acids for *de novo* myofibrillar protein synthesis (Glover *et al.*, 2008; Symons *et al.*, 2009a; Tanner *et al.*, 2015; Wall *et al.*, 2016b). The role of MPB is less clear but may be implicated in the initial stages of muscle disuse (Abadi *et al.*, 2009; Wall *et al.*, 2014; Tanner *et al.*, 2015). The resultant negative NBAL leads to rapid and substantial loss of muscle mass, which can be observed after as little as five days of lower limb immobilisation (Wall *et al.*, 2016b). Evidently, day-to-day alterations in muscular contractile activity play a central role in determining muscle mass and physical function in later life.

1.2.1 Regulation of muscle protein turnover

Alterations in muscular contractile activity, the ingestion of food, and/or other factors (e.g., ageing) control MPS through up/down-regulation of molecular signalling pathways within skeletal muscle. Of these pathways, the mechanistic target of rapamycin complex 1 (mTORC1) pathway is arguably the most characterised in response to nutritional and contractile alterations. Indeed, it has been well documented that mTORC1 is activated following resistance exercise and/or protein ingestion (Cuthbertson *et al.*, 2005; Burd *et al.*, 2010b; Churchward-Venne *et al.*, 2012; Mitchell *et al.*, 2014) and that pharmacological suppression of mTORC1 activation (via its inhibitor rapamycin) blunts the muscle protein synthetic response to both resistance exercise (Drummond *et al.*, 2009) and EAA ingestion (Dickinson *et al.*, 2011). Thus, mTORC1 appears to play a key role in initiating the muscle protein synthetic machinery.

In contrast, muscle disuse has been shown to blunt the feeding-induced activation of mTORC1 and subsequent muscle protein synthetic response (Drummond *et al.*, 2012; Tanner *et al.*, 2015; Wall *et al.*, 2016b). This blunting of feeding-induced mTORC1 activation

following muscle disuse is accompanied by and may be related to heightened expression of myostatin, a known inhibitor of mTORC1 (Dirks *et al.*, 2014a; Dirks *et al.*, 2014b). Similarly, ageing has been associated with blunted post-exercise mTORC1 signalling (Kumar *et al.*, 2009; Fry *et al.*, 2011) and a reduced muscle protein synthetic response (i.e., anabolic resistance) following resistance exercise (Kumar *et al.*, 2009; Fry *et al.*, 2011; Shad *et al.*, 2016) and protein ingestion (Cuthbertson *et al.*, 2005; Wall *et al.*, 2015b; Shad *et al.*, 2016).

Downstream of mTORC1, ribosomal protein S6 kinase beta-1 (p70S6K) and 4E-binding protein 1 (4E-BP1) promote the synthesis of muscle proteins by activating ribosomal protein S6 (rpS6) and releasing inhibition of eukaryotic translation initiation factor 4E (eIF4E) respectively **(Figure 1.1)**, facilitating the formation of the eukaryotic initiation complex. The eukaryotic initiation complex, composed of several eukaryotic initiation factors, is required for the assembly of a ribosomal subunit, which can subsequently bind to a messenger RNA (mRNA) strand and initiate mRNA translation. mTORC1 activation following resistance exercise and/or protein ingestion is generally accompanied by the phosphorylation of p70S6K and 4E-BP1 in conjunction with elevated MPS rates (Atherton *et al.*, 2010; Burd *et al.*, 2010b; Churchward-Venne *et al.*, 2012). Conversely, the phosphorylation of p70S6K and 4E-BP1 and muscle protein synthetic response following amino acid provision is reduced after bed rest (Drummond *et al.*, 2012), limb immobilisation (Wall *et al.*, 2016b) and in older individuals (Guillet *et al.*, 2004; Cuthbertson *et al.*, 2005). While the upstream signalling events that culminate in the activation of mTORC1 following resistance exercise and/or protein ingestion in human skeletal muscle are less characterised in comparison to events downstream of mTORC1, recent evidence implicates co-localisation and cellular trafficking of mTORC1 and the lysosome to the cell membrane **(Figure 1.1)** (Hodson *et al.*, 2017; Song *et al.*, 2017). Specifically, it has been shown that in the immediate hours following resistance

exercise and/or protein ingestion, the association of mTORC1 (but not mTORC2) and the lysosome with the cell membrane increases significantly (Hodson *et al.*, 2017; Song *et al.*, 2017). Evidently, the mTORC1 signalling pathway holds a central role in initiating and controlling rates of MPS in response to contractile and/or nutritional alterations.

While translational efficiency (i.e., protein synthesis per unit RNA) has an established role in controlling MPS following contractile and/or nutritional alterations, translational capacity (i.e., ribosomal biogenesis) has received considerably less attention (Brook *et al.*, 2019; Figueiredo & McCarthy, 2019). Ribosomal biogenesis occurs in the nucleolus and is the process of transcribing ribosomal DNA (rDNA) to ribosomal RNA (rRNA). The first step (i.e., transcription of rDNA into 45S pre-rRNA by RNA polymerase I) requires binding of the rDNA promotor by a number of transcription factors including upstream binding factor (UBF) and c-Myc which form part of the pre-initiation complex (PIC). The formation and activation of proteins that form the PIC is partly driven by phosphorylation of eIF4E which promotes translation of cyclin D1. Cyclin D1 subsequently binds to and activates cyclin-dependent kinase 4 (CDK4) which ultimately results in the phosphorylation of UBF and subsequent rDNA transcription **(Figure 1.1)**. Once transcribed, 45S pre-rRNA is processed and forms the mature rRNAs (i.e., 5.8S, 18S and 28S) which eventually compose the major subunits of the ribosome.

Emerging evidence has implicated ribosomal biogenesis in resistance training induced skeletal muscle hypertrophy (Figueiredo *et al.*, 2015; Reidy *et al.*, 2017; Mobley *et al.*, 2018) as well as age-related anabolic resistance to muscular contraction (Brook *et al.*, 2016). Indeed, acute resistance exercise has been shown to induce the phosphorylation of eIF4E, increase cyclin D1 and UBF protein expression and elevate 45S pre-rRNA levels over the 48 hour post-exercise period (Figueiredo *et al.*, 2016). Likewise, two recent studies have

demonstrated a significant positive correlation between RNA synthesis (i.e., ribosomal biogenesis) and MPS in response to prolonged resistance training (Brook *et al.*, 2017b; Sieljacks *et al.*, 2019) and there is mounting evidence that markers of ribosomal biogenesis correlate strongly with skeletal muscle hypertrophy (Figueiredo *et al.*, 2015; Reidy *et al.*, 2017). In contrast, older individuals appear to exhibit blunted ribosomal biogenesis, MPS and skeletal muscle hypertrophy compared to younger adults following resistance training (Brook *et al.*, 2016). Collectively, these data highlight an emerging role for ribosomal biogenesis in skeletal muscle hypertrophy that requires further investigation.

Studies that have directly measured MPB rates *in vivo* have typically shown MPB to be less responsive to contractile and/or nutritional alterations than MPS. Nevertheless, the breakdown of skeletal muscle proteins in humans involves a number of proteolytic processes. The ubiquitin – proteasome pathway is one of the primary processes implicated in the ubiquitination and degradation of skeletal muscle proteins. Through the addition of ubiquitin (i.e., ubiquitination), muscle-specific E3 ubiquitin ligases (e.g., muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1)) selectively target muscle proteins for degradation via the 26S proteasome (Bodine & Baehr, 2014).

The response of MAFbx and MuRF1 to an acute bout of resistance exercise is equivocal, with an up-regulation (Borgenvik *et al.*, 2012; Fry *et al.*, 2013), no change (Fry *et al.*, 2013; Stefanetti *et al.*, 2015) and down-regulation (Kostek *et al.*, 2007) all reported. Similarly, the sensitivity of MAFbx and MuRF1 to muscle disuse remains unclear, with some observing an up-regulation (Jones *et al.*, 2004; Tanner *et al.*, 2015; Wall *et al.*, 2016b) and others no change (Wall *et al.*, 2013c; Dirks *et al.*, 2014a). These discrepancies are likely explained by the length of disuse, with up-regulation of the ubiquitin-ligases typically seen in first few days (Wall *et al.*, 2014; Wall *et al.*, 2016b). Clearly, further research is required to define

whether, and to what extent, proteolytic pathways are involved in regulating muscle mass following perturbations in muscular contractile activity and in older age.

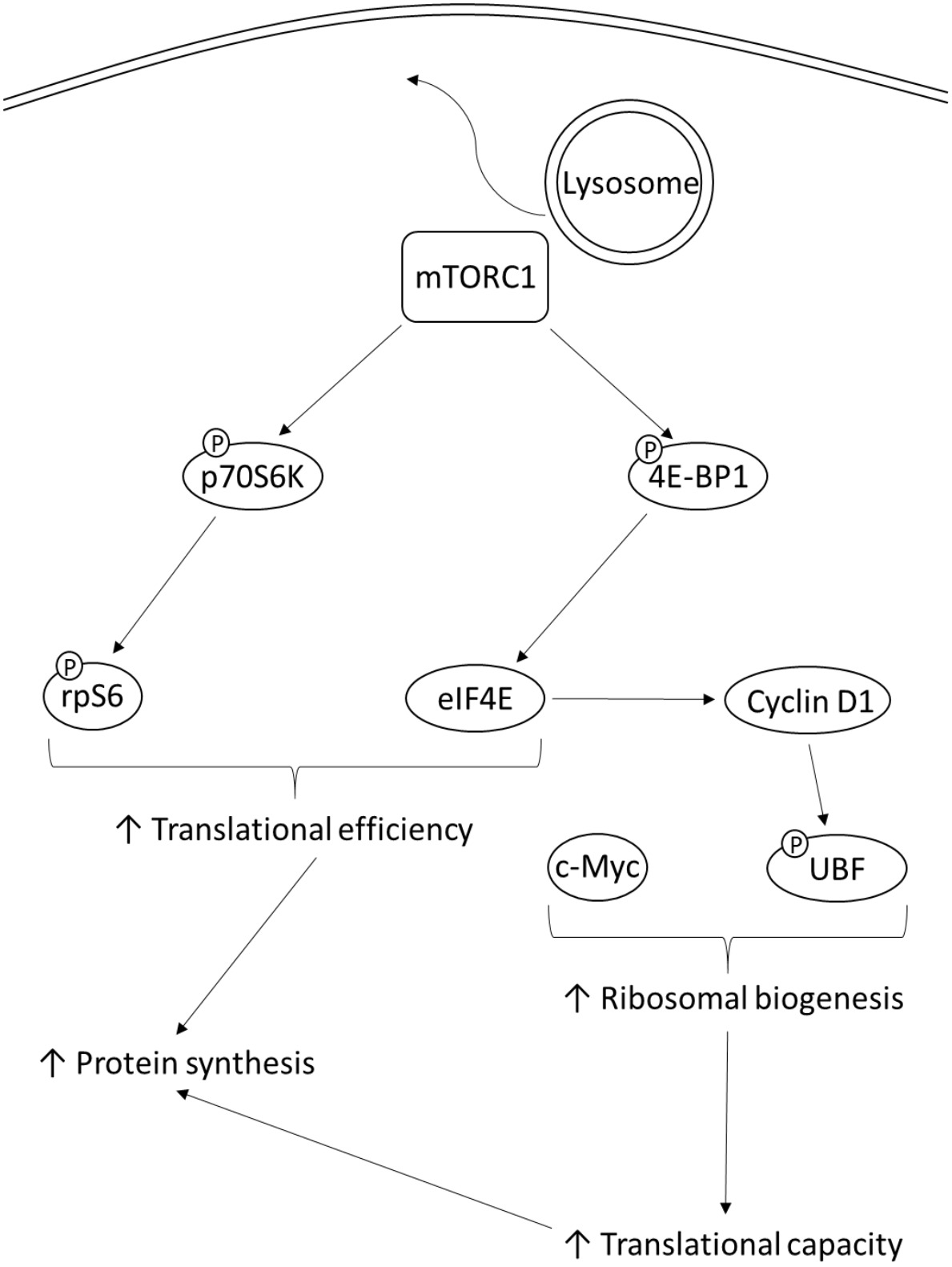

Figure 1.1 Schematic outlining skeletal muscle signalling pathways and events that facilitate an increase in MPS rates following muscular contraction and/or protein/amino acid ingestion.

1.3 The physical activity spectrum

Different modes of physical activity and inactivity can be placed broadly along a spectrum based on variables such as energy requirement and muscular contractile activity **(Figure 1.2)**. Resistance exercise, aerobic exercise and other strenuous physical activities, which have a relatively high energy requirement and involve significant muscular contractile activity, can be placed at the physical activity end of the spectrum. At the opposite end, muscle disuse (i.e., bed rest and limb immobilisation), which requires minimal muscular contractile activity, sits at the physical inactivity end of the spectrum.

To date, the vast majority of studies assessing the impact of physical (in)activity on muscle mass regulation have focussed on the extremes of the spectrum. For example, it has been well established that MPS rates are accelerated following resistance (Chesley *et al.*, 1992; Kumar *et al.*, 2009; Fry *et al.*, 2011) and/or aerobic exercise (Harber *et al.*, 2010; Di Donato *et al.*, 2014) and reduced in response to muscle disuse/unloading in young and older individuals (Glover *et al.*, 2008; Drummond *et al.*, 2012; Wall *et al.*, 2016b). However, the muscle and metabolic responses that occur in response to activities that lie in the middle of the spectrum (i.e., sedentary behaviour and activities of daily living) are far less established. Current recommendations suggest that adults minimise the amount of time spent sedentary for extended periods of time. However, the negative consequences of sedentariness need to become better understood before more specific guidelines on minimising sedentary behaviour can be developed (Hamilton *et al.*, 2008). This is especially important with the knowledge that a large proportion of the global population spend the majority of day-to-day life sedentary.

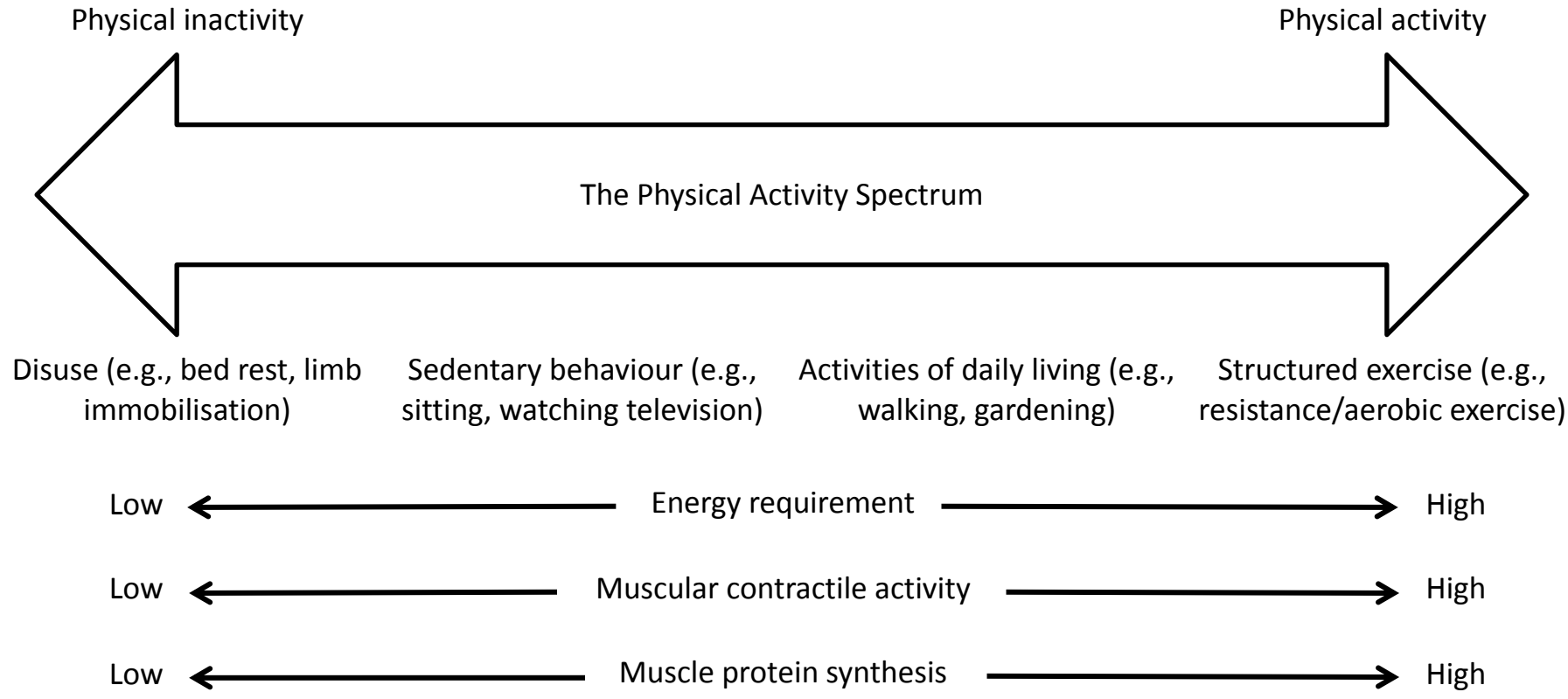

Figure 1.2 Representation of the relative energy requirement and muscular contractile activity required for activities across the physical activity spectrum. MPS rates are increased following a bout of physical activity and reduced following a period of physical inactivity.

1.3.1 Muscle disuse as a model of physical inactivity and sedentary behaviour

It is important to note that much of the evidence used in support of the detrimental health effects of reduced physical activity and increased sedentary behaviour has often been inferred from extreme experimental models (i.e., muscle disuse). Bed rest and single limb immobilisation are the two most commonly used models of muscle disuse/unloading. Bed rest is typically used to study the impact of whole-body disuse on muscle and metabolic health and can be of great clinical relevance when applied over an acute period (<seven days) as it closely mimics the length of stay experienced by patients due to hospitalisation (Fisher *et al.*, 2010). Moreover, bed rest can be applied in a head-down manner to simulate zero-gravity experienced during spaceflight and thus is highly useful for agencies interested in developing combative strategies against muscle and metabolic health deterioration induced by weightlessness. Whereas the bed rest model induces whole-body muscle disuse, limb immobilisation induces muscle disuse locally. Typically, a cast/brace is applied to a single leg while the other is left free to ambulate with the use of crutches. This allows an internal comparison to be made between the immobilised leg and ambulatory leg, allowing the impact of local muscle disuse upon muscle and metabolic parameters to be examined.

1.3.2 Influence of muscle disuse on metabolic health

Both acute (<seven days) and long-term (>10 days) bed rest induces insulin resistance at the whole-body level (Stuart *et al.*, 1988; Yanagibori *et al.*, 1994; Sonne *et al.*, 2010; Dirks *et al.*, 2016b). Defects within skeletal muscle are considered the primary drivers of bed rest-induced insulin resistance, with reductions in mitochondrial content, oxidative capacity and the activity of key glucose uptake/storage regulating proteins (e.g., glucose transporter 4 and hexokinase II) all reported (Bienso *et al.*, 2012; Dirks *et al.*, 2016b). Impaired microvascular

function may also contribute to the development of insulin resistance following bed rest, with decreased reactive hyperaemia observed following five days of bed rest (Hamburg *et al.*, 2007). Other factors purported to mediate bed rest-induced insulin resistance include the accumulation of lipids within skeletal muscle and oxidative stress, although recent evidence fails to support this thesis, with no change observed in either of these parameters following seven days of bed rest (Dirks *et al.*, 2016b).

Although less studied, limb immobilisation can also induce insulin resistance locally, with decreased glucose uptake reported across the thigh following seven days of leg immobilisation (Richter *et al.*, 1989). Similar to bed rest, this appears to be mediated primarily by changes in the activity and expression of genes related to skeletal muscle oxidative capacity and mitochondrial biogenesis in both young and older individuals (Richter *et al.*, 1989; Vigelso *et al.*, 2015; Wall *et al.*, 2015a). Taken together, it is clear that whole-body and local muscle disuse/unloading induce unfavourable alterations within skeletal muscle which may contribute to the development of insulin resistance. These findings highlight the central role that day-to-day muscular contractile activity plays in maintaining metabolic health (Hamilton, 2018).

1.3.3 Influence of muscle disuse on muscle mass and strength

Aforementioned disuse-induced defects within skeletal muscle are mirrored by rapid and substantial loss of muscle mass and declines in muscular strength. The impact of protracted muscle disuse on muscle mass and strength is well recognised, with observed reductions of ~8-18% and ~20-60% in thigh cross-sectional area (CSA) and leg muscle strength, respectively, after one-to-three months bed rest (Convertino *et al.*, 1989; LeBlanc *et al.*, 1992; Berg *et al.*, 1997; Alkner & Tesch, 2004; Brooks *et al.*, 2008). These changes are

mirrored at the muscle fibre level, with ~18-31% atrophy of type I and II fibres generally observed following long-term bed rest (Trappe *et al.*, 2004a; Borina *et al.*, 2010). Similarly, prolonged (i.e., two-to-three weeks) immobilisation induces a ~5-10% and ~15-25% decline in thigh CSA and leg strength in parallel with ~7-30% type I and II fibre atrophy (Hortobagyi *et al.*, 2000; Suetta *et al.*, 2009; Hvid *et al.*, 2010; Oates *et al.*, 2010; Wall *et al.*, 2013c; Wall *et al.*, 2014). The greater relative decline in muscle strength, compared with muscle mass, following bed rest and limb immobilisation is likely a consequence of paralleled declines in neuromuscular function (Seki *et al.*, 2001; Clark *et al.*, 2006; Clark *et al.*, 2008).

More recently, it has become apparent that substantial muscle atrophy and loss of strength also occurs over much shorter timeframes than previously recognised. Indeed, a ~2-4% reduction in thigh CSA and a ~7-10% decline in muscle strength is observed after just five-to-seven days of bed rest or leg immobilisation across varying ages, representing a ~0.6%·d^{-1} leg muscle mass loss (Ferrando *et al.*, 1995; Dirks *et al.*, 2014a; Wall *et al.*, 2014; Tanner *et al.*, 2015; Dirks *et al.*, 2016b; Wall *et al.*, 2016b). The rapid loss of muscle mass and strength that occurs over short periods of disuse, typical of hospitalisation, is of great clinical concern particularly for older adults who are likely to have lower muscle mass, limited physical function and impaired recovery compared to younger individuals. Reductions in muscle mass and strength appear to occur in the absence of discernible type I or II muscle fibre atrophy, suggesting that prolonged (>seven days) muscle unloading is required to induce a substantial reduction in muscle fibre size (Dirks *et al.*, 2014a; Wall *et al.*, 2014; Dirks *et al.*, 2016b). As highlighted by Dirks and colleagues, although loss of leg muscle mass is analogous following bed rest and single leg immobilisation of the same duration, whole-body muscle mass loss is substantially greater following bed rest due to the larger amount of muscle subjected to unloading (Dirks *et al.*, 2016a). Regardless, it is clear that removal of muscular contractile

activity instigates rapid and substantial loss of muscle mass and strength in individuals of all ages.

1.3.4 Influence of muscle disuse on muscle protein turnover

Disuse-induced muscle atrophy must be precipitated by an alteration in muscle protein turnover which shifts NBAL into negative for a sustained period. There is considerable debate as to whether disuse-induced muscle atrophy in humans is primarily caused by decreased MPS, accelerated MPB, or a combination of the two (Phillips & McGlory, 2014; Reid *et al.*, 2014).

Seminal studies established that postabsorptive MPS rates are significantly lowered following prolonged (i.e., five-to-six weeks) single leg immobilisation as a result of tibial fracture (Gibson *et al.*, 1987; Gibson *et al.*, 1988). However, it is difficult to discern whether these findings are a result of muscle disuse *per se*, tibial fracture (and the accompanying inflammatory healing process), or a combination of the two. Since then, it has become clear that prolonged bed rest and single limb immobilisation triggers substantial depression of postabsorptive MPS in young and older adults (Ferrando *et al.*, 1997; Kortebein *et al.*, 2007; Symons *et al.*, 2009a; English *et al.*, 2016). In fact, recent evidence has shown that even short-term (i.e., five days) single leg immobilisation produces large (41%) declines in postabsorptive myofibrillar protein synthesis rates, at least in young individuals (Wall *et al.*, 2016b). Clearly the depression of postabsorptive MPS plays a significant role in short-term and prolonged disuse-induced human muscle atrophy.

Less clear is the response of MPB rates following muscle disuse. This is largely explained by the technical challenges encountered in accurately determining dynamic MPB rates in humans *in vivo*. The single study that has determined postabsorptive MPB rates (i.e.,

fractional breakdown rate (FBR)) suggests that MPB is unaltered by protracted bed rest (Symons *et al.*, 2009a). However, it should be noted that increased mRNA expression of a number of molecular markers of MPB (e.g., MuRF1 and MAFbx) has been observed following 10-14 days muscle disuse in some, but not all studies (Jones *et al.*, 2004; de Boer *et al.*, 2007; Wall *et al.*, 2013c; Wall *et al.*, 2014). Interestingly, evidence suggests that MPB may play a more central role in the early phase of disuse muscle atrophy, with a growing number of studies observing up-regulated mRNA expression of the ubiquitin-ligases following two-to-five days muscle disuse (Abadi *et al.*, 2009; Dirks *et al.*, 2014a; Wall *et al.*, 2014; Tanner *et al.*, 2015; Wall *et al.*, 2016b). Further research will be required to define the precise role that MPB plays in short-term and prolonged disuse-induced human muscle atrophy.

Maintenance of a robust and sustained muscle protein synthetic response to protein-containing nutrition during periods of muscle disuse is essential in minimising muscle loss. However, growing evidence suggests that muscle disuse/unloading induces the rapid development of 'anabolic resistance' to protein/amino acid provision. Indeed, 14 days of single leg immobilisation blunts the muscle protein synthetic response to intravenous amino acid provision and dietary protein ingestion in young individuals (Glover *et al.*, 2008; Wall *et al.*, 2013c). Likewise, short-term muscle disuse reduces the EAA-induced increase in MPS in older individuals subjected to five-to-seven days bed rest (Drummond *et al.*, 2012; Tanner *et al.*, 2015) and in young individuals, the myofibrillar protein synthetic response to dietary protein ingestion is lowered by 53% following only five days of single leg immobilisation (Wall *et al.*, 2016b). Mechanistically, the development of anabolic resistance following disuse is thought to be, in part, explained by blunting of the feeding-induced activation of mTORC1 and downstream signalling proteins (i.e., p70S6K and 4E-BP1) (Drummond *et al.*,

2012; Tanner *et al.*, 2015; Wall *et al.*, 2016b), although other defects such as impaired amino acid transport (Drummond *et al.*, 2012) and reduced nutritive blood flow (Timmerman *et al.*, 2012) may also be implicated.

Collectively, the available evidence suggests that human disuse muscle atrophy is primarily brought about by reductions in postabsorptive MPS rates and blunting of the muscle protein synthetic response to protein/amino acid provision, although accelerated MPB may be implicated in the early muscle disuse atrophy phase.

1.4 Step reduction as a model of physical inactivity and sedentary behaviour

Whilst disuse models provide invaluable information on the impact of severe muscle unloading, their extreme nature may not accurately reflect typical physically inactive and sedentary lifestyles (Perkin *et al.*, 2016). Even those who are highly inactive and sedentary complete low amounts of ambulation (i.e., activities of daily living), and thus muscular contractions, on a daily basis. As such, the step reduction model, which typically requires habitually active participants to reduce their daily step count to a specified number (generally 1500-2000 steps·d^{-1}) for a period of one-to-two weeks, has been proposed as an alternative model which may more accurately reflect the underlying physiology of physically inactive and sedentary individuals (Perkin *et al.*, 2016).

One major advantage of the step reduction model is that it allows the consequences of a highly inactive and sedentary lifestyle to be examined using a within-subject design, controlling for genetic and environmental (e.g., dietary intake, sleep quality) variability which may confound results when comparing habitually active and sedentary individuals in a cross-sectional manner. In addition, the step reduction model closely mimics the reduction in physical activity and increase in sedentary time that commonly occurs during the transition

from adolescence to adulthood and during various other significant life events (e.g., education, employment, parenthood, marriage) (Bell & Lee, 2005; Zick *et al.*, 2007; Brown *et al.*, 2009; Larouche *et al.*, 2012). What impact these life events that reduce physical activity levels and increase engagement in sedentary behaviours have on muscle and metabolic processes remains relatively unknown.

1.4.1 Influence of step reduction on metabolic health

Step reduction has consistently been shown to induce the development of whole-body insulin resistance and/or reduce skeletal muscle insulin sensitivity. Indeed, 14 days of step reduction increases postprandial glucose and insulin responses, irrespective of age (Olsen *et al.*, 2008; Breen *et al.*, 2013; Bowden Davies *et al.*, 2018; McGlory *et al.*, 2018; Reidy *et al.*, 2018). These metabolic alterations may be related to a reduction in skeletal muscle insulin sensitivity as opposed to increased hepatic insulin resistance, at least when assessed in young individuals using a hyperinsulinaemic-euglycaemic clamp (Krogh-Madsen *et al.*, 2010). This link between inactivity and insulin resistance is of great clinical concern and is likely to be implicated in the development of type 2 diabetes, obesity and other chronic diseases (Thyfault & Krogh-Madsen, 2011).

More recently, it has become clear that even short-term (one-to-seven days) step reduction reduces markers of insulin sensitivity in young and older individuals (Stephens *et al.*, 2011; Mikus *et al.*, 2012; Dixon *et al.*, 2013; Lyden *et al.*, 2015). In contrast to longer-term step reduction, this reduced insulin sensitivity appears to be associated solely with an increased postprandial insulin response, whereas the postprandial glucose response remains largely unchanged (Mikus *et al.*, 2012; Lyden *et al.*, 2015; Reynolds *et al.*, 2015). Interestingly, the aforementioned metabolic alterations have been observed with modest reductions (50-70%)

in daily step count (Mikus *et al.*, 2012; Walhin *et al.*, 2013; Lyden *et al.*, 2015; Reynolds *et al.*, 2015) and may take a significant amount of time to return back to baseline levels (Reynolds *et al.*, 2015; Bowden Davies *et al.*, 2018; McGlory *et al.*, 2018; Reidy *et al.*, 2018). Taken together, it is clear that the transition to a physically inactive and sedentary lifestyle induces rapid declines in insulin sensitivity that may take extended periods of time to fully recover from.

Promisingly, emerging evidence suggests that regular breaks during prolonged bouts of sedentary behaviour (e.g., sitting) can improve glycaemic control in both healthy and 'at risk' populations. For example, it has been shown that breaking up sitting with two minute bouts of light or moderate intensity walking every 20-30 minutes lowers postprandial plasma glucose and insulin responses in healthy, normal-weight adults as well as those that are overweight/obese and/or have diabetes (Dunstan *et al.*, 2012; Peddie *et al.*, 2013; Dempsey *et al.*, 2016). Interestingly, breaking up sitting with bouts of standing appears to be less effective than breaking sitting up with walking, suggesting that muscular contractile activity is an important determinant of the observed improvements in insulin sensitivity and glycaemic control (Bailey & Locke, 2015; Henson *et al.*, 2015; Hamilton, 2018). Indeed, this notion is supported by recent evidence showing similar improvements in postprandial glucose and insulin responses when prolonged sitting is broken up with either walking or resistance exercise in overweight/obese individuals with type two diabetes (Dempsey *et al.*, 2016). Collectively, these findings highlight the importance of maintaining a minimum amount of daily muscular contractile activity in order to avoid the negative health consequences associated with prolonged sedentary behaviour (Hamilton, 2018).

1.4.2 Influence of step reduction on muscle mass and strength

Whereas muscle disuse leads to rapid and substantial loss of muscle mass and strength, the impact of step reduction on these parameters is less clear. Whilst some studies have observed significant loss of total-body lean/fat-free mass (FFM) following 14 days of step reduction (Olsen *et al.*, 2008; Bowden Davies *et al.*, 2018), others have failed to observe any change (Breen *et al.*, 2013; Devries *et al.*, 2015; McGlory *et al.*, 2018; Reidy *et al.*, 2018). When compartmentalised, prolonged step reduction induces significant loss of lower but not upper-body lean mass, presumably as the lower limb muscles are subjected to greater unloading (Krogh-Madsen *et al.*, 2010; Breen *et al.*, 2013; Devries *et al.*, 2015; Bowden Davies *et al.*, 2018; Reidy *et al.*, 2018). However, it is important to note that all of the aforementioned studies used dual-energy x-ray absorptiometry (DXA) to assess muscle mass changes following step reduction. As all of the observed changes in muscle mass are within the error of measurement for DXA (Wosje *et al.*, 2006), more sensitive measures of muscle mass (e.g., magnetic resonance imaging (MRI)) may be required to further our understanding of the impact of reduced physical activity and increased sedentary behaviour on muscle loss.

At the muscle fibre level, type I and II fibre CSA are unaffected by prolonged step reduction (McGlory *et al.*, 2018; Reidy *et al.*, 2018), which is unsurprising given that 14 days of muscle disuse is generally required to detect significant muscle fibre atrophy (Wall *et al.*, 2014; Dirks *et al.*, 2016b). In relation to muscular strength, studies to date have generally shown that step reduction does not lead to loss of isometric or isokinetic muscular strength or indices of physical function. Overall, reduced physical activity and increased time spent sedentary induced by prolonged step reduction generally leads to a small loss of muscle mass, specific to the lower limbs, whereas muscular strength and physical function remain unaffected, at least in a short-term setting.

1.4.3 Influence of step reduction on muscle protein turnover

Step reduction-induced muscle loss must be underpinned by a reduction in MPS, an increase in MPB, or a combination of the two. Breen and colleagues were the first to demonstrate that postabsorptive myofibrillar protein synthesis rates are unaffected by 14 days of step reduction in the older population (Breen *et al.*, 2013). However, it was also shown that step reduction lead to the development of 'anabolic resistance' to protein ingestion in conjunction with reduced feeding-induced activation of 4E-BP1 (Breen *et al.*, 2013). This is in contrast to disuse-induced muscle atrophy, which is underpinned by declines in both postabsorptive and postprandial MPS rates (Drummond *et al.*, 2012; Tanner *et al.*, 2015; English *et al.*, 2016; Wall *et al.*, 2016b). Why step reduction only appears to affect postprandial MPS rates is unclear but may be related to the severity of inactivity imposed in comparison to complete muscle disuse. A follow-up study from Devries et al. compared older males who had one leg undergo step reduction whilst the other leg underwent step reduction with the addition of low-load resistance exercise three times per week (Devries *et al.*, 2015). Postabsorptive and postprandial myofibrillar protein synthesis rates were found to be lower in the step reduction only condition compared to the step reduction plus resistance exercise condition (Devries *et al.*, 2015). However, as myofibrillar protein synthesis rates were only assessed at the end of the step reduction period, it is difficult to discern whether this was due to a decline in MPS rates following step reduction or an increase in MPS rates due to the resistance exercise.

Whilst the two aforementioned studies measured MPS rates acutely over a period of hours, a recent study utilised deuterated water (^2H_2O) to assess myofibrillar protein synthesis rates continuously throughout a 14 day period of step reduction in older males and females, showing that integrated myofibrillar protein synthesis rates were depressed throughout the step reduction period and failed to return back to baseline following 14 days of ambulatory recovery (McGlory *et al.*, 2018). To date, no study has assessed the impact of step reduction

on dynamic rates of MPB, although gene expression of proteolytic markers was found to be unchanged after 14 days of step reduction in the elderly (McGlory *et al.*, 2018). The aforementioned studies describe responses in older, overweight individuals but young and older individuals have distinct responses to muscle disuse (Suetta *et al.*, 2009; Tanner *et al.*, 2015). The influence of step reduction on skeletal muscle mass regulation in young individuals has not been studied but is important to characterise in order to understand the impact of reduced physical activity and increased sedentary time on skeletal muscle mass regulation across different stages of the lifespan. Accordingly, the research conducted within this PhD sought to determine the impact of a short-term (i.e., one week) reduction in physical activity and increase in sedentary time on muscle mass regulation in a healthy, young population. This study is presented in **Chapter 3**.

1.5 The case for novel resistance exercise-based strategies

Current physical activity guidelines recommend individuals engage in some form of resistance exercise at least two times per week in order to maintain or improve strength and physical function (World Health Organization, 2010). Indeed, resistance exercise is a highly potent stimulus for increasing and/or maintaining muscle mass and functional independence. However, most people fail to achieve these recommendations. Reasons for this are multifactorial but can be influenced by many factors including body mass index (BMI), age, culture, gender and one's self-efficacy to engage in exercise (Moschny *et al.*, 2011; Ashton *et al.*, 2017). Commonly cited internal barriers to physical activity participation include tiredness, lack of motivation/willpower, laziness and self-identifying as not being an active or 'sporty' person (Ziebland *et al.*, 1998; Justine *et al.*, 2013; Ashton *et al.*, 2017). Similarly, there are many external barriers to physical activity participation including poor health, lack of accessibility/transport, cost of equipment/facilities and lacking the time to exercise

(Moschny *et al.*, 2011; Justine *et al.*, 2013; Ashton *et al.*, 2017). The low adherence to current physical activity guidelines highlights the need to design resistance exercise-based strategies that are effective, realistic and time-efficient for physically inactive and/or sedentary populations, as this may help to minimise muscle loss and decrements in metabolic health associated with a physical inactive and/or sedentary lifestyle.

1.5.1 Manipulation of resistance exercise variables to maximise muscle mass and strength

A number of variables can be manipulated in order to maximise muscle mass and strength gains in response to resistance exercise. Commonly manipulated variables include absolute load (Burd *et al.*, 2010b; Mitchell *et al.*, 2012; Schoenfeld *et al.*, 2017a), total exercise volume (Burd *et al.*, 2010a; Burd *et al.*, 2010b), proximity to failure (Burd *et al.*, 2010b; Nobrega & Libardi, 2016) and rest interval between sets (McKendry *et al.*, 2016; Schoenfeld *et al.*, 2016b).

Absolute load represents the amount of weight lifted and is often expressed relatively (e.g., as a percentage of one repetition maximum (1RM)). When the absolute load lifted each repetition is multiplied by the number of repetitions performed (i.e., absolute load (kg) x *n* repetitions), total exercise volume can be calculated. It has now become clear that a comparable myofibrillar protein synthetic response and degree of muscle hypertrophy can be achieved using low (i.e., 30% 1RM) or high (80% 1RM) loads in both untrained and trained individuals (Burd *et al.*, 2010b; Schoenfeld *et al.*, 2015a; Morton *et al.*, 2016; Schoenfeld *et al.*, 2017a). However, this is provided that the exercise is taken close to the point of muscular failure, otherwise low load resistance exercise produces a suboptimal stimulation of myofibrillar protein synthesis and muscle hypertrophic response (Holm *et al.*, 2008; Burd *et*

al., 2010b; Holm *et al.*, 2010). These findings suggest that when resistance exercise is completed to the point of or close to muscular/contractile failure, type II muscle fibres are adequately recruited and a similar degree of muscle hypertrophy can be achieved, independent of absolute load or total exercise volume. It is important to note that strength adaptations are typically greater when high load resistance exercise is completed, although this can be largely made up for with periodic practice of the chosen strength outcome when using lower loads (Mitchell *et al.*, 2012; Schoenfeld *et al.*, 2015a; Morton *et al.*, 2016).

A less studied resistance exercise variable that can be manipulated is the frequency with which each bout of resistance exercise is completed (i.e., the number of sessions completed per muscle group over a given length of time). Some authors have speculated that low volume, high frequency resistance exercise may be an effective strategy to maximise muscular adaptations to training (Schoenfeld *et al.*, 2015b; Dankel *et al.*, 2017). This would require minimal daily time commitment and could also be completed in a non-gym-based setting (e.g., at home). As such, low volume, high frequency resistance exercise could be of particular relevance to sedentary/physically inactive individuals who do not have the time, ability and/or inclination to exercise in a gym-based setting (Justine *et al.*, 2013; Richardson *et al.*, 2017). The rationale for low volume, high frequency resistance exercise will be presented below.

1.5.2 Rationale for considering more frequent resistance exercise

While exercise volume influences muscular adaptations to resistance exercise, a relatively low volume (~three sets) of high load resistance exercise appears to maximise post-exercise myofibrillar protein synthesis rates, at least in young individuals (Burd *et al.*, 2010a; Kumar *et al.*, 2012). As such, the completion of particularly high exercise volumes may be of little

value if the primary goal is to maximally stimulate the post-exercise myofibrillar protein synthetic response and may simply delay subsequent recovery time (Gomes *et al.*, 2018).

It is generally recommended that each muscle group be targeted two-to-three times per week in order to maximise muscular adaptations to training (American College of Sports Medicine, 2009). However, a recent survey in competitive bodybuilders showed that all respondents trained each muscle group one-to-two times per week (Hackett *et al.*, 2013), and this is likely to be even lower in those who recreationally participate in resistance exercise. This practice may be suboptimal as it has been shown that resistance exercise sensitises skeletal muscle to the anabolic properties of protein-based nutrition for approximately 24 hours and allows more of the dietary protein-derived amino acids to be incorporated into muscle protein (Burd *et al.*, 2011; Holwerda *et al.*, 2016; Wall *et al.*, 2016a). Thus, low volume, high frequency resistance exercise could, theoretically, provide an optimal strategy to regularly stimulate myofibrillar protein synthesis and sensitise skeletal muscle to subsequent protein feeding, whilst avoiding 'wasted' exercise volume that fails to further stimulate MPS rates. This hypothetical concept is depicted in **Figure 1.3**.

Some authors have posited that a higher resistance exercise frequency may be more relevant to trained individuals as the muscle protein synthetic response becomes of shorter duration with prolonged resistance training and thus more frequent stimulation of MPS may be particularly effective in this population (Dankel *et al.*, 2017). However, this notion is based primarily on findings in mixed-muscle protein (Kim *et al.*, 2005; Tang *et al.*, 2008); whereas the duration of the myofibrillar protein synthetic response to resistance exercise is less altered by training (Kim *et al.*, 2005; Wilkinson *et al.*, 2008; Damas *et al.*, 2016). As such, low volume, high frequency resistance exercise may prove to be effective at increasing and/or maintaining muscle mass in various populations.

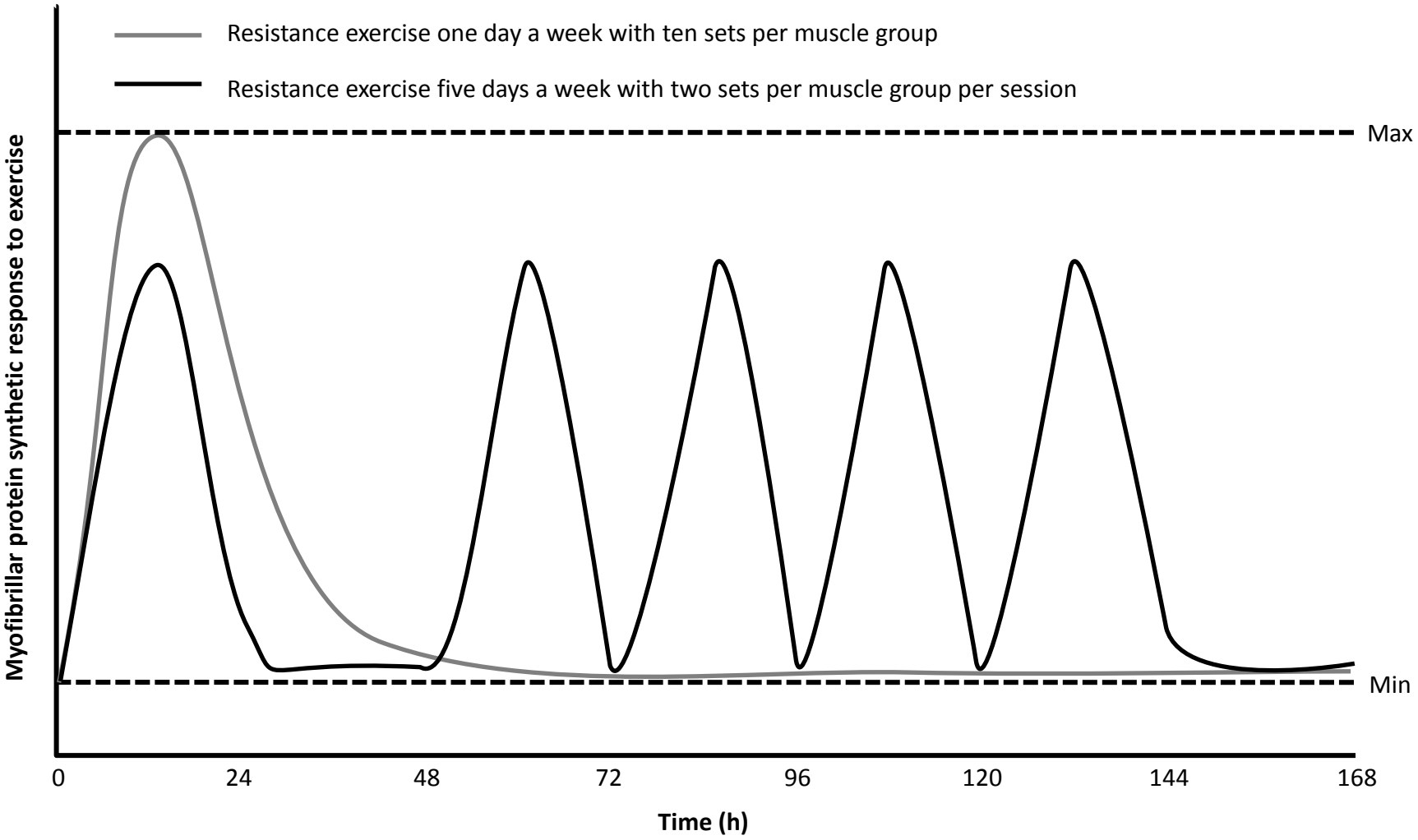

Figure 1.3 Hypothetical depiction of the myofibrillar protein synthetic response to volume-matched low frequency (grey line) or high frequency (black line) resistance exercise over a one-week period. Adapted from (Dankel *et al.*, 2017).

1.5.3 Influence of exercise frequency on muscle mass and strength adaptations to resistance exercise

Early studies examining resistance exercise frequency demonstrated that more frequent resistance exercise is associated with greater muscular strength adaptations. For example, following seven weeks of total-body resistance training, bench press 1RM increased significantly more in young untrained subjects training with a higher frequency (Hunter, 1985). Similarly, McLester and colleagues found that leg press 1RM increased significantly more in young individuals training three times per week compared to those training once per week, even when matched for total exercise volume (McLester *et al.*, 2000). While these early findings are promising, more recent studies have failed to replicate these findings (Schoenfeld *et al.*, 2015b; Barcelos *et al.*, 2018; Gomes *et al.*, 2018). Similarly, most studies have found no difference in the muscle hypertrophic response to low and high frequency resistance training (Brigatto *et al.*, 2018; Gomes *et al.*, 2018; Ochi *et al.*, 2018). This would suggest that manipulating resistance exercise frequency may be an ineffective strategy to maximise muscular adaptations to resistance training.

However, there are a number of possible caveats that should be considered. Firstly, the training status and age of subjects in these studies varied widely. It is well known that it becomes more challenging to increase muscle mass and strength as an individual becomes more accustomed to resistance training (Ahtiainen *et al.*, 2003; Ogasawara *et al.*, 2013; Brook *et al.*, 2015) and/or older (Greig *et al.*, 2011; Brook *et al.*, 2016). As such, it is conceivable that some of the discrepancies in the literature are related to differences in the population studied.

Secondly, all studies to date have used ultrasound or DXA to assess changes in muscle mass following low or high frequency resistance training. Whilst these measures are valuable, it may be that larger sample sizes and/or more sensitive measures of muscle mass (i.e., MRI) are needed to detect subtle differences between groups.

Finally, a large amount of studies failed to control for total exercise volume (Fisher *et al.*, 2013; Thomas & Burns, 2016; Barcelos *et al.*, 2018; Gomes *et al.*, 2018). Some authors have argued that the potential superiority of high frequency resistance exercise lies in that it allows for a greater amount of exercise volume to be completed (Dankel *et al.*, 2017; Grgic *et al.*, 2018). Whilst true, failing to match total exercise volume precludes conclusions from being made about frequency *per se*, as any differences between groups could be due to differences in the amount of resistance exercise undertaken. Indeed, evidence suggests that exercise volume influences the magnitude of the myofibrillar protein synthetic and subsequent hypertrophic response to resistance exercise (Burd *et al.*, 2010a; Schoenfeld *et al.*, 2017b).

Acknowledging a number of these caveats, several recent meta-analyses have provided further insight on the impact of resistance exercise frequency on muscle strength and hypertrophy. Interestingly, high frequency resistance training is not associated with enhanced strength adaptations when total exercise volume is equated (Grgic *et al.*, 2018). On the other hand, high frequency resistance training may (Schoenfeld *et al.*, 2016a) or may not (Grgic *et al.*, 2019) promote superior muscle hypertrophy when total exercise volume is matched. It is worth nothing that the vast majority of studies have examined the impact of resistance exercise frequencies in the range of one-to-three times per week. Far less research has been conducted on the impact of higher frequency resistance training (e.g., five times per week) on muscle adaptations to resistance training. Nevertheless, it appears that high frequency

resistance training *per se* may be of more benefit if the goal is to maximise muscle hypertrophy rather than strength adaptations.

1.5.4 Influence of exercise frequency on the muscle protein turnover response to resistance exercise

MPS rates are elevated following the completion of resistance exercise for a given muscle group (MacDougall *et al.*, 1995; Trappe *et al.*, 2004b; Kumar *et al.*, 2009). Theoretically then, it follows that higher frequency resistance training would result in more frequent elevations in MPS and enhance the sensitivity of skeletal muscle to subsequent protein feeding, resulting in a greater overall NBAL. However, no study has yet determined MPS rates when using different resistance exercise frequencies.

It is possible that very high resistance exercise frequencies are required to enhance MPS rates and subsequent muscle hypertrophy. For example, two recent studies compared the effect of whole-body resistance training on muscle hypertrophy and strength adaptations when training a muscle group either one or five times per week (Gomes *et al.*, 2018; Zaroni *et al.*, 2018). In both studies, all subjects completed resistance training five times per week over an eight week period. However, one group trained using a split training routine (i.e., each muscle group was trained once per week) whilst the other group trained using a total-body routine (i.e., each muscle group was trained five times per week). Whilst Gomes et al. found no difference between groups in muscle hypertrophic or strength outcomes, Zaroni and colleagues found that the group that trained each muscle group five times per week had a superior muscle hypertrophic response than the group training each muscle group once per week (Gomes *et al.*, 2018; Zaroni *et al.*, 2018). Thus, it remains unclear whether relatively

high frequency resistance exercise offers a hypertrophic and/or strength adaptation advantage over considerably lower frequency resistance exercise.

It is worth noting that both studies were conducted over an eight week period and in trained individuals. It generally becomes more challenging to increase muscle mass and strength as an individual becomes accustomed to resistance exercise (Ahtiainen *et al.*, 2003; Brook *et al.*, 2015) and thus it is possible that these studies were of insufficient length to detect differences between groups. Considering these limitations, and the proposition that high frequency resistance exercise leads to greater overall MPS rates, the research conducted within this PhD sought to compare daily myofibrillar protein synthesis rates over a seven-day period of low frequency and high frequency resistance exercise in young individuals. This study is presented in **Chapter 4**.

1.6 Summary and overall aims

To summarise, physical inactivity and sedentariness is highly prevalent and a growing public health concern. Extreme physical inactivity (i.e., muscle disuse) results in rapid and substantial loss of muscle mass and strength. However, disuse models do not accurately reflect typical physically inactive and sedentary lifestyles. Recent observational evidence has linked physical inactivity and increased sedentary time with the age-related loss of muscle mass and strength. The impact of physical inactivity and increased time spent sedentary on muscle mass regulation has begun to be addressed, but the physiological processes which may contribute to the negative consequences of physical inactivity and high levels of sedentary time on skeletal muscle mass and function are poorly defined. This is important to understand in order to develop strategies to maintain muscle mass, physical function and quality of life in highly inactive and sedentary populations.

Accordingly, **Chapter 3** describes a study with the primary purpose of using a step reduction model to determine the impact of one week of reduced physical activity and increased sedentary time on daily myofibrillar protein synthesis rates in healthy young men. The secondary purpose of this study was to explore the effects of reduced physical activity and increased sedentary time on glucose tolerance and estimated whole-body insulin sensitivity, and the skeletal muscle gene expression of selected proteins related to muscle mass regulation and oxidative metabolism. It was hypothesised that daily myofibrillar protein synthesis rates would be reduced following one week of step reduction.

The majority of individuals in highly developed nations are physically inactive and this is linked to accelerated loss of muscle mass and strength over the lifespan. As such, there is a need to design resistance exercise-based strategies that are attainable for physically inactive populations. Although there are many barriers to physical activity participation, time is often cited as a major barrier. Low volume, high frequency resistance exercise could be valuable as it involves minimal daily time commitment and has also been suggested as a potential strategy to maximise the muscle protein synthetic response to resistance exercise. This hypothesis remains to be directly tested but if proven to be effective, low volume, high frequency resistance exercise could be used as a novel strategy to increase physical activity levels and ultimately slow the gradual decline in muscle mass and physical function across the lifespan. Therefore, **Chapter 4** describes a study which aimed to compare daily myofibrillar protein synthesis rates over a seven-day period of volume-matched, low frequency and high frequency resistance exercise in young, untrained men.

Chapter 5 provides a discussion and critical overview of findings from these studies in the context of the existing literature. Avenues for future research and the practical implications of these findings are presented and discussed.

CHAPTER 2

GENERAL METHODS

2.1 Introduction

The aim of this chapter is to outline the principles and underlying assumptions of the key methods adopted during data collection for this thesis, and provide a rationale for their use. The specific procedures employed in each study can be found in the methods section of the relevant experimental chapter.

2.2 Quantification of physical activity and sedentary behaviour

In **Chapter 3**, free-living sedentary, standing and ambulatory activity was quantified using an ActivPAL3^{TM} accelerometer (PAL Technologies Ltd., Glasgow, UK) to confirm that the step reduction intervention was successful at inducing an increase in sedentary time and a reduction in daily step count.

Physical activity levels and sedentary time were objectively assessed, as self-reported measures of these parameters are highly subjective, susceptible to bias and show low agreement with objective measurements (Busschaert *et al.*, 2015; Chastin *et al.*, 2018). In the absence of direct observation, which is highly impractical and laborious, the ActivPAL3^{TM} accelerometer is generally considered as the gold standard to estimate physical activity and sedentary parameters (Kozey-Keadle *et al.*, 2011; Lyden *et al.*, 2012; Sellers *et al.*, 2016). Using accelerometer-based feedback on thigh position, the ActivPAL3^{TM} device can estimate postural changes and thus estimate the amount of time spent sitting, standing and ambulatory.

2.3 Body composition assessment

In **Chapter 3**, body composition was assessed at baseline using DXA (Discovery QDR W series; Hologic). The use of DXA to estimate body composition relies on differentiating the distinct densities of fat and fat-free (bone and muscle) tissue. The DXA scanner transmits

low-energy x-ray beams from two different sources to the subject. As the density of fat and FFM differ (i.e., bone tissue is considerably denser than fat tissue), the amount of radiation attenuated by tissues of the body can be measured and distinguished to quantify the relative mass of bone, fat and muscle tissue. DXA was chosen as it is a quick (~seven minutes) and non-invasive method to estimate body composition and is considered a valid tool for estimating skeletal muscle mass and adiposity in a wide range of populations (Glickman *et al.*, 2004; Chen *et al.*, 2007). Although participants are exposed to radiation, the amount is extremely small and of minimal concern.

2.4 Estimation of habitual energy and macronutrient intake

In **Chapters 3** and **4**, habitual energy and macronutrient intakes were estimated using self-reported weighed four-day food diaries. Food diaries were completed twice during each study to assess energy and macronutrient intake across the interventions.

A number of methods exist to assess dietary intake. Three of the most commonly used methods in experimental research are weighed dietary records, food frequency questionnaires and 24-hour dietary recalls. Weighed dietary records are often considered advantageous as they provide a quantitative means of estimating habitual energy and macronutrient intake. However, the prospective nature of weighed dietary records and burdensome process of weighing and recording all dietary intake often leads to changes in habitual eating behaviour (Rebro *et al.*, 1998). Reasons for this include guilt about eating certain foods, embarrassment about portion sizes and becoming more conscious of the general composition of one's diet (Macdiarmid & Blundell, 1997). As such, most individuals significantly under-or-overestimate energy intake regardless of the recording method (Livingstone *et al.*, 1990; Poslusna *et al.*, 2009).

A four-day period of dietary recording was chosen to maintain participant adherence and to minimise changes in eating patterns that have been reported with relatively long recording timeframes (Rebro *et al.*, 1998). Participants were required to include two week days and both weekend days in their recordings, as eating behaviour has been reported to change on the weekend and is associated with increased energy and fat intake (An, 2016).

2.5 Determination of maximal strength

In **Chapter 4**, maximal isotonic strength of both the right and left leg was determined via leg press and leg extension exercises using a standardised, progressive 1RM protocol adapted from Mayhew *et al.* (1992). This allowed the determination of the corresponding load (~70% 1RM) to be used during subsequent resistance exercise sessions. A training load of ~70% 1RM (~10 repetitions) was chosen as previous studies have shown this stimulus to be sufficient to induce an increase in mixed-muscle and myofibrillar protein synthesis (Kumar *et al.*, 2009; Burd *et al.*, 2010a; Holwerda *et al.*, 2018).

2.6 Measurement of glycaemic control and insulin sensitivity

In **Chapter 3**, an oral glucose tolerance test (OGTT) was conducted following one week of habitual physical activity and after one week of step reduction to assess glycaemic control and insulin sensitivity. Whole-body insulin sensitivity was estimated from fasting and postprandial glucose and insulin concentrations using the Matsuda index as previously described (Matsuda & DeFronzo, 1999). Although the hyperinsulinaemic-euglycaemic clamp technique is widely considered the gold standard method of measuring insulin sensitivity, it was not feasible to apply this technique during the conduct of this study. Nevertheless, the Matsuda index has been shown to have a relatively strong association ($r = 0.73$) with the rate of whole-body glucose disposal calculated during the hyperinsulinaemic-euglycaemic clamp

technique (Matsuda & DeFronzo, 1999). Thus, it provided a convenient and valid means of estimating changes in whole-body insulin sensitivity following one week of step reduction.

2.7 Calculation of muscle protein synthesis: principles, assumptions and measurement

The primary outcome in **Chapters 3** and **4** was the measurement of MPS using the stable isotope tracer $^{2}H_{2}O$. Measurement of muscle protein turnover rates in humans is heavily reliant on stable isotope tracer methodology (Wolfe & Chinkes, 2004; Kim *et al.*, 2016). A stable isotope (e.g., ^{13}C or ^{2}H) is a naturally occurring, less abundant variant of the highly abundant, lighter isotope of that element (e.g., ^{12}C or ^{1}H). Stable isotopes are heavier as they contain one or more additional neutrons and it is this subtle difference that allows them to be distinguished and detected in a tissue of interest. As stable isotopes are considered to be functionally identical to their more common isotope, amino acids of interest (e.g., phenylalanine) can be isotopically labelled (e.g., L-[1-^{13}C]phenylalanine), allowing amino acid and protein metabolism to be measured *in vivo* (Wolfe & Chinkes, 2004).

Stable isotope labelled amino acids can be used to determine protein synthesis, breakdown, oxidation and NBAL at the whole-body level. However, these measurements fail to accurately capture protein turnover in skeletal muscle as the turnover of other tissues (e.g., brain, gut) are measured simultaneously (Holwerda *et al.*, 2016). The arteriovenous (AV) balance technique overcomes this issue by combining infused isotopically labelled amino acids, artery and vein catheterisations and blood flow measurements to provide estimates of MPS, MPB and NBAL. The rate of disappearance (Rd) of the labelled amino acid from the artery provides a proxy of MPS, whilst the rate of appearance (Ra) of the unlabelled amino acid in the vein provides a proxy of MPB (Wolfe & Chinkes, 2004). By calculating the net

AV difference in unlabelled amino acid concentrations, NBAL can be determined. However, without the collection of skeletal muscle biopsies (i.e., a two-pool model), the Rd can only provide an estimate of MPS as it is not certain that all of the amino acids taken up from the artery into the muscle will be used for MPS. With the addition of muscle tissue samples (i.e., a three-pool model), intracellular free amino acid concentrations can be measured, allowing direct calculation of the utilisation of amino acids for MPS and release of amino acids into the free pool from MPB.

Due to the highly invasive nature of the AV balance technique and the need to accurately calculate blood flow, the preferred method to directly measure MPS is the precursor-product approach. Typically, a stable isotope labelled amino acid (e.g., L-[1-^{13}C]phenylalanine) is administered (via a primed, continuous infusion) and combined with serial blood samples and muscle biopsies (Phillips *et al.*, 1997; Tipton *et al.*, 1999; Burd *et al.*, 2015). By measuring the change in the muscle protein-bound enrichment of the labelled amino acid over time between two muscle biopsies, and the mean precursor enrichment in plasma and/or muscle, the fractional synthetic rate (FSR) of skeletal muscle proteins can be determined (Wolfe & Chinkes, 2004; Kim *et al.*, 2016). This approach is typically used to determine MPS rates acutely over the one-to-six hours following exercise and/or nutritional interventions but can be extended for up to 24 hours (Kim *et al.*, 2015). One major advantage of this approach over the aforementioned methods is the ability to determine muscle protein synthetic responses in subcellular protein fractions of interest (e.g., mitochondrial and/or myofibrillar) (Wilkinson *et al.*, 2008; Di Donato *et al.*, 2014). Indeed, studies implementing this approach have provided valuable insight into the mechanisms that underpin contraction specific adaptive responses, demonstrating that myofibrillar protein synthesis is highly responsive to resistance

exercise (Wilkinson *et al.*, 2008; Kumar *et al.*, 2009), whilst mitochondrial protein synthesis is primarily stimulated by aerobic exercise (Wilkinson *et al.*, 2008; Di Donato *et al.*, 2014).

Whilst the infusion of labelled amino acid tracers is a highly sensitive method to determine MPS responses to contractile, nutritional and/or pharmacological interventions, it is limited as measurements are confined to highly controlled laboratory settings and MPS rates can only be determined relatively acutely (1-24 hours). As such, acute MPS responses measured in the hours following resistance exercise may not accurately predict long-term muscle hypertrophy and remodelling (Mitchell *et al.*, 2014).

$^{2}H_2O$ has recently been introduced as a method to measure MPS rates over longer time frames (i.e., days to weeks) than previously permitted (Wilkinson *et al.*, 2014; Brook *et al.*, 2016; Holwerda *et al.*, 2018). $^{2}H_2O$ is a non-substrate specific tracer which quickly equilibrates with body water upon oral ingestion, labelling metabolic substrates within the body through exchange with carbon-bound hydrogens. The non-substrate specific nature of $^{2}H_2O$ enables the turnover of multiple pools (e.g., protein, lipid and glucose) of interest to be determined simultaneously without the need for multiple tracers (Brook *et al.*, 2017a).

Although any deuterium (^{2}H) labelled amino acid can theoretically be used to determine MPS, alanine is generally used as it has four possible sites for incorporation of ^{2}H (Oshima & Tamiya, 1961), allowing MPS rates to be calculated with relatively low ^{2}H enrichments (~0.1-1%) in body water (Brook *et al.*, 2016; Murphy *et al.*, 2016; Holwerda *et al.*, 2018). This is particularly important as the high costs associated with administering $^{2}H_2O$ can be minimised and possible side effects (e.g., vertigo and nausea) previously reported at higher enrichment levels (Holm *et al.*, 2013) can be essentially eliminated. Similar to infused labelled amino acids, the change in muscle protein-bound enrichment of a labelled amino

acid (i.e., ^{2}H-alanine) between two muscle biopsies and the mean precursor enrichment is calculated over time. Importantly, ^{2}H-alanine (precursor) enrichments are minimally affected by feeding (Belloto *et al.*, 2007) and easily calculated from ^{2}H enrichments in body water (^{2}H-alanine is ~3.7 times ^{2}H enrichment in body water), allowing precursor enrichments to be obtained non-invasively (e.g., saliva samples) (Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018).

The $^{2}H_2O$ dosing protocol implemented in **Chapters 3** and **4** was optimised and adapted from the pioneering work of the Hellerstein laboratory (Busch *et al.*, 2006) and others (Robinson *et al.*, 2011) by collaborators of this PhD researcher, Professor Luc van Loon and Dr Andrew Holwerda at Maastricht University, and has successfully been used by this group to measure MPS rates in humans *in vivo* (Holwerda *et al.*, 2018). The $^{2}H_2O$ dosing protocol was identical in **Chapters 3** and **4** and consisted of one dosing day and 16 maintenance days. A dosing day followed by 16 maintenance days was utilised in order to achieve and maintain a steady state body water ^{2}H enrichment of 0.5-1% throughout the study interventions. Approximately 60-90 minutes was allowed between each bolus on the dosing day to negate side effects (e.g., vertigo, nausea) previously reported upon consumption of large volumes of $^{2}H_2O$ over short periods of time (Holm *et al.*, 2013).

2.8 Saliva collection and analysis

In **Chapters 3** and **4**, daily saliva samples were collected to measure ^{2}H enrichment in body water. During *de novo* synthesis, ~3.7 ^{2}H moieties are incorporated per alanine (Belloto *et al.*, 2007; Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018). As such, the mean ^{2}H enrichment in body water (corrected by a factor of 3.7) can be used as the surrogate precursor in the precursor-product calculation of myofibrillar protein synthesis rates. While plasma samples

would have provided a direct measurement of ^{2}H-alanine, saliva samples provided greater temporal resolution as precursor kinetics could easily be followed on a day-to-day basis in a free-living environment. Participants were instructed to provide their saliva sample at least two hours following their last $^{2}H_2O$ bolus to allow near-plateau values to be achieved (Belloto *et al.*, 2007). Whether a change in saliva flow rate alters ^{2}H body water enrichment values has not been determined. Nevertheless, indirect evidence demonstrating that ^{2}H enrichment values measured in saliva and plasma water are essentially identical (Wilkinson *et al.*, 2014) suggests that ^{2}H body water enrichment values measured in saliva are valid.

2.8.1 Analysis of saliva samples

i) Sample preparation: Dr Andrew Holwerda and this PhD researcher prepared the saliva samples collected in **Chapters 3** and **4** prior to analysis at Maastricht University via gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). To measure body water ^{2}H enrichment, saliva samples were centrifuged at high speed and then diluted seventy-fold with ddH_2O. This dilution was required to bring the enrichment within the detection limits of the GC-C-IRMS. This method has been validated in a recently published study from collaborators at Maastricht University, where body water ^{2}H enrichment measurements correlated highly ($r = 0.778$, $P < 0.0001$) with plasma ^{2}H-alanine enrichment measurements (Holwerda *et al.*, 2018).

Subsequently, small plastic cups holding catalyst were placed inside glass vials and 300 uL of diluted saliva was then added. Air in each vial was simultaneously evacuated and replaced by hydrogen gas. Once prepared, the vials were left at 21 °C for 24 hours. This allows for ^{2}H equilibration to occur between the hydrogen gas and the saliva samples.

ii) Sample measurement: The ^{2}H enrichment of the hydrogen gas was measured in duplicate on a GC-C-IRMS. Standard regression curves were applied to assess the linearity of the mass spectrometer and to account for ^{2}H loss during equilibration.

2.9 Blood collection and analysis

In **Chapter 3**, venous blood samples were collected at baseline and 30, 60, 90 and 120 minutes following an OGTT to assess the impact of step reduction on glycaemic control, estimated whole-body insulin sensitivity and metabolic health parameters. This PhD researcher collected all blood samples. Upon arrival, participants had a 20G cannula inserted into an antecubital vein due to its ease of access and relative comfort for the participant. A three-way stop cock was then attached to the cannula to allow repeated blood sampling and saline flushing throughout the OGTT. Blood samples were drawn into a 5 mL syringe, transferred to an EDTA-containing Vacutainer (BD, New Jersey, USA) to prevent blood clotting, and then placed immediately on ice until centrifugation. All blood samples were centrifuged at 1500 g for 15 minutes at 4 °C and the plasma component then aliquoted and stored at -80 °C for later analysis.

2.9.1 Analysis of plasma glucose and NEFA concentrations

i) Sample preparation: This PhD researcher completed all of the plasma metabolite analyses in **Chapter 3**. The following enzymatic colorimetric assays were used for glucose (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK) and NEFA (NEFA kit, Randox, London, UK). These assays work on the principle that the absorbance of light, generated by the enzymatic reaction at a given wavelength, is directly proportional to the concentration of the metabolite of interest within the sample.

Plasma glucose concentrations were quantified based on the Trinder method which involves oxidation of glucose by glucose oxidase to form gluconic acid and hydrogen peroxide. The hydrogen peroxide produced by this reaction then reacts with phenol and aminoantipyrine to form red quinoneimine. The intensity of the colour of the quinoneimine, measured at 540 nm, is proportional to the glucose concentration within the sample.

Plasma NEFA concentrations were quantified using a colorimetric method. First, NEFA is converted to Acyl-CoA by Acyl-CoA synthetase. The resulting Acyl-CoA is then oxidised by Acyl-CoA oxidase producing hydrogen peroxide. In a reaction catalysed by peroxidase, a purple adduct is formed with the intensity of the colour, measured at 540 nm, being proportional to NEFA concentrations within the sample.

ii) Sample measurement: Prior to analysis, a calibration and quality check were conducted for each metabolite and checked against known values provided by the supplier. Glucose and NEFA concentrations were determined in duplicate using a spectrophotometer (iLAB 650 Clinical Chemistry Analyser, Instrumentation Laboratory, Warrington, UK). The average intra-assay coefficient of variation (based on all plasma samples analysed) for glucose was 0.9% and for NEFA was 5.0%. The average inter-assay coefficient of variation (based on analysis of 20 duplicate plasma samples ran across two assays) for glucose was 5.9% and for NEFA was 3.1%.

2.9.2 Analysis of plasma insulin concentrations

i) Sample preparation: This PhD researcher completed all plasma insulin analyses. Plasma insulin concentrations were analysed in duplicate using a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, UK). Each well of the ELISA plate is coated with a capture antibody that is targeted against the antigen of insulin. Each sample,

along with standards and controls, was first added in duplicate to the wells. This allows any antigen present in the samples to bind to the capture antibody. Subsequently, a detecting antibody directly conjugated to horseradish peroxidase (i.e., anti-insulin horseradish peroxidase conjugate) was added to each well. Following incubation, any unbound enzyme was removed by washing and substrate solution (i.e., tetramethylbenzidine) was added to each well. Stop solution (i.e., 1.0 N HCl) was then added which results in a colour change – the intensity of which is directly proportional to the concentration of the metabolite in the sample.

ii) Sample measurement: All microtiter plates were read using a Biotek 800 Absorbance Reader (Biotek Instruments, USA) with absorbance read at a wavelength of 450 nm. Standards run on each plate were used to generate the best standard curve fit. The standard curve was then used to calculate plasma insulin concentrations in the experimental samples. The average intra-assay coefficient of variation (based on all plasma samples analysed) was 8.6%, while the inter-assay coefficient of variation (average of high and low control samples analysed in duplicate on each plate) was 14.9%.

2.10 Muscle biopsy collection and analysis

Muscle biopsy samples collected during **Chapters 3** and **4** were performed by Dr Gareth Wallis and collected and stored for later analysis by this PhD researcher. Dr Andrew Holwerda and this PhD researcher prepared the muscle samples used for the calculation of myofibrillar protein synthesis rates at Maastricht University prior to analysis by gas chromatography–pyrolysis–isotope ratio mass spectrometry. This PhD researcher prepared and analysed the muscle samples used for gene expression and western blot analysis at University of Birmingham.

In **Chapters 3** and **4**, three and six muscle biopsies were collected from each participant, respectively. Muscle biopsies (~100 mg) were collected from the vastus lateralis muscle using the Bergström needle technique (Bergstrom, 1975) with manual suction under local anaesthesia (1% lidocaine). Muscle biopsy samples were quickly (~20-30 seconds) blotted and freed from any visible fat, blood or connective tissue before snap freezing in liquid nitrogen and storing at -80 °C for later analysis.

2.10.1 Measurement of myofibrillar protein synthesis rates

(i) Sample extraction: To measure myofibrillar protein synthesis rates from muscle samples collected in **Chapters 3** and **4**, myofibrillar protein bound ^{2}H-alanine enrichment was determined. To achieve this, ~50 mg wet muscle tissue was hand-homogenised on ice using a Teflon pestle in a standard extraction buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrosphospate, 100 mM NaF, 2 mM Na3VO4, and 1% Nonidet P-40, pH 7.4, 10 μL·mg^{-1}) supplemented with the following protease and phosphatase inhibitors: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine, and 1 mM PMSF. The addition of protease and phosphatase inhibitors was used in order to prevent degradation of proteins and maintain protein phosphorylation sites.

Once homogenised, the aim of the next step is to begin extraction of the myofibrillar proteins from other cellular proteins. This involved an initial centrifugation step at high speed. The resulting supernatant contained the sarcoplasmic proteins and was removed. This can be discarded or retained for western blot analysis (as was done in **Chapter 4**). The remaining pellet, containing myofibrillar protein and collagen, was then washed with ddH_2O and centrifuged.

The next step is to solubilise the myofibrillar proteins. This was achieved by adding 1 mL of 0.3 M NaOH to the samples which were subsequently heated at 50 ºC for 30 minutes with vortex mixing every 10 minutes. Following this step, samples were centrifuged and the supernatant containing the myofibrillar proteins was collected. This step was completed twice to ensure sufficient extraction of myofibrillar proteins. The remaining collagen pellet was discarded. An important step in the extraction process is the removal of interfering proteins from the samples. This was achieved by precipitation of the myofibrillar proteins by adding 1 mL of 1 M PCA. The samples were then centrifuged and the supernatant discarded. This was following by a washing step with 70% ethanol.

Next, the free amino acids need to be released from the samples. This was achieved by heating the samples in a strong acid (i.e., 2 mL of 6 M HCL) at 110 ºC overnight. The next morning, free amino acids from the hydrolysed myofibrillar protein pellet were dried under nitrogen stream while being heated to 120 ºC. To achieve purification, the free amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns, eluted with 2 M NH_4OH and dried.

(ii) Sample measurement: In preparation for analysis using a gas chromatography-isotope ratio mass spectrometer (GC-IRMS), the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters to make the samples volatile and stable for analysis. Once derivatized, samples were measured in quadruplicate using a GC-IRMS equipped with a pyrolysis oven and a 60 m DB-17MS column and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the $^{2}H/^{1}H$ ratios of myofibrillar protein bound alanine. A series of known standards were applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

(iii) Calculation of myofibrillar protein synthesis rates: Myofibrillar protein FSR is typically determined using the incorporation of ^{2}H-alanine into myofibrillar protein and the mean ^{2}H enrichment in body water between sequential biopsies, corrected by a factor of 3.7, as the surrogate precursor based upon ^{2}H labelling during *de novo* alanine synthesis (Busch *et al.*, 2006; Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018). The standard precursor-product method used to calculate FSR was:

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

where E_{m1} and E_{m2} are the myofibrillar protein-bound ^{2}H-alanine enrichments between muscle biopsies. $E_{precursor}$ represents the mean body water ^{2}H enrichment between biopsies corrected by a factor of 3.7 based upon the ^{2}H labelling of alanine during *de novo* synthesis (Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018). *t* represents the time between biopsies in days.

2.10.2 Measurement of skeletal muscle gene expression

(i) RNA isolation: In **Chapter 3**, the skeletal muscle gene expression of selected proteins was determined using real-time quantitative polymerase chain reaction (RT-qPCR). The first requirement is to isolate total RNA from the muscle samples. This was achieved by a phenol-chloroform extraction step. First, ~20 mg of powdered muscle tissue was homogenised in an RNA isolation reagent (i.e., TRI Reagent). This reagent contains phenol and guanidine isothiocyanate. Phenol solubilises the proteins whilst guanidine isothiocyanate acts as an effective protein denaturant which helps to inactive RNases. To achieve phase separation, 200 μL of chloroform was added to each sample followed by vigorous shaking and incubation at ambient temperature. The addition of chloroform is important as phenol by

itself will retain some water (containing RNA). Chloroform helps to separate phenol from the water and thus improves total RNA yield. Following the addition of chloroform, samples were centrifuged at high speed. The result of this process is the formation of an upper aqueous phase (containing RNA) and a lower organic phase (containing the DNA).

The RNA-containing aqueous phase was then removed and mixed with an equal volume of 2-propanol to achieve precipitation of the RNA. RNA was then further purified using spin columns which included a DNase treatment step to remove/degrade potentially contaminating genomic DNA. Following this, the RNA concentration and purity of each sample was determined using spectrophotometric analysis via a FLUOstar Omega microplate reader (LVis function). RNA purity is determined by assessing the ratio of absorbance at 260 nm and 280 nm, with a ratio of ~2.0 considered to be indicative of pure RNA. A 260/280 ratio lower than this is suggestive of the presence of protein, phenol or other contaminants. The ratio of absorbance at 260 nm and 280 nm was ≥ 2.0 for all samples. The mean (SD) RNA concentration of the muscle samples was 149.8 ± 73.9 ng/µL which was of sufficient quantity to allow reverse transcription of 900 ng of total RNA to cDNA. As such, no repeats of RNA isolation were required.

(ii) Reverse transcription: Following isolation of total RNA, the next step is to reverse transcribe the RNA into complementary DNA (cDNA) to use as the template for qPCR analysis. This requires two steps, an annealing step and an extension step. The annealing step involves the binding of a chosen reverse transcription primer to the denatured RNA. This allows polymerases to move freely along the RNA template. At this point, it is important to decide whether to use oligo-dT primers, random nonamer primers, or a mix of the two for reverse transcription. A mix of oligo-dT and random nonamer primers was chosen for the annealing step in order to target transcription of both mRNA and ribosomal RNA (rRNA).

This was an important consideration as one of the internal control genes (18S) is rRNA and thus using only oligo-dT would have resulted in suboptimal transcription of this gene. The extension step involved addition of a buffer to the samples which contains enzymes which initiate the synthesis of cDNA to be used as the template for qPCR analysis. All samples were diluted to a concentration of 10 ng/µL prior to qPCR analysis.

(iii) qPCR analysis: In order to quantify the expression of genes of interest, qPCR was used. This technique involves the amplification of cDNA which is achieved by a denaturation, annealing and extension step using a thermal cycler or PCR machine. The first step (denaturation) involved heating the cDNA at a high temperature (95 ºC). This allows the double stranded cDNA to be separated into single strands. Subsequently, the temperature was lowered (60 ºC) (annealing step) which allows the primer of choice to bind to the target of interest. A forward and reverse primer (sequences are presented for all primers in **Table 3.2)** was used to specify which region of the target of interest should be copied. Binding of the primer is required before the DNA polymerase enzyme can bind to the target of interest and create a complementary copy of the single DNA strand (extension step). The result of this process is that twice the number of copies of the target of interest now exists. The three steps outlined above were then repeated for multiple (i.e., 40) cycles with each cycle resulting in a doubling of the number of copies.

Using a qPCR machine, the amplification process was monitored in real time. To achieve this, fluorescence must be generated and detected by a camera in the PCR machine during each qPCR cycle. This was achieved by adding an intercalating dye (i.e., SYBR Green) into the qPCR reaction. Prior to thermal cycling, the dye intercalates with the double stranded DNA and fluoresces. As each thermal cycle is completed, additional copies of the DNA are produced and thus the dye has more DNA that it can bind to and more fluorescence is

emitted. Thus, the more amplified DNA there is the more fluorescence that is produced. It is important to note that a potential drawback of using intercalating dyes is that that they are not specific to the target of interest. This can be problematic as an increase in the fluorescence will still be detected if the wrong target is amplified. This was circumvented by running a post qPCR melt curve to ascertain the specificity of each primer.

(iv) Calculation of mRNA expression: The most common method to quantify mRNA expression, the $2^{-\Delta\Delta CQ}$ method (Livak & Schmittgen, 2001), was used for the calculations of mRNA expression. The exponential phase of the qPCR reaction was used during quantification as it is the phase where exact doubling of product occurs with each cycle and thus the reaction is most precise. The cycle where fluorescence reaches the threshold (i.e., becomes detectable above background) is known as the quantitation cycle (CQ) and was quantified for each sample. Thus the lower the CQ value is the higher the amount of target that is present in the qPCR reaction.

To control for RNA input, it is essential to use an internal control for normalisation. Whilst a single internal control gene has often been used, it has been shown that this approach results in large error due to higher variability (Vandesompele *et al.*, 2002). A more robust approach is to assess the stability of multiple internal controls and use the geometric mean of the three most stable genes across all samples. Accordingly, the stability of five internal control genes was assessed prior to quantification in **Chapter 3 (Table 2.1)**. As suggested, the geometric mean of the CQ values for TOP1, B2M and ACTB **(Table 2.1)** was used as an internal control as these were found to be the three most stable genes across all samples using RefFinder (Vandesompele *et al.*, 2002; Xie *et al.*, 2012).

Table 2.1 CQ values of five internal control genes.

	18S		GAPDH		ACTB		B2M		TOP1		Geometric Mean	
	HPA	**SR**	**HPA**	**SR**	**HPA**	**SR**	**HPA**	**SR**	**HPA**	**SR**	**HPA**	**SR**
P01	9.1	10.5	16.9	17.6	21.8	21.8	20.1	20.1	26.6	27.2	22.7	22.8
P03	9.7	9.3	17.3	17.5	22.9	22.2	21.5	21.1	27.2	27.6	23.7	23.5
P04	10.0	8.8	17.5	16.9	22.3	22.7	20.5	20.8	27.3	27.6	23.2	23.5
P05	9.4	9.2	17.1	17.3	22.7	21.8	21.8	20.1	27.6	26.8	23.9	22.7
P08	10.0	12.2	17.0	17.4	22.6	23.3	21.3	21.5	27.8	29.7	23.7	24.6
P12	9.8	9.3	17.4	17.2	22.6	23.4	21.7	21.9	27.4	27.9	23.8	24.3
P13	10.3	10.0	17.0	18.2	22.9	22.7	21.2	20.9	27.9	27.2	23.8	23.4
P14	8.7	8.6	16.1	15.8	22.1	22.2	21.0	20.8	27.2	27.0	23.3	23.2
P15	9.4	9.4	16.9	17.0	21.8	22.0	21.0	21.1	27.1	27.3	23.1	23.3
P16	9.6	9.3	16.5	16.7	21.4	22.0	20.9	20.9	26.6	26.9	22.8	23.2
Mean	9.6	9.7	17.0	17.1	22.3	22.4	21.1	20.9	27.3	27.5	23.4	23.5
SD	0.5	1.0	0.4	0.6	0.5	0.6	0.5	0.6	0.4	0.8	0.4	0.6

2.10.3 Measurement of skeletal muscle protein expression

(i) Sample preparation: In **Chapter 4**, the skeletal muscle protein expression of selected targets implicated in ribosomal biogenesis was determined using western blotting. This analysis was performed on the sarcoplasmic fraction collected during myofibrillar protein extraction. The first requirement is to quantify the amount of protein in each sample. This is an essential step to ensure equal loading of protein when adding samples to each well. Protein quantification was achieved using the detergent compatible (DC) protein assay. This is a modified form of the Lowry method (Lowry *et al.*, 1951), a colorimetric assay which relies on the biuret reaction. An alkaline copper tartate solution was added which allows the peptide bonds of proteins to form a complex with copper. A Folin reagent was also added, which results in the reduction of copper and colour change which is directly proportional to the number of peptide bonds (and thus protein) present in the sample. Following 15 minutes of

incubation, absorbance was measured at 750 nm using a microplate reader and the protein concentration of each sample was calculated.

In preparation for sample loading, all samples were diluted to a protein concentration of 2 μg/μl. As will be discussed below, the separation of proteins by their molecular mass (i.e., gel electrophoresis) requires that proteins be negatively charged. This was be achieved by adding Laemlli sample buffer and boiling the samples for five minutes at 97 °C. Sodium dodecyl sulfate (SDS) contained within the Laemlli sample buffer not only coats proteins with a negative charge but also unfolds and denatures the proteins. This eliminates the impact of protein structure on movement through the gel and allows protein separation based on molecular mass.

(ii) SDS-PAGE: Once all samples have been prepared for western blotting, the next step is to separate proteins based on their molecular mass using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). First, 15 μl (30 μg protein) of sample was loaded onto 10% polyacrylamide gels. This percentage polyacrylamide gel was chosen to allow sufficient separation and band resolution based on the known molecular mass of each protein of interest. Following sample loading, gels were placed in an electrophoresis tank with running buffer and an electric voltage was then applied until sufficient separation was achieved (~60 minutes). As the gel has a negative charge at one end and a positive charge at the other, the negatively charged proteins are strongly attracted towards the positively charged end and migrate down the gel. Small pores contained in the gel allow proteins with a small molecular mass to migrate quickly through the gel whereas larger proteins migrate more slowly.

(iii) Membrane transfer: Following separation, proteins need to be transferred to a membrane. This immobilises proteins in place and allows subsequent detection through antibody recognition. Electrotransfer utilises similar principles to SDS-PAGE in that negatively charged proteins migrate from the gel towards the positively charged anode in the direction of the membrane. Proteins were wet transferred to polyvinylidene fluoride (PVDF) membranes in a transfer tank (surrounded by ice) containing transfer buffer for 60 minutes at 100 V. A constant voltage and active cooling was used in order to maintain strength of the electrical field which is essential for successful transfer. PVDF membranes were chosen over nitrocellulose membranes as they bind higher amounts of protein and thus provide greater sensitivity (Bass *et al.*, 2017). Following completion of the transfer, membranes were checked for transfer efficiency and protein loading using Ponceau S staining. This reversibly stains and binds to protein on the membrane which can then be imaged and quantified later to correct for total protein content.

(iv) Blocking: In order to reduce background and eliminate non-specific binding of the primary and/or secondary antibodies, a blocking step is required. This was achieved by rocking the membranes for 60 minutes at room temperature in non-fat dried milk diluted in Tris-buffered saline Tween 20 (TBST). This method has been shown to be effective at reducing background and non-specific binding (Bass *et al.*, 2017). Once blocking was complete, membranes were washed in TBST in preparation for antibody recognition.

(v) Antibody incubation: To allow detection of the protein of interest, a primary and secondary antibody are required. The primary antibody is specific to the protein of interest. Thus, when the membrane is incubated in the primary antibody, the antigen-binding site of the antibody binds to the antigen of the protein of interest. Any unbound primary antibody is then washed away using TBST and a secondary antibody linked to a reporter function (e.g.,

horseradish peroxidase) is then added. This secondary antibody recognises and attaches to a region of the primary antibody which enables subsequent detection and quantification. Primary antibodies were prepared in bovine serum albumin (BSA) diluted in TBST to further minimise background and non-specific binding. Membranes were then incubated in the required primary antibody on a shaker overnight at 4 °C to allow sufficient signal for detection. All primary antibodies were generated from rabbit as a host species and thus an anti-rabbit secondary antibody was used. The secondary antibody was diluted in TBST and membranes incubated in the secondary antibody for 60 minutes at room temperature to allow sufficient binding time.

(vi) Detection: Secondary antibodies are conjugated to a label which allows the protein of interest to be detected. As described above, an anti-rabbit secondary antibody conjugated with horseradish peroxidase was used. When using enzyme-linked secondary antibodies, either colorimetric or chemiluminescent detection can be used. Chemiluminescent detection was utilised for detection as it offers the greatest sensitivity and allows the exposure time to be manipulated and repeated, which is useful in case of unintentional overexposure or underexposure of the membrane (Bass *et al.*, 2017). Upon addition of a chemiluminescent reagent containing substrate, luminol becomes oxidised which results in the release of light at 425 nm, which can then be visualised and captured using an imager. The incubation time varied from one-to-five minutes dependent on the protein of interest to facilitate optimal detection conditions.

(vii) Quantification: A number of steps were completed in order to ensure accurate quantification of protein expression. First, all images were checked for oversaturation prior to quantification. This is important to avoid misinterpretation of protein expression changes. In addition, a background correction was performed for each single band. This corrects for any

background which often varies across the image and thus could lead to erroneous interpretation if not accounted for. Finally, and perhaps most importantly, all samples were corrected for total protein content to account for any errors when preparing or loading samples. This is essential as any observed change in protein expression could be due to differences in the amount of protein loaded as opposed to the intervention that is being investigated. As described above, correction for total protein content was achieved by using Ponceau S staining of the membrane which was then imaged and quantified. This method was chosen over the use of a 'housekeeping' protein such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as this method assumes that the expression of the housekeeping protein is unaffected by intervention which may not always be the case.

CHAPTER 3

ONE WEEK OF STEP REDUCTION LOWERS MYOFIBRILLAR PROTEIN SYNTHESIS RATES IN YOUNG MEN

The work contained within this chapter is published:

Shad BJ, Thompson JL, Holwerda AM, Stocks B, Elhassan YS, Philp A, van Loon LJC, Wallis GA. One week of step reduction lowers myofibrillar protein synthesis rates in young men. Medicine & Science in Sports & Exercise, 2019 May, PMID: 31083048

3.1 Abstract

Across the lifespan, physical activity levels decrease and time spent sedentary typically increases. However, little is known about the impact that these behavioural changes have on skeletal muscle mass regulation. The primary aim of this study was to use a step reduction model to determine the impact of reduced physical activity and increased sedentary time on daily myofibrillar protein synthesis rates in healthy young men. Eleven men (22±2 y) completed 7 days of habitual physical activity (HPA) followed by 7 days of step reduction (SR). Myofibrillar protein synthesis rates were determined during HPA and SR using the $^{2}H_2O$ method combined with the collection of skeletal muscle biopsies and daily saliva samples. Gene expression of selected proteins related to muscle mass regulation and oxidative metabolism were determined via real time RT-qPCR. Daily step count was reduced by approximately 91% during SR (from 13054±2763 to 1192±330 steps·d^{-1}; $P<0.001$) and this led to an increased contribution of sedentary time to daily activity (73±6 to 90±3%; $P<0.001$). Daily myofibrillar protein synthesis decreased by approximately 27% from 1.39±0.32 %·d^{-1} during HPA to 1.01±0.38 %·d^{-1} during SR ($P<0.05$). MAFbx and myostatin mRNA expression were up-regulated whereas mTOR, p53 and PDK4 mRNA expression were down-regulated following SR ($P<0.05$). One week of reduced physical activity and increased sedentary time substantially lowers daily myofibrillar protein synthesis rates in healthy young men.

3.2 Introduction

Skeletal muscle mass, physical function and metabolic health progressively decline with advancing age. This could be attributed to the gradual reduction in levels of physical activity and/or the increase in sedentary behaviour that typically occurs across the lifespan (Loyen *et al.*, 2017). The importance of physical activity in maintaining skeletal muscle mass and function is well appreciated and recent evidence has implicated sedentariness, distinct from physical activity, as a risk factor for age-related loss of skeletal muscle mass and strength (Gianoudis *et al.*, 2015). However, the physiological processes which may contribute to the negative consequences of physical inactivity and sedentary behaviour on skeletal muscle mass and function are relatively unknown.

Skeletal muscle mass is governed by overall protein balance, which is determined by rates of MPS and MPB. Any loss of muscle mass must be explained by an overall negative protein balance (i.e., MPB must exceed MPS). Extreme muscle disuse (i.e., bed rest or limb immobilisation), where voluntary muscular contractile activity is essentially removed, results in substantial loss of skeletal muscle mass (Dirks *et al.*, 2016b; Wall *et al.*, 2016b). This is associated with reductions in postabsorptive MPS rates and the development of 'anabolic resistance'; that is, a reduced stimulation of postprandial MPS (Wall *et al.*, 2016b). On the other hand, the impact of disuse on MPB rates in humans is poorly defined. Whilst disuse models provide invaluable information on the impact of severe muscle unloading, their extreme nature may not accurately reflect typical physically inactive and sedentary lifestyles (Perkin *et al.*, 2016).

Step reduction has been proposed as a model to more accurately examine the underlying physiology of physically inactive and sedentary individuals and also explore the

physiological changes that occur when inactivity is enforced by injury, illness and/or other significant life events (Fisher *et al.*, 2011; Tipton, 2015; Perkin *et al.*, 2016). Step reduction has been shown to reduce insulin sensitivity (Krogh-Madsen *et al.*, 2010; Reidy *et al.*, 2018), but few studies have evaluated its impact on skeletal muscle mass regulation. Breen and colleagues reported reduced postprandial myofibrillar protein synthesis rates following 14 days of step reduction in older, overweight individuals (Breen *et al.*, 2013). These findings are supported by more recent data showing reduced integrated myofibrillar protein synthesis rates throughout a two-week period of step reduction in older, overweight adults (McGlory *et al.*, 2018).

While these findings are important, there are currently no data available on MPS rates over shorter periods of time typically associated with recovery from injury and/or acute illness (i.e., ≤ one week). The above studies also describe responses in older, overweight individuals, but young and older individuals have distinct responses to muscle disuse (Suetta *et al.*, 2009; Tanner *et al.*, 2015). The influence of step reduction on skeletal muscle mass regulation in young individuals has yet to be studied but is important to characterise to enhance our understanding of the impact of reduced physical activity and increased sedentary behaviour on skeletal muscle mass regulation at various stages across the lifespan.

Accordingly, the primary purpose of the present study was to use the step reduction model to determine the impact of short-term reduced physical activity and increased sedentary time on myofibrillar protein synthesis rates in healthy young men. The $^{2}H_{2}O$ approach was used as it allows myofibrillar protein synthesis rates to be measured under free-living conditions over time frames where quantifiable changes in muscle mass are unlikely to occur, providing important insight into longer-term muscle mass regulation (Robinson *et al.*, 2011; Wilkinson

et al., 2014; Holwerda *et al.*, 2018). It was hypothesised that one week of reduced physical activity and increased sedentary time would reduce daily myofibrillar protein synthesis rates.

3.3 Methods

3.3.1 Participants and ethical approval

Eleven healthy young men participated in the present study which took place between June 2016 and February 2018. All participants were recreationally active and self-reported engaging in structured physical activity ≥ 3 times/week for > 6 months prior to inclusion. Five of the participants reported undertaking only aerobic-based exercise, four of the participants reported undertaking only resistance-based exercise and two of the participants reported undertaking both aerobic and resistance-based exercise. None of the participants were competitive endurance and/or power athletes. The participants' baseline characteristics are presented in **Table 3.1**. Prior to providing informed written consent, each volunteer was informed of the experimental procedures and potential risks associated with the experimental intervention. Participants were screened prior to inclusion in the study and deemed healthy based on their responses to a general health questionnaire. Exclusion criteria included being a current or recent (last 6 months) smoker, hypertensive (≥ 140/90 mmHg), diagnosed with diabetes and/or suffering from a recent injury. Participants deemed eligible were subsequently fitted with an ActivPAL3^{TM} accelerometer (see *Accelerometry* section below) for 7 days to objectively assess daily step count. Any individual completing <7000 steps·d^{-1} was excluded from participating in the study. The study was approved by the National Research Ethics Service Committee West Midlands, Edgbaston, United Kingdom (Reference: 16/WM/0011) and conformed to standards for the use of human participants in

research as outlined in the Declaration of Helsinki. The intervention was registered at clinicaltrials.gov prior to data collection (Identifier: NCT02624011).

Table 3.1 Participant characteristics at baseline.

Variable	Value
Age (y)	22.2 ± 2.2
Height (m)	1.77 ± 0.08
Body mass (kg)	74.0 ± 11.0
BMI ($kg \cdot m^{-2}$)	23.4 ± 2.4
Body fat (%)	18.6 ± 3.2
Whole body FFM (kg)	60.0 ± 7.2
Leg FFM (kg)	20.2 ± 2.4

Values are mean±SD. n=11.

3.3.2 Study overview

An overview of the study is presented in **Figure 3.1**. Following an initial ^2H_2O dosing day (day -2) and one maintenance day (see *^2H_2O dosing protocol* section below), participants completed 7 days of HPA followed by 7 days of SR. For the first 7 days, participants were instructed to maintain their habitual physical activity levels (i.e., regular ambulation and structured physical activity). During SR, participants were instructed to reduce their step count to ~1500 $steps \cdot d^{-1}$, be as sedentary as possible and refrain from any form of structured physical activity for the remaining 7 days. A target daily step count of ~1500 $steps \cdot d^{-1}$ was set during SR as large-scale global data suggest that the average daily step count for adults is <5000 $steps \cdot d^{-1}$ (Althoff *et al.*, 2017). An ActivPAL3^{TM} accelerometer (see *Accelerometry* section below) was worn throughout HPA and SR to objectively assess physical activity

levels and sedentary time. As the ActivPAL3^{TM} accelerometer does not provide visual feedback on daily step count, participants were also provided with a pedometer during the SR period to help prevent their daily step count exceeding the 1500 steps·d^{-1} threshold. A member of the investigative team was on call throughout the SR period to help participants with activities of daily living (e.g., food shopping) that were not practical within the step count parameters set out. Weighed four-day food diaries were completed during HPA and SR (see *Dietary intake* section below). Muscle biopsies were collected on days 0, 7 and 14, saliva samples were collected daily and an OGTT was conducted on days 7 and 14.

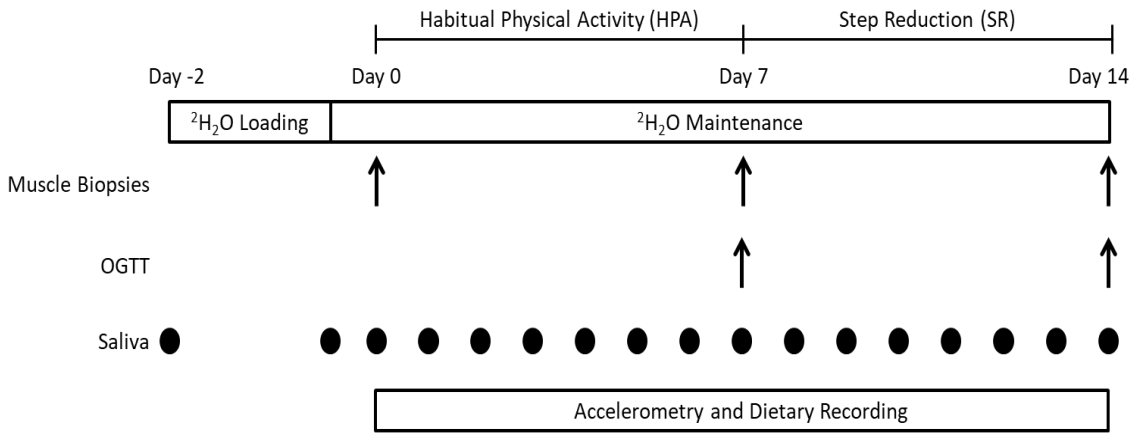

Figure 3.1 Study overview.

3.3.3 Experimental visits

On the morning of day 0, participants arrived at the laboratory at 08:00 in a fasted state from 22:00 the evening before. After voiding, participants were weighed in light clothing to the nearest 0.1 kg (OHaus, Champ II scales, USA) and height measured to the nearest centimetre (Stadiometer, Seca, UK). Body composition (whole-body FFM and body fat percentage) was subsequently determined by DXA (Discovery QDR W series; Hologic). Following the DXA scan, a saliva sample (see *^2H_2O dosing protocol* section below) was obtained before

collection of a muscle biopsy from the *vastus lateralis* muscle. Muscle biopsies were collected using the Bergström needle technique with manual suction under local anaesthesia (1% lidocaine). Muscle biopsy samples were blotted and any visible fat, blood or connective tissue removed before snap freezing in liquid nitrogen and storing at -80 °C for later analysis. Subsequent muscle biopsies (days 7 and 14) were taken from separate incisions in an alternating pattern between legs. Participants then consumed a single maintenance bolus of $^{2}H_2O$ (see *$^{2}H_2O$ dosing protocol* section below) before being fitted with an ActivPAL3^{TM} accelerometer prior to leaving the laboratory.

Following 7 days of HPA, participants returned to the laboratory at 08:00 on day 7, again in a fasted state from 22:00 the evening before. Participants were weighed prior to insertion of a 20G cannula into an antecubital vein to allow for repeated blood sampling during the OGTT. A saliva sample was subsequently obtained before collection of the second muscle biopsy. Following the muscle biopsy, a baseline blood sample was then drawn before participants completed an OGTT. Participants consumed 75 g dextrose as a 25% solution with subsequent blood samples drawn at 30, 60, 90 and 120 minutes to assess postprandial blood glucose, insulin and non-esterified fatty acid (NEFA) concentration responses. NEFA responses to an OGTT were measured as the development of insulin resistance may be associated with reduced insulin-mediated suppression of fatty acid mobilisation from adipose tissue (Karpe *et al.*, 2011). Blood samples were collected into EDTA-containing Vacutainers (BD, New Jersey, USA) prior to centrifugation at 1500 g for 15 minutes at 4 °C. Aliquots containing plasma were stored at -80 °C. Participants remained in a semi-supine position throughout the OGTT and once completed, consumed a single maintenance bolus of $^{2}H_2O$ before leaving the laboratory. Following 7 days of SR, participants arrived at 08:00 in a fasted state for the final

laboratory visit (i.e., day 14) which was identical to the experimental protocol completed on day 7.

3.3.4 $^{2}H_2O$ dosing protocol

The $^{2}H_2O$ dosing protocol consisted of one dosing day and 16 maintenance days (Holwerda *et al.*, 2018). On day -2, participants completed a $^{2}H_2O$ loading day. Following collection of a background saliva sample, participants were provided with 8 x 50 mL boluses of 70% $^{2}H_2O$ (Cambridge Isotope Laboratories, Massachusetts, USA) to increase ^{2}H enrichment in body water to 0.5-1%. Approximately 60-90 minutes was allowed between each bolus to negate side effects (e.g., vertigo, nausea) previously reported upon consumption of large volumes of $^{2}H_2O$ over short periods of time. The $^{2}H_2O$ protocol was well tolerated with none of the participants reporting any adverse effects. For each subsequent day, participants were provided with a daily 50 mL maintenance bolus of $^{2}H_2O$ to consume. Participants were instructed to consume the daily bolus upon waking up to ensure consistency and minimise the risk of missed doses. The time at which each bolus was consumed was recorded and participants were instructed to bring the empty bottles back in on each laboratory visit to measure compliance. All boluses were returned void, suggesting full compliance with the $^{2}H_2O$ protocol.

To measure ^{2}H enrichment in body water, saliva samples were collected daily. Participants lightly chewed a cotton swab until completely saturated with saliva (~2-3 minutes). On days -2, 0, 7 and 14, swabs were collected in the laboratory, immediately placed in a 5 mL syringe and the saliva compressed into sample tubes and stored at -80 °C for later analysis. On the remaining days when participants were not in the laboratory, daily saliva samples were collected at home and stored in pre-labelled falcon tubes in the fridge until the next

laboratory visit where samples were stored as described above. Participants were instructed to provide their saliva sample at least 2 hours following their last $^{2}H_2O$ bolus and at least 30 minutes after their last meal or drink and to record the time at which the sample was collected.

3.3.5 Accelerometry

During the screening process, participants were fitted with an ActivPAL3^{TM} accelerometer (PAL Technologies Ltd., Glasgow, UK) to assess daily step count. Participants were also fitted with an ActivPAL3^{TM} accelerometer during HPA and SR to objectively assess physical activity levels and sedentary time. The ActivPAL3^{TM} accelerometer was attached to the anterior of the upper thigh using waterproof dressing. Participants were required to wear the accelerometer at all times except when bathing. Complete 14-day accelerometry data were obtained from all 11 participants over the experimental intervention. During the seven-day period of SR, participants were also provided with a hip-worn pedometer (Yamax Digi-Walker SW-200) which provided visual feedback on their step count to aid compliance with the 1500 steps·d^{-1} requirement. Daily step count from the hip-worn pedometer was recorded by participants before bed. Accelerometry data were downloaded from devices using ActivPAL3^{TM} analysis software (PAL Technologies Ltd., Glasgow, UK, v7.2.32).

3.3.6 Dietary intake

The evening prior to each experimental visit on days 0, 7 and 14, participants received the same standardised meal (~689 kcal, providing ~55 energy% (En%) carbohydrate, ~20 En% protein, and ~25 En% fat). A weighed four-day food diary was completed over the first seven-day period of HPA and over the second seven-day period of SR to evaluate energy and macronutrient intake. Participants were required to include two week-days and both weekend

days in their recordings. Dietary records were analysed using Dietplan software (Forestfield Software Ltd., v6.70.67).

3.3.7 Plasma analyses

Plasma glucose (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK) and NEFA (NEFA kit, Randox, London, UK) concentrations were analysed in duplicate using enzymatic colorimetric assays using an ILAB 650 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). Plasma insulin concentrations were determined in duplicate using commercially available ELISA kits (Invitrogen, California, United States, KAQ1251).

3.3.8 Body water ^{2}H enrichment

Body water ^{2}H enrichment was analysed from daily saliva samples collected throughout the study as previously described (Holwerda *et al.*, 2018). Briefly, samples were centrifuged at 10000 g and then diluted 70-fold with ddH_2O. Subsequently, small plastic cups holding 4 mg of catalyst (5% platinum on alumina, 325 mesh, Sigma-Aldrich, St. Louis, USA) were placed inside 3 mL glass vials (Labco Exetainer, Labco limited, Lampeter, UK) and 300 uL of diluted saliva was then added. Air in each vial was simultaneously evacuated and replaced by hydrogen gas. Once prepared, the vials were left at 21 °C for 24 hours for ^{2}H equilibration to occur between the hydrogen gas and the saliva samples. The ^{2}H enrichment of the hydrogen gas was then measured in duplicate on a GC-C-IRMS (Micromass 205 Optima IRMS fitted with a Multiprep and Gilson autoinjector, Micromass UK Limited, 206 Manchester, UK). Standard regression curves were applied to assess the linearity of the mass spectrometer and to account for ^{2}H loss during equilibration.

3.3.9 Myofibrillar bound ^{2}H-alanine enrichment

For measurement of ^{2}H-alanine enrichment in the myofibrillar fractions, ~50 mg wet muscle tissue was hand-homogenised on ice using a pestle in a standard extraction buffer (10 μL·mg^{-1}). The samples were then spun at 2500 g for 5 minutes at 4 ºC. The pellet was washed with 500 μL of ddH_2O and centrifuged at 250 g for 10 minutes at 4 ºC. The myofibrillar protein was solubilised by adding 1 mL of 0.3 M NaOH and heating at 50 ºC for 30 minutes with vortex mixing every 10 minutes. Samples were centrifuged at 9500 g for 5 minutes at 4 ºC, the supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and spinning at 700 g for 10 minutes at 4 ºC. The myofibrillar protein was washed twice with 70% ethanol and hydrolysed overnight in 2 mL of 6 M HCL at 110 °C. The free amino acids from the hydrolysed myofibrillar protein pellet were dried under a nitrogen stream while being heated to 120 ºC. The free amino acids were then dissolved in 25% acetic acid solution, 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. Thereafter, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. The derivatized samples were measured using a GC-IRMS (Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60 m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE, USA) and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the ^{2}H/^{1}H ratios of myofibrillar protein bound alanine. A series of known standards were applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

3.3.10 Gene expression analysis

Total RNA was isolated from ~20 mg of frozen powdered muscle tissue by homogenising in 1 mL of TRI Reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRA-

TURRAX homogeniser (IKA, Oxford, UK). To achieve phase separation, 200 µL of chloroform was added to each sample followed by vigorous shaking for 15 seconds, 15 minutes at ambient temperature and subsequent centrifugation at 12000 g for 15 minutes at 4 °C. The RNA-containing supernatant was then removed and mixed with an equal volume of 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturer's instructions, which includes a DNase treatment step. A FLUOstar Omega microplate reader (LVis function) was used to determine the RNA concentration and purity of each sample. The ratio of absorbance at 260 nm and 280 nm was ≥ 2.0 for all samples. 900 ng of total RNA was reverse-transcribed to cDNA in 20 µL volumes using the nanoScript 2 RT kit and a combination of oligo(dT) and random primers (Primerdesign, Southampton, UK) as per the manufacturer's instructions. The resultant cDNA was diluted to 10 ng/mL prior to RT-qPCR analysis. All analysis was performed in triplicate using Primerdesign custom designed (**Table 3.2**) or commercially available (18S, GAPDH, TOP1, B2M and ACTB, Primerdesign Southampton, UK) primers and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign Southampton, UK) on a QuantStudio3 Real-Time PCR System (Applied Biosystems, Thermo Fisher, UK). Dependent on the gene of interest, 10-50 ng of cDNA was added to each well in a 20 uL reaction volume. Thermal cycling conditions were 2 minutes at 95 °C and 40 cycles of 10 seconds at 95 °C and 60 seconds at 60 °C. A post qPCR run melt curve (Applied Biosystems, Thermo Fisher, UK) was used to ascertain the specificity of each primer. qPCR results were analysed using Experiment Manager (Thermo Fisher). mRNA expression values are expressed as fold change relative to the average baseline (i.e., HPA) ΔCQ value using the $2^{-\Delta\Delta CQ}$ method (Livak & Schmittgen, 2001). To control for RNA input, the geometric mean of the CQ values for TOP1, B2M and ACTB was used as an internal control as these were

found to be the three most stable genes across all samples using RefFinder (RefFinder, RRID:SCR_000472) (Xie *et al.*, 2012). All gene expression data are presented for n=10 as insufficient muscle tissue was available for RNA isolation for one participant. Statistical analysis was performed on the $2^{-\Delta\Delta CQ}$ transformed data.

Table 3.2 RT-qPCR primer sequences.

Gene	Forward primer	Reverse primer
MuRF1	5'-GACGCCCTGAGAGCCATT-3'	5'-CCTCTTCCTGATCTTCTTCTTCAAT-3'
MAFbx	5'-AACTCAAATACAAAATAGGACGCTTT-3'	5'-CCTTCGCCTTCTCAAAACAAAC-3'
Myostatin	5'-GTCGAGACTCCTACAACAGTG-3'	5'-TCCAGTATACCTTGTACCGTCTT-3'
mTOR	5'-CTGATGCTGGACCGTCTGA-3'	5'-TCTTGTTAGTCTAAATGGAATCTTCTC-3'
p70S6K	5'-GCAAGCTGGACAAACTATCACA-3'	5'-CCACTGAGATAATACTTGTGCTATAATG-3'
p53	5'-GTGGAGTATTTGGATGACAGAAAC-3'	5'-GTAGTTGTAGTGGATGGTGGTAC-3'

PDK4	5’-GAGGGACACTCAGGACACTTTAC-3’	5’-TGGAGGAAACAAGGGTTCACAC-3’
PGC-1α	5’-TTGCTAAACGACTCCGAGAAC-3’	5’-GACCCAAACATCATACCCCAAT-3’

3.3.11 Calculations

Total area under the curve (AUC) for plasma glucose and insulin concentrations was calculated using the trapezoidal method. The Matsuda index, an index of whole-body insulin sensitivity, was calculated as previously described (Matsuda & DeFronzo, 1999). Myofibrillar protein FSR was determined using the incorporation of ^{2}H-alanine into myofibrillar protein and the mean ^{2}H enrichment in body water between sequential biopsies, corrected by a factor of 3.7, as the surrogate precursor based upon ^{2}H labelling during *de novo* alanine synthesis (Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018). The standard precursor-product method was used to calculate FSR:

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

where E_{m1} and E_{m2} are the myofibrillar protein-bound ^{2}H-alanine enrichments between sequential muscle biopsies. $E_{precursor}$ represents the mean body water ^{2}H enrichment between sequential biopsies corrected by a factor of 3.7 based upon the ^{2}H labelling of alanine during *de novo* synthesis (Holwerda *et al.*, 2018). *t* represents the time between sequential biopsies in days.

3.3.12 Statistics

Based on previous research (Wilkinson *et al.*, 2014), sample size calculations showed that n=9 would be sufficient to detect a difference in daily myofibrillar protein synthesis rates between HPA and SR conditions using a two-tailed paired samples t-test (95% power, α-level of 0.05, G*power version 3.1.9.2). Allowing for a 20% dropout rate, eleven participants were recruited. All statistical analyses were performed using SPSS 22.0 (SPSS, RRID:SCR_002865, Chicago, IL, USA). Differences between conditions (HPA vs. SR) for accelerometry, dietary intake, plasma insulin and glucose AUC, Matsuda index, myofibrillar protein FSR and gene expression were compared using paired sample t-tests. Body water ^{2}H enrichment was analysed using a one-factor repeated measures ANOVA with time as the within-subjects factor. A two-factor repeated measures ANOVA (condition x time) with condition (HPA vs. SR) and time (0, 30, 60, 90 and 120 minutes) as within-subjects factors was performed for analysis of plasma glucose, insulin and NEFA concentrations. Bonferroni post-hoc tests were conducted to correct for multiple comparisons when a significant condition x time interaction was identified. All data are presented as mean±SD.

3.4 Results

3.4.1 Accelerometry

Daily step count was reduced by approximately 91% during SR (13054±2763 to 1192±330 steps·d^{-1}; P<0.001). Self-reported pedometer-derived daily step count during SR (1312±297 steps·d^{-1}) was highly correlated with accelerometer-derived daily step count (r = 0.851; P=0.001). Percentage of total time spent sedentary (73±6 to 90±3%; P<0.001) increased and percentage of total time spent standing (17±6 to 8±3%; P<0.001) and ambulatory (10.0±1.0

to 1.0±0.5%; P<0.001) decreased during SR. The number of daily transitions from a sitting to standing position was also significantly reduced during SR (46±8 to 31±10; P<0.001).

3.4.2 Body weight and dietary intake

Body weight was not different following HPA and SR (75.3±11.0 to 75.1±10.8 kg; P>0.05). Dietary intake during HPA and SR is presented in **Table 3.3**. Daily energy intake tended to decrease (P=0.07) whereas both daily protein intake (P<0.01) and protein intake relative to body weight (P<0.01) significantly decreased during SR. However, absolute carbohydrate and fat intake and the relative contribution of protein, carbohydrate and fat to overall energy intake were unchanged across the intervention (P>0.05).

Table 3.3 Dietary intake during HPA and SR.

Variable	HPA	SR
Energy intake ($kcal \cdot d^{-1}$)	2625 ± 732	2380 ± 864
Protein ($g \cdot kg^{-1} \cdot d^{-1}$)	2.1 ± 0.7	1.8 ± 0.6*
Protein intake ($g \cdot d^{-1}$)	156 ± 51	133 ± 45*
Carbohydrate intake ($g \cdot d^{-1}$)	297 ± 142	279 ± 165
Fat intake ($g \cdot d^{-1}$)	83 ± 34	77 ± 33
Protein (En%)	26 ± 13	24 ± 12
Carbohydrate (En%)	46 ± 13	46 ± 12
Fat (En%)	28 ± 9	29 ± 10

Values are mean±SD. n=11. *(P<0.01) indicates a significant difference between HPA and SR conditions.

3.4.3 Oral glucose tolerance

Following SR, fasting plasma glucose concentrations were unaltered (P>0.05; **Figure 3.2A**), whereas fasting plasma insulin concentrations increased (P<0.05; **Figure 3.2C**). In response to the OGTT, a significant main effect for time (P<0.001) was observed, with plasma glucose concentrations elevated at 30 minutes compared to all other time points (P<0.01), at 60 minutes compared to baseline and 120 minutes (P<0.05), and at 90 minutes compared to 120 minutes (P<0.05; **Figure 3.2A**). Plasma glucose AUC (703±118 to 788±79 mmol·120 min·L^{-1}) was not significantly altered by 7 days of SR (P>0.05; **Figure 3.2B**). In contrast, a significant condition x time interaction (P<0.01) was observed for plasma insulin, with greater plasma insulin concentrations at 60, 90 and 120 minutes of the OGTT following 7 days of SR (P<0.05; **Figure 3.2C**). In line with these findings, plasma insulin AUC (4590±1817 to 6287±1363 µIU·120 min·mL^{-1}) was significantly greater following SR (P<0.01; **Figure 3.2D**), corresponding with a decrease in the Matsuda index (6.5±1.8 to 4.5±0.7) (P<0.01; **Figure 3.2F)**. A significant main effect for time (P<.001) was also observed for plasma NEFA concentrations, with baseline values being significantly higher at baseline compared to all other time points (P<0.05), at 30 minutes compared to 60, 90 and 120 minutes (P<0.001) and at 60 minutes compared to 90 minutes (P<0.05; **Figure 3.2E**).

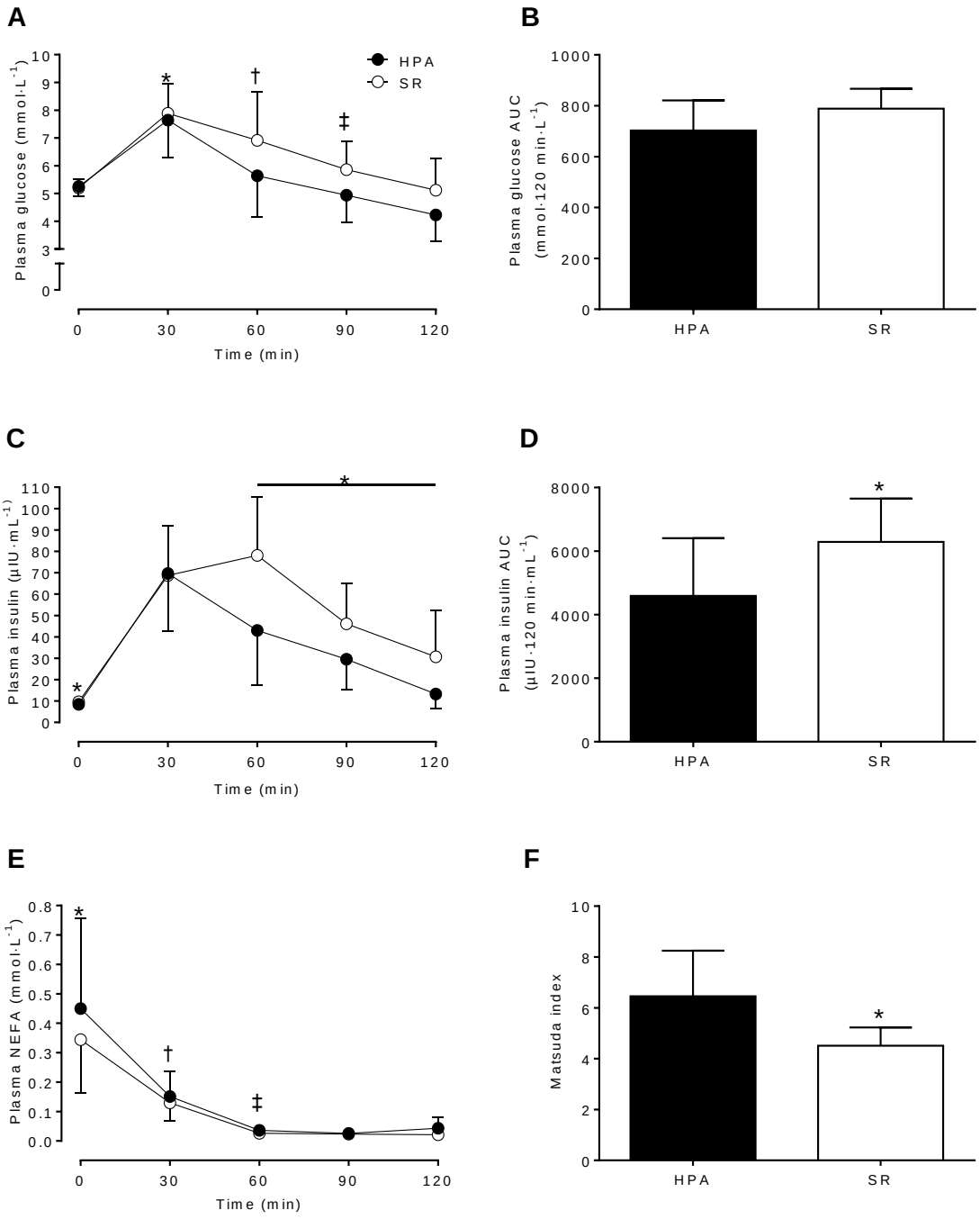

Figure 3.2 Plasma metabolite responses to an OGTT following 7 days of HPA and 7 days of SR in young males (n=11). Data are mean±SD. A: significant main effect for time (P<0.001), *P<0.01 compared to all other time points, †P<0.05 compared to 0 and 120 minutes, ‡P<0.05 compared to 120 minutes. B: no significant effect. C: significant condition x time interaction (P<0.01), *P<0.05 compared with corresponding HPA value. D: *P<0.01 compared with

HPA. E: significant main effect for time (P<0.001), *P<0.05 compared to all other time points, †P<0.001 compared to 60, 90 and 120 minutes, ‡P<0.05 compared to 90 minutes. F: *P<0.01 compared with corresponding HPA value.

3.4.4 Body water ^{2}H enrichment

Figure 3.3A presents the mean body water ^{2}H enrichment on a day-by-day basis. Following the loading phase on day -2 and a single maintenance day on day -1, body water ^{2}H enrichment reached 0.54±0.09% (day 0). Body water ^{2}H enrichment did not change significantly over the duration of the study with an average body water ^{2}H enrichment of 0.59±0.12% during HPA and 0.64±0.17% during SR (P>0.05).

3.4.5 Myofibrillar protein synthesis

As shown in **Figure 3.3B**, daily myofibrillar protein synthesis rates decreased by approximately 27% from 1.39±0.32 $\%\cdot d^{-1}$ during HPA to 1.01±0.38 $\%\cdot d^{-1}$ during SR (P<0.05).

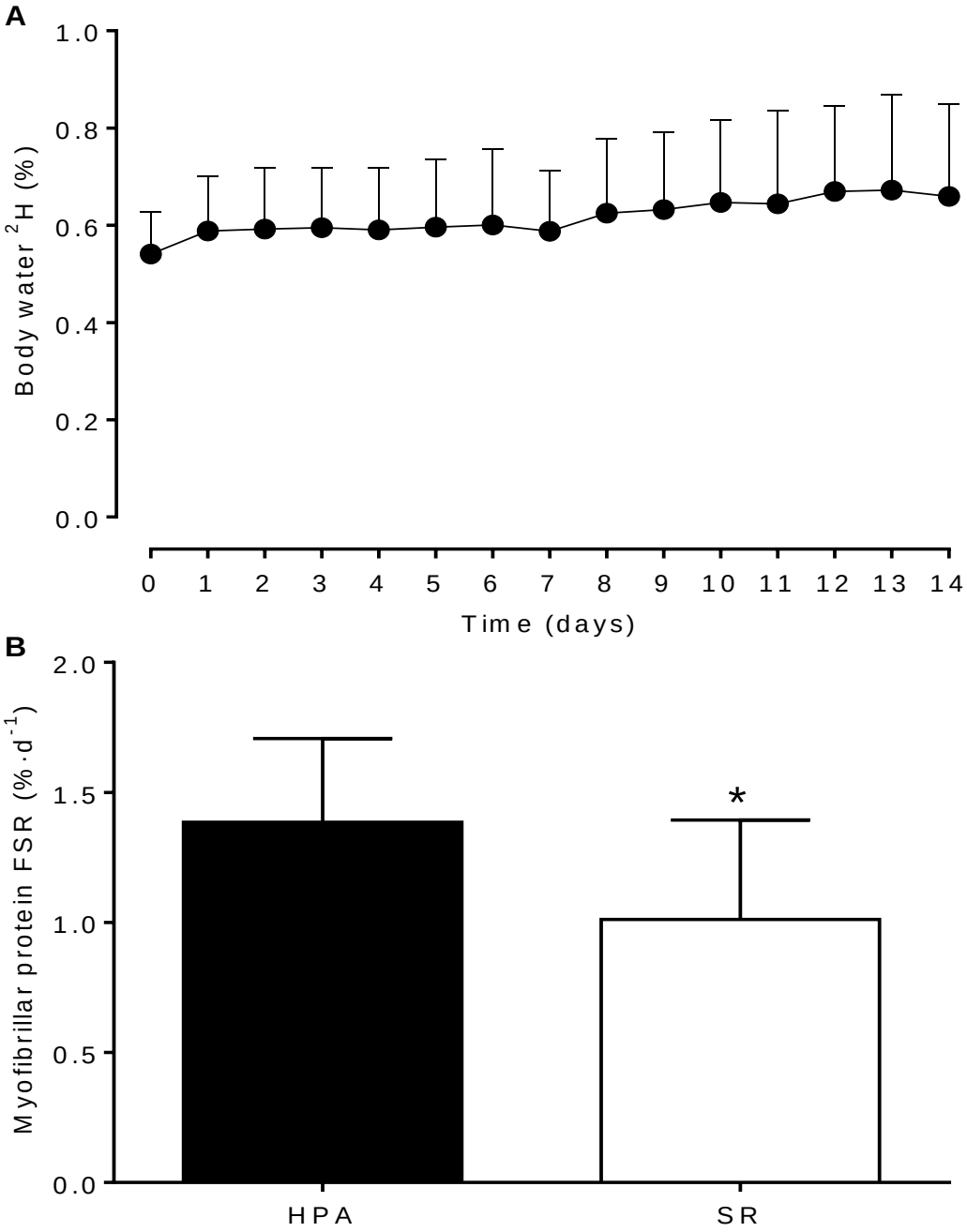

Figure 3.3 Body water ^{2}H enrichment and daily myofibrillar protein FSR during 7 days of HPA and 7 days of SR in young males (n=11). Data are mean±SD. Body water ^{2}H enrichment remained in steady state for the duration of the study ($P>0.05$). *($P<0.05$) indicates a significant difference between HPA and SR conditions.

3.4.6 Gene expression

The skeletal muscle mRNA expression of genes implicated in muscle mass regulation and oxidative metabolism is presented in **Figure 3.4**. In relation to the regulation of MPS, myostatin mRNA expression was increased following SR and this was paralleled by reduced mTOR mRNA expression (both $P<0.05$; **Figures 3.4C and 3.4D**). However, p70S6K mRNA expression was unchanged following SR ($P>0.05$; **Figure 3.4E**). In regards to MPB, MuRF1 mRNA expression was unchanged ($P>0.05$; **Figure 3.4A**), whereas MAFbx mRNA expression was up-regulated following SR ($P<0.05$; **Figure 3.4B**). p53 and PDK4 mRNA expression both decreased following SR (both $P<0.05$; **Figures 3.4F and 3.4G**) with no change in PGC-1α mRNA expression ($P>0.05$; **Figure 3.4H**).

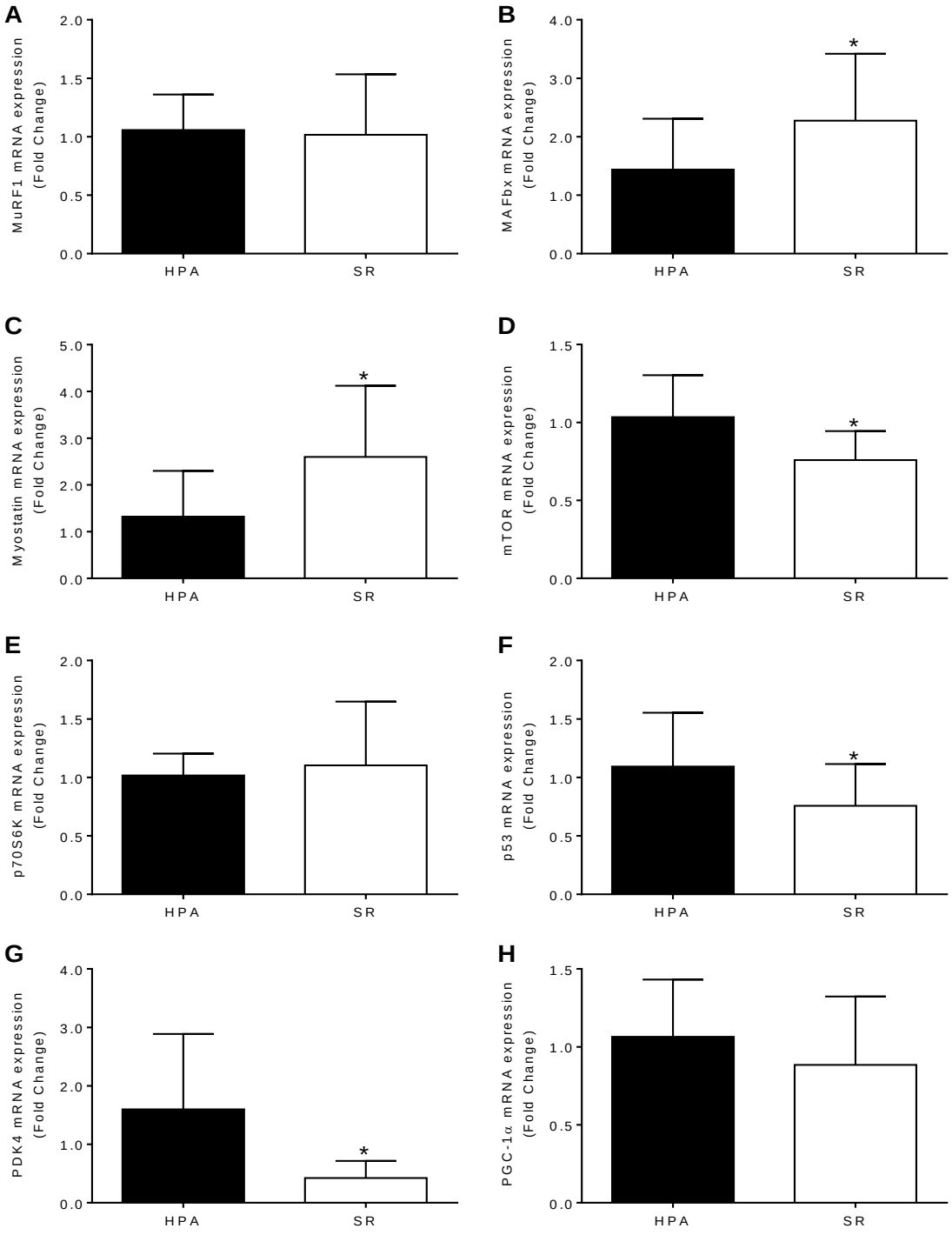

Figure 3.4 Skeletal muscle mRNA expression of MuRF1 (A), MAFbx (B), myostatin (C), mTOR (D), p70S6K (E), p53 (F), PDK4 (G) and PGC-1α (H) following 7 days of HPA and 7 days of SR in young males (n=10). Data are mean±SD. *($P<0.05$) indicates a significant difference between HPA and SR conditions.

3.5 Discussion

The major novel finding of the present study was that one week of reduced physical activity and increased sedentary time led to a substantial (~27%) decline in daily myofibrillar protein synthesis rates. This decline in myofibrillar protein synthesis was associated with increased skeletal muscle mRNA expression of myostatin and MAFbx and decreased mRNA expression of mTOR. The present findings also show that one week of reduced physical activity and increased sedentary time led to a decline in whole-body insulin sensitivity, in addition to decreasing skeletal muscle mRNA expression of selected genes related to oxidative metabolism (i.e., PDK4 and p53). Together, these findings provide direct evidence that reduced physical activity and increased sedentary time alters the physiological processes which regulate skeletal muscle in healthy young individuals.

Across the lifespan, physical activity levels generally decrease and time spent sedentary typically increases (Loyen *et al.*, 2017). Likewise, injury, illness and/or other significant life events often necessitate short periods (typically 2-7 days) of reduced physical activity and increased sedentariness (Fisher *et al.*, 2011; Tipton, 2015). The findings of the present study demonstrate for the first time that just one week of reduced physical activity and increased sedentary time leads to significant (~27%) declines in daily myofibrillar protein synthesis rates in young healthy individuals (**Figure 3.3B**). These findings extend previous observations of reduced postprandial and integrated myofibrillar protein synthesis rates following two weeks of step reduction in older, overweight adults (Breen *et al.*, 2013; McGlory *et al.*, 2018) and highlights the central role that day-to-day muscular contractile activity plays in regulating MPS rates. Promotion of regular physical activity and minimising sedentariness throughout the lifespan should be considered as integral to the maintenance of skeletal muscle health.

The findings of McGlory and colleagues are most comparable as they also applied $^{2}H_2O$ to measure daily myofibrillar protein synthesis rates (McGlory *et al.*, 2018). The ~27% decline in daily myofibrillar protein synthesis observed in the present study is substantially greater than the ~12% decline in integrated myofibrillar protein synthesis rates observed by McGlory and colleagues (McGlory *et al.*, 2018). This may be related to the greater relative change in daily step count induced by this step reduction intervention (~91%) compared to McGlory et al. (~70%) (McGlory *et al.*, 2018). Alternatively, this discrepancy could be explained by the duration of step reduction or differences in the populations studied (i.e., younger vs. older adults). For example, some, but not all, human muscle disuse studies have shown that 5-14 days of limb immobilisation results in greater loss of muscle mass in younger individuals when compared to older individuals (Suetta *et al.*, 2009; Wall *et al.*, 2015a). Thus, it could be hypothesised that a similar pattern of response is seen from the perspective of MPS, whereby younger individuals are more susceptible to changes in physical activity status than older individuals. In this regard, an older comparator group to directly assess age-related differences in the present study would have been informative and is an important avenue for future research.

Whilst one week of step reduction was sufficient to induce a substantial decrease in daily myofibrillar protein synthesis rates in the present study, bed rest of the same duration was recently shown to have no impact on daily myofibrillar protein synthesis rates (Dirks *et al.*, 2019). This is surprising given that bed rest has previously been shown to reduce postabsorptive and postprandial MPS rates when measured acutely (Ferrando *et al.*, 1997; Drummond *et al.*, 2012; Tanner *et al.*, 2015). Whilst this discrepancy is different to reconcile, it should be noted that the participants in the present study were highly active. Detailed baseline physical activity characteristics of the participants in the study by Dirks and

colleagues (Dirks *et al.,* 2019) are not reported but it is possible that a greater relative change in physical activity levels in the present study compared to Dirks *et al.* (2019) explains these discrepant findings.

A number of factors including habitual physical activity (Breen *et al.,* 2013), diet composition (van Vliet *et al.,* 2017) and energy balance (Murphy *et al.,* 2015) can influence day-to-day MPS rates. It is also well established that dietary protein/amino acid administration robustly stimulates MPS (Volpi *et al.,* 1999; Bohe *et al.,* 2003; Witard *et al.,* 2014). Whilst dietary protein intake decreased from habitual levels during SR, it is important to note that participants were still consuming 133±13 $g \cdot d^{-1}$ of dietary protein during the SR period **(Table 3.2)**. When expressed relative to body weight, this equates to a protein intake of 1.8±0.2 $g \cdot kg^{-1} \cdot d^{-1}$. This intake is well above the established recommended dietary allowance for protein of 0.8 $g \cdot kg^{-1}$ of body weight and is also greater than recently proposed changes to those recommendations (i.e., 1.2-1.6 $g \cdot kg^{-1}$ of body weight) (Phillips *et al.,* 2016). Energy balance can also influence MPS rates, with studies showing that energy restriction reduces myofibrillar protein synthesis rates in young and older individuals (Areta *et al.,* 2014; Murphy *et al.,* 2015; Oikawa *et al.,* 2018). In the present study, daily energy intake tended (P=0.07) to decrease during SR but body weight remained stable, suggesting that participants were not in negative energy balance. Whilst it is unlikely that modifications in dietary protein and/or energy intake contributed to the decline in daily myofibrillar protein synthesis rates during one week of SR, these factors and other dietary related variables (e.g., protein distribution across the day) cannot be completely ruled out and thus future studies should investigate the independent and combined impact of these variables on muscle mass regulation.

Previous studies that employed stable isotope infusion protocols within a laboratory setting provide some insight into what could explain the reduction in daily myofibrillar protein synthesis rates observed herein. For example, the reduction in regular muscular contractile activity undoubtedly contributed given that physical activity acts synergistically to enhance the muscle protein synthetic response to dietary protein/amino acids (Pennings *et al.*, 2011; Timmerman *et al.*, 2012; Holwerda *et al.*, 2016). In addition, two weeks of step reduction has previously been shown to induce the development of 'anabolic resistance' in older adults and thus it is possible that a similar phenomenon was captured in the long-term measurement of myofibrillar protein synthesis rates in the present study (Breen *et al.*, 2013).

The precise acute metabolic mechanisms underpinning the step reduction-induced decline in myofibrillar protein synthesis rates remain to be confirmed in a younger population. Nonetheless, in the present study, a coordinated up-regulation of myostatin expression and down-regulation of mTOR expression was observed in skeletal muscle following one week of SR **(Figures 3.4C and 3.4D)**. These findings are relatively consistent with previous studies that have observed increased myostatin expression following human muscle disuse (Dirks *et al.*, 2014a; Wall *et al.*, 2014). Myostatin negatively regulates muscle mass in part via inhibition of mTOR, a key regulator of MPS (Amirouche *et al.*, 2009). Heightened mRNA expression of myostatin in conjunction with lowered mRNA expression of mTOR is therefore entirely consistent with the observed reduction in myofibrillar protein synthesis rates.

To gain further insight into the impact of short-term reduced physical activity and increased sedentary time on muscle mass regulation, the gene expression of putative markers of MPB was also determined. Muscle-specific E3 ubiquitin ligases (e.g., MAFbx and MuRF1) selectively target muscle proteins for degradation via the 26S proteasome (Bodine & Baehr,

2014). In the present study, an increase in MAFbx expression was observed whereas MuRF1 expression remained unchanged **(Figures 3.4A and 3.4B)**. The disparity in the responsiveness of the E3 ubiquitin ligases to step reduction is intriguing but has been reported previously following bed rest and limb immobilisation in humans (Dirks *et al.*, 2014a; Tanner *et al.*, 2015). It is possible that the observed decrease in myofibrillar protein synthesis rates was matched by a similar decrease in MPB, reflecting a reduced muscle protein turnover, although the increase in MAFbx expression following step reduction does not support this notion. However, this observation represents a single time point and may not necessarily reflect dynamic changes that occurred throughout the entire step reduction period. Clearly further research is required to provide greater insight into the relative importance of MPB in the context of reduced physical activity and increased sedentary time.

In line with previous findings, one week of reduced physical activity and increased sedentary time led to a decline in whole-body insulin sensitivity **(Figure 3.2)**. The increased plasma insulin response **(Figures 3.2C and 3.2D)**, without a significant change in the plasma glucose response to the OGTT **(Figure 3.2A and 3.2B)**, supports previous findings in young individuals (Lyden *et al.*, 2015) and likely represents a compensatory mechanism in order to maintain glycaemic control. This is in contrast to longer-term (2 weeks) step reduction, where both plasma glucose and insulin concentrations appear to be elevated in response to an OGTT (McGlory *et al.*, 2018).

PDK4 is a key enzyme that regulates skeletal muscle fuel selection by modulating the activity of the pyruvate dehydrogenase complex (Tsintzas *et al.*, 2007). Following SR, PDK4 expression was markedly down-regulated **(Figure 3.4G).** Given that PDK4 expression is up-regulated by physical activity (Pilegaard *et al.*, 2000; Latouche *et al.*, 2013), the observed down-regulation of PDK4 expression in the present study may be related to reduced muscular

contractile activity during the period of step reduction. Interestingly, whole-body insulin sensitivity declined in the present study but skeletal muscle PDK4 expression is typically up-regulated in insulin resistant states (Majer *et al.*, 1998; Tsintzas *et al.*, 2007). As such, the decrease in PDK4 expression does not appear to align with what might be expected in skeletal muscle insulin resistance, and is more likely to be a direct consequence of reduced muscular contractile activity during the period of step reduction.

The absence of muscle mass measures following step reduction may be considered a limitation of the present investigation. However, it is unlikely that DXA would have been sensitive enough to detect any change in muscle mass that may have occurred over one week of step reduction (Krogh-Madsen *et al.*, 2010; Buehring *et al.*, 2014). Importantly, recent evidence has shown that myofibrillar protein synthesis rates measured using $^{2}H_2O$ are predictive of long-term changes in skeletal muscle mass (Damas *et al.*, 2016). Thus, it is possible that the observed decline in daily myofibrillar protein synthesis would contribute to loss of muscle mass with chronic reduced physical activity and increased sedentary time. It should also be noted that structured physical activity was reduced *and* sedentary time was increased in the present study, precluding any conclusions being made on the independent impact of either of these distinct behaviours. However, given that a large proportion of the global population are both physically inactive and highly sedentary (Loyen *et al.*, 2017), the present findings are highly relevant. It should also be acknowledged that the order of the interventions was not randomised and thus future studies should ensure that the interventions being examined are randomised and counterbalanced to control for order effects. Finally, physical activity levels tend to be lower in women compared to men and thus future research utilising a similar study design in women is warranted (Althoff *et al.*, 2017).

In conclusion, one week of step reduction lowers daily myofibrillar protein synthesis rates and alters the expression of several genes within skeletal muscle related to muscle mass regulation and oxidative metabolism in healthy young men. Promotion of regular physical activity and minimising sedentariness throughout the lifespan should be considered as essential to the preservation of skeletal muscle health.

CHAPTER 4

DAILY MYOFIBRILLAR PROTEIN SYNTHESIS RATES IN RESPONSE TO LOW AND HIGH FREQUENCY RESISTANCE EXERCISE IN YOUNG MEN

4.1 Abstract

High resistance exercise frequencies have been posited to maximise MPS rates and muscle hypertrophy. However, the impact of resistance exercise frequency on MPS rates remains unknown. The primary aim of this study was to compare daily myofibrillar protein synthesis rates over a seven-day period of volume-matched, low frequency and high frequency resistance exercise. Nine young men (21±2 y) completed a seven-day period of habitual physical activity (BASAL). This was followed by a seven-day exercise period of volume-matched, low frequency (once per week; LF) or high frequency (five times per week; HF) resistance exercise. Participants had one leg randomly allocated to LF and the other to HF. Skeletal muscle biopsies and daily saliva samples were collected to determine daily myofibrillar protein synthesis rates using $^{2}H_2O$ and intracellular signalling. Daily myofibrillar protein synthesis rates were not different between LF (1.46±0.26 %·d^{-1}) and HF (1.48±0.33 %·d^{-1}) conditions over the seven-day exercise period (P>0.05). Moreover, there were no significant differences between LF and HF conditions over the first two days (1.45±0.41 vs. 1.25±0.46 %·d^{-1}) or over the last five days (1.47±0.30 vs. 1.50±0.41 %·d^{-1}) of the exercise period (P>0.05). Daily myofibrillar protein synthesis rates were not different from BASAL at any time point during LF or HF (P>0.05). The phosphorylation status and total protein content of selected proteins implicated in skeletal muscle ribosomal biogenesis were not different between LF and HF (P>0.05). Under the conditions of the present study, resistance exercise frequency does not appear to modulate daily myofibrillar protein synthesis rates in young men.

4.2 Introduction

Skeletal muscle mass and strength are essential components of sport performance, functional independence and overall health. One of the most effective methods to increase muscular strength and induce skeletal muscle hypertrophy is long-term resistance training. The muscle hypertrophic response to resistance exercise can be modulated by manipulating variables such as absolute load (Burd *et al.*, 2010b; Mitchell *et al.*, 2012), total exercise volume (Burd *et al.*, 2010a), proximity to failure (Burd *et al.*, 2010b) and rest interval between sets (McKendry *et al.*, 2016; Schoenfeld *et al.*, 2016b). Less clear is the impact that resistance exercise frequency (i.e., the number of times a muscle group is exercised over a given period of time) has on muscle hypertrophy. Understanding the relative importance of exercise frequency is necessary to optimise the skeletal muscle adaptive response to resistance training.

Whilst some studies have shown muscle hypertrophy to be enhanced by a higher (i.e., two or more times per week) resistance exercise frequency (Schoenfeld *et al.*, 2015b; Zaroni *et al.*, 2018), most studies have shown no differences (Gentil *et al.*, 2015; Thomas & Burns, 2016; Barcelos *et al.*, 2018; Gomes *et al.*, 2018; Ochi *et al.*, 2018). Indeed, a recent meta-analysis suggests that under volume-matched conditions, resistance exercise frequency does not significantly impact muscle hypertrophy (Schoenfeld *et al.*, 2018). However, most studies to date have examined the impact of resistance exercise frequencies in the range of one-to-three times per week. In contrast, far less research has been conducted on the impact of higher frequency resistance exercise (e.g., five times per week) under volume-matched conditions. It is possible that very high resistance exercise frequencies are required to enhance MPS rates and subsequent muscle hypertrophy. The evidence currently available is equivocal, with one study (Zaroni *et al.*, 2018) showing greater muscle hypertrophy with a relatively high (five

times per week) resistance exercise frequency and the other no difference (Gomes *et al.*, 2018). As such, the impact of very high resistance exercise frequencies on muscle hypertrophy remains unclear.

Muscle hypertrophy following prolonged resistance training is the product of sustained elevations in MPS that exceed MPB. It has recently been posited that relatively high resistance exercise frequencies could maximise muscle hypertrophy by regularly stimulating the acute myofibrillar protein synthetic response to a single bout of resistance exercise (Dankel *et al.*, 2017). Following an acute bout of resistance exercise, myofibrillar protein synthesis remains elevated for approximately 24 hours before returning to basal levels (Burd *et al.*, 2011; Damas *et al.*, 2016). Furthermore, a relatively low volume (~three sets) of high load resistance exercise appears to maximise post-exercise myofibrillar protein synthesis rates, at least in young individuals (Burd *et al.*, 2010a; Kumar *et al.*, 2012). On this basis, it has been speculated that low-volume, more frequent resistance exercise could induce more regular elevations in myofibrillar protein synthesis rates which in the long-term would lead to greater muscle hypertrophy (Dankel *et al.*, 2017). Given that time is often cited as a major barrier to physical activity participation, minimal daily time commitment associated with low volume, high frequency resistance exercise could also provide a novel strategy to increase physical activity levels. Whilst the proposed hypothesis is plausible, it has yet to be directly tested and is based on the acute myofibrillar protein synthetic response which does not necessarily reflect long-term muscle hypertrophy and remodelling (Mitchell *et al.*, 2014).

Accordingly, the primary purpose of the present study was to compare daily myofibrillar protein synthesis rates over a seven-day period of volume-matched, low frequency and high frequency resistance exercise. To achieve this, young men completed volume-matched, low frequency (once per week; LF) and high frequency (five times per week; HF) resistance

exercise over a seven-day period. $^{2}H_2O$ was ingested throughout to allow myofibrillar protein synthesis rates to be measured in a free-living environment, providing important insights into longer-term muscle mass regulation (Damas *et al.*, 2016). As MPS is partly regulated by translational capacity (i.e., the number of ribosomes available for protein synthesis) (Figueiredo & McCarthy, 2019), a secondary aim was to assess whether resistance exercise frequency impacts the phosphorylation status and total protein content of selected proteins implicated in ribosomal biogenesis.

4.3 Methods

4.3.1 Participants and ethical approval

Nine healthy young men participated in the present study which took place between February 2018 and August 2018. Participant characteristics are presented in **Table 4.1**. Participants were recreationally active and untrained (i.e., performed activities of daily living and recreation but had completed no regular lower body resistance exercise in the last year). Prior to providing informed written consent, each volunteer was informed of the experimental procedures and potential risks associated with the experimental intervention. Participants were screened prior to inclusion in the study and deemed healthy based on their responses to a general health questionnaire. The study was approved by the National Research Ethics Service Committee West Midlands, Edgbaston, United Kingdom (Reference: 17/WM/0430) and conformed to standards for the use of human participants in research as outlined in the Declaration of Helsinki. The intervention was registered at clinicaltrials.gov prior to data collection (Identifier: NCT03275779).

Table 4.1 Participant characteristics at baseline.

Variable	Value
Age (y)	21.0 ± 2.3
Height (m)	1.79 ± 0.07
Body mass (kg)	72.4 ± 7.1
BMI ($kg \cdot m^{-2}$)	22.7 ± 2.6
LF leg press 1RM (kg)	104 ± 22
HF leg press 1RM (kg)	106 ± 22
LF leg extension 1RM (kg)	82 ± 11
HF leg extension 1RM (kg)	81 ± 12

Values are mean±SD. n=9.

4.3.2 Pretesting

During the initial screening visit, participants underwent maximal strength testing and a familiarisation session. Prior to strength testing, participants completed a five minute warm-up of self-paced cycling at ~100 W. Maximal leg strength was then determined for each leg on a plate loaded 45° leg press (Cybex, 5321). This process was then repeated on a weight-stacked leg extension machine (Element Fitness, 9328). This exercise order was chosen to replicate the order of exercises to be completed during subsequent resistance exercise session. Participants first performed a submaximal warm-up set of eight-to-ten repetitions and had their lifting form critiqued and corrected when necessary. This was followed by sets at progressively increasingly loads until only one valid repetition could be completed. The load for each set was chosen by the investigator based on the participant's rating of perceived exertion following the previous set. A three minute rest interval was provided between each

set. Exercise machine positions for each participant were recorded and replicated in subsequent sessions. Once completed, the corresponding load (~70% 1RM) to be used during the subsequent familiarisation session and resistance exercise sessions was calculated.

In order to familiarise participants with the exercise volume to be completed during the experimental trials, and to avoid the muscle damage response associated with an unfamiliarised bout of resistance exercise (Damas *et al.*, 2016), participants completed five sets of bilateral leg press followed by five sets of bilateral leg extension at ~70% 1RM with two minutes of rest allowed between each set. Total exercise volume completed during the familiarisation (12121±2206 kg) was similar to that completed in total by both legs during the experimental resistance exercise sessions (11952±2700 kg). Pretesting and the first experimental trial (day 0) were separated by at least seven days.

4.3.3 Study overview

An overview of the study is presented in **Figure 4.1**. The study was designed to assess whether resistance exercise frequency modulates daily myofibrillar protein synthesis rates under free-living conditions. To achieve this, participants undertook a $^{2}H_2O$ dosing protocol (see *$^{2}H_2O$ dosing protocol* section below). An initial $^{2}H_2O$ dosing day was commenced on day -2 and maintenance doses were then consumed daily for the duration of the study. Daily saliva samples were also collected to assess body water ^{2}H enrichment.

Participants arrived at the laboratory at ~08:00 in a fasted state on day 0 and had a muscle biopsy collected. All muscle biopsies were collected from the *vastus lateralis* muscle using the Bergström needle technique with manual suction under local anaesthesia (1% lidocaine). Muscle biopsy samples were blotted and any visible fat, blood or connective tissue removed before snap freezing in liquid nitrogen and storing at -80 °C for later analysis. Participants

then completed a seven-day basal period (BASAL) where they were instructed to maintain habitual physical activity (i.e., activities of daily living and recreation without structured physical activity). Participants returned to the laboratory on day 7 and had a second muscle biopsy collected from the alternate leg. Following the muscle biopsy, participants had each leg randomly allocated to one of LF or HF resistance exercise (see *Resistance exercise sessions* section below). A bout of LF and HF was completed on day 7. Approximately, 48 hours later (day 9), participants returned to the laboratory and had one muscle biopsy collected from each leg. This was followed by the second bout of HF. Additional bouts of HF were completed on days 10, 11 and 12. Participants returned to the laboratory on day 14 (~48 hours after the final HF bout) and had the final muscle biopsies collected from each leg, signifying the end of the study. A pedometer was worn throughout and weighed four-day food diaries were completed to assess daily step count and dietary intake, respectively, across the study.

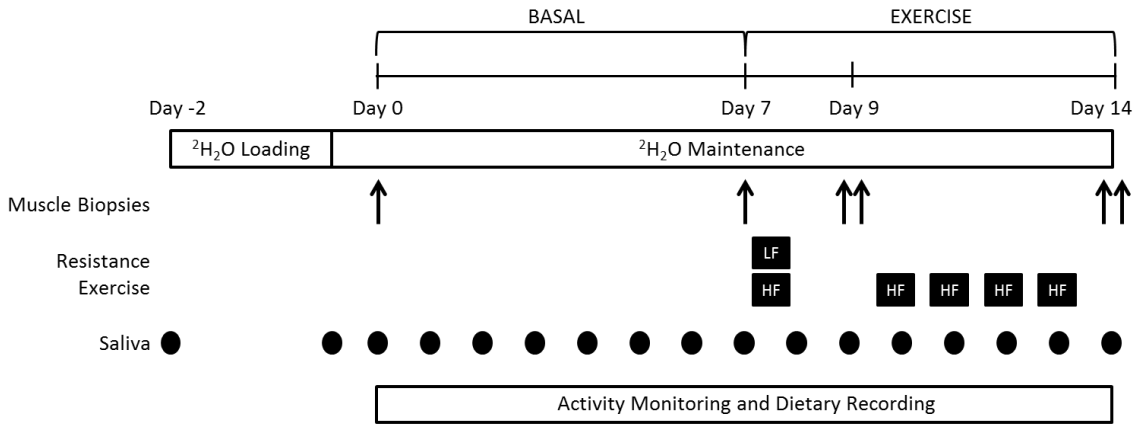

Figure 4.1 Study overview.

4.3.4 ^2H_2O dosing protocol

The ^2H_2O dosing protocol consisted of one dosing day and 16 maintenance days (Holwerda *et al.*, 2018; Shad *et al.*, 2019). On day -2, participants completed a ^2H_2O loading day.

Following collection of a background saliva sample, participants were provided with 8 x 50 mL boluses of 70% $^{2}H_2O$ (Cambridge Isotope Laboratories, Massachusetts, USA) to increase ^{2}H enrichment in body water to 0.5-1%. Approximately 60-90 minutes was allowed between each bolus to negate side effects (e.g., vertigo, nausea) previously reported upon consumption of large volumes of $^{2}H_2O$ over short periods of time (Holm *et al.*, 2013). The $^{2}H_2O$ protocol was well tolerated with none of the participants reporting any adverse effects. For each subsequent day, participants were provided with a daily 50 mL maintenance bolus of $^{2}H_2O$ to consume. Participants were instructed to consume the daily bolus upon waking to ensure consistency and minimise the risk of missed doses. The time at which each bolus was consumed was recorded and participants were instructed to bring the empty bottles back in on each laboratory visit to measure compliance. All boluses were returned void, suggesting full compliance with the $^{2}H_2O$ protocol.

To measure ^{2}H enrichment in body water, saliva samples were collected daily. Participants lightly chewed a cotton swab until completely saturated with saliva (~two-to-three minutes). On days that participants were in the laboratory, swabs were collected immediately and placed in a 5 mL syringe and the saliva compressed into sample tubes and stored at -80 °C for later analysis. On the remaining days when participants were not in the laboratory, daily saliva samples were collected at home and stored in pre-labelled falcon tubes in the refrigerator until the next laboratory visit where samples were stored as described above. Participants were instructed to provide their saliva sample at least two hours following their last $^{2}H_2O$ bolus and at least 30 minutes after their last meal or drink and to record the time at which the sample was collected.

4.3.5 Dietary intake and physical activity

The evening prior to each experimental visit involving muscle biopsies on days 0, 7, 9 and 14, participants received the same standardised meal (~689 kcal, providing ~55 energy% (En%) carbohydrate, ~20 En% protein, and ~25 En% fat). A weighed four-day food diary was completed over the first seven-day period of habitual physical activity (BASAL) and over the second seven-day period of LF and HF resistance exercise to evaluate energy and macronutrient intake. Participants were required to include two week-days and both weekend days in their recordings. Dietary records were analysed using Dietplan software (Forestfield Software Ltd., v6.70.67). Participants were instructed to refrain from structured physical activity throughout the study other than the prescribed resistance exercise completed as part of the study. Participants were also provided with a hip-worn pedometer (Yamax Digi-Walker SW-200) to wear throughout the study to assess daily step count. Daily step count was recorded by participants in a log before bed.

4.3.6 Resistance exercise sessions

Using a within-subject design, participants had one leg randomised to complete LF and the other to HF. Prior to all resistance exercise sessions, participants completed a five-minute warm-up of self-paced cycling at ~100 W. On day 7, a single bout of unilateral high volume LF resistance exercise was completed. This consisted of five sets of 10 repetitions at ~70% 1RM on the 45° leg press machine followed by five sets of 10 repetitions at ~70% 1RM on the weight-stacked leg extension machine. A single bout of unilateral low volume HF resistance exercise was also completed on day 7 using the opposite leg. This consisted of one set of 10 repetitions at ~70% 1RM on the 45° leg press machine followed by one set of 10 repetitions at ~70% 1RM on the weight-stacked leg extension machine. A further four bouts of unilateral low volume HF resistance exercise were completed on days 9, 10, 11 and 12. This design ensured that total exercise volume and the number of sets completed were

matched between the LF and HF conditions. Total exercise volume was intentionally matched as exercise volume has an established role in determining the magnitude of the myofibrillar protein synthetic and subsequent hypertrophic response to resistance exercise (Burd *et al.*, 2010a; Schoenfeld *et al.*, 2017b). All sets were performed close to or to the point of muscular failure; this varied depending on the set number (i.e., later sets tended to be closer to the point of muscular failure due to fatigue from the earlier sets). Two minutes of rest was allowed between all sets and five minutes of rest was allowed between the bouts of LF and HF on day 7. Following all resistance exercise sessions, participants ingested 25 g of whey protein powder (Impact Whey Protein; Myprotein), containing 21 g of protein, dissolved in water.

4.3.7 Body water ^{2}H enrichment

Body water ^{2}H enrichment was analysed from daily saliva samples collected throughout the study as previously described (Holwerda *et al.*, 2018; Shad *et al.*, 2019). Briefly, samples were centrifuged at 10000 g and then diluted 70-fold with ddH_2O. Subsequently, small plastic cups holding 4 mg of catalyst (5 % platinum on alumina, 325 mesh, Sigma-Aldrich, St. Louis, USA) were placed inside 3 mL glass vials (Labco Exetainer, Labco limited, Lampeter, UK) and 300 uL of diluted saliva was then added. Air in each vial was simultaneously evacuated and replaced by hydrogen gas. Once prepared, the vials were left at 21 °C for 24 hours for ^{2}H equilibration to occur between the hydrogen gas and the saliva samples. The ^{2}H enrichment of the hydrogen gas was then measured in duplicate on a GC-C-IRMS (Micromass 205 Optima IRMS fitted with a Multiprep and Gilson autoinjector, Micromass UK Limited, 206 Manchester, UK). Standard regression curves were applied to assess the linearity of the mass spectrometer and to account for ^{2}H loss during equilibration.

4.3.8 Myofibrillar bound ^{2}H-alanine enrichment

For measurement of ^{2}H-alanine enrichment in the myofibrillar fractions, ~50 mg wet muscle tissue was hand-homogenised on ice using a pestle in a standard extraction buffer (10 μL·mg^{-1}). The samples were then spun at 2500 g for 5 minutes at 4 ºC. The pellet was washed with 500 μL of ddH_2O and centrifuged at 250 g for 10 minutes at 4 ºC. The myofibrillar protein was solubilised by adding 1 mL of 0.3 M NaOH and heating at 50 ºC for 30 minutes with vortex mixing every 10 minutes. Samples were centrifuged at 9500 g for 5 minutes at 4 ºC, the supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and spinning at 700 g for 10 minutes at 4 ºC. The myofibrillar protein was washed twice with 70% ethanol and hydrolysed overnight in 2 mL of 6 M HCL at 110 °C. The free amino acids from the hydrolysed myofibrillar protein pellet were dried under a nitrogen stream while being heated to 120 ºC. The free amino acids were then dissolved in 25% acetic acid solution, 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. Thereafter, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. The derivatized samples were measured using a gas chromatography-isotope ratio mass spectrometer (GC-IRMS) (Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60 m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE, USA) and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the ^{2}H/^{1}H ratios of myofibrillar protein bound alanine. A series of known standards were applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

4.3.9 Western blotting

Western blot analyses were performed on the sarcoplasmic fraction obtained during myofibrillar protein extraction. The total protein concentration of each sample was determined using a DC^{TM} protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Subsequently, samples were prepared to a concentration of 2 µg/µL in 4X Laemmli sample buffer and sucrose lysis buffer and boiled at 97 ºC for 5 minutes. An equal amount (30 µg) of protein was separated on 10% gels by SDS-PAGE for ~60 minutes and subsequently wet transferred to PVDF membranes (Pall Laboratory, Portsmouth, UK) for 60 minutes at 100 V. Membranes were then stained with Ponceau S (Sigma, Gillingham, UK) and imaged to correct for sample loading. Membranes were blocked in 2.5% skimmed milk solution in Tris-buffered saline with Tween 20 (TBST) for 60 minutes and then washed 3 times for 5 minutes in TBST prior to overnight incubation at 4 ºC in the following primary antibodies (1:1000) in 2.5% bovine serum albumin (BSA): total eIF4E (ab33766), phospho-eIF4E Ser209 (ab76256), total cyclin D1 (ab16663) and total UBF (ab244287) all purchased from Abcam (Abcam, Cambridge, U.K). The following morning, membranes were washed 3 times for 5 minutes in TBST before incubation in the appropriate secondary antibody (Anti-Rabbit IgG, HRP-linked Antibody (#7074)) (1:10000) for 60 minutes at room temperature. This was followed by a final wash 3 times for 5 minutes in TBST. Antibody detection was achieved via enhanced chemiluminescence horseradish peroxidase substrate detection (Merck Millipore, Watford, UK). Imaging was undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK) and bands were quantified using Image Studio Lite (Li-Cor, Lincoln, Nebraska, U.S).

4.3.10 Calculations

Myofibrillar protein FSR was determined using the incorporation of ^{2}H-alanine into myofibrillar protein and the mean ^{2}H enrichment in body water between sequential biopsies,

corrected by a factor of 3.7, as the surrogate precursor based upon ^{2}H labelling during *de novo* alanine synthesis (Belloto *et al.*, 2007; Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018). The standard precursor-product method was used to calculate FSR:

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

where E_{m1} and E_{m2} are the myofibrillar protein-bound ^{2}H-alanine enrichments between sequential muscle biopsies. $E_{precursor}$ represents the mean body water ^{2}H enrichment between sequential biopsies corrected by a factor of 3.7 based upon the ^{2}H labelling of alanine during *de novo* synthesis (Belloto *et al.*, 2007; Holwerda *et al.*, 2018). *t* represents the time between sequential biopsies in days.

4.3.11 Statistics

All statistical analyses were performed using SPSS 25.0 (SPSS, RRID:SCR_002865, Chicago, IL, USA). Differences between the seven-day basal period and seven-day exercise period (i.e., BASAL vs. LF/HF) for daily step count and dietary intake were compared using paired sample t-tests. Differences between exercise conditions (LF vs. HF) for exercise variables (i.e., maximal strength and total exercise volume) were compared using a paired sample t-test. Body water ^{2}H enrichment was analysed using a one-factor repeated measures ANOVA with time as the within-subjects factor. Myofibrillar protein FSR was analysed using two-factor repeated measures ANOVAs (condition x time) with condition (BASAL vs. LF vs. HF) and time (days 0-7, 7-9, 9-14 and 7-14) as within-subjects factors. Intracellular signalling was analysed using a two-factor repeated measures ANOVA (condition x time) with condition (BASAL vs. LF vs. HF) and time (days 7, 9 and 14) as within-subjects factors. A biopsy sample for one participant could not be collected on day 9, and thus myofibrillar

protein FSR data for days 7-9 and 9-14 and all intracellular signalling data were analysed on n=8. When a significant main effect or interaction was found, t-tests with Bonferroni correction for multiple comparisons were performed. Significance was set at $P<0.05$. All data are presented as mean±SD.

4.4 Results

4.4.1 Exercise variables

Maximal strength (i.e., 1RM) values at baseline were not different between the LF and HF conditions for the leg press (P=0.397) and leg extension (P=0.650) exercises (**Table 4.1**). By design, total exercise volume completed was not different between the LF (5933±1357 kg) and HF (6019±1347 kg) conditions (**Figure 4.2**; P=0.121).

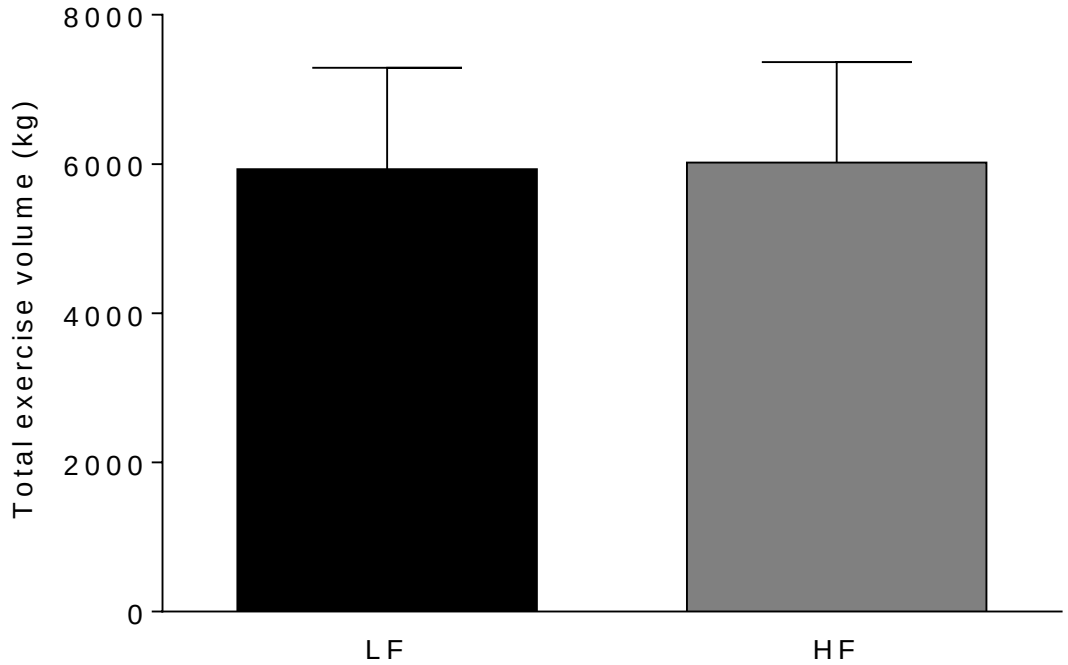

Figure 4.2 Total exercise volume completed in LF and HF resistance exercise conditions (n=9). Data are mean±SD.

4.4.2 Daily step count and dietary intake

Daily step count and dietary intake are presented in **Table 4.2.** Daily step count was not different between BASAL and the seven-day period of LF and HF resistance exercise (P=0.167). The relative contribution of dietary fat to overall energy intake significantly decreased during the period of LF and HF resistance exercise (P=0.041). There was also a trend for daily protein intake (P=0.061) and protein intake relative to body weight (P=0.089) to increase during the period of LF and HF resistance exercise. This trend for daily protein intake to increase during the period of LF and HF resistance exercise is likely explained by the provision of 21 g of protein following each resistance exercise bout during this period. All other dietary variables were unchanged across the study.

Table 4.2 Daily step count and dietary intake during the seven-day period of habitual physical activity (BASAL) and seven-day period of LF and HF resistance exercise.

Variable	BASAL	LF/HF	*P* Value
Daily step count	10000 ± 2420	11458 ± 1871	0.167
Energy intake ($kcal \cdot d^{-1}$)	2253 ± 316	2336 ± 208	0.477
Protein ($g \cdot kg^{-1} \cdot d^{-1}$)	1.3 ± 0.4	1.5 ± 0.2	0.089
Protein intake ($g \cdot d^{-1}$)	93 ± 25	104 ± 15	0.061
Carbohydrate intake ($g \cdot d^{-1}$)	278 ± 53	280 ± 43	0.931
Fat intake ($g \cdot d^{-1}$)	82 ± 12	82 ± 8	0.906
Protein (En%)	16 ± 5	18 ± 2	0.402
Carbohydrate (En%)	51 ± 7	52 ± 4	0.602
Fat (En%)	32 ± 3	30 ± 4*	0.041

Values are mean±SD. n=9. *($P<0.05$) indicates a significant difference between BASAL and LF/HF conditions.

4.4.3 Body water ^{2}H enrichment

Figure 4.3A presents the mean body water ^{2}H enrichment on a day-by-day basis. Following the loading phase on day -2 and a single maintenance day on day -1, body water ^{2}H enrichment reached 0.55±0.05% (day 0). Body water ^{2}H enrichment did not change significantly over the duration of the study with an average body water ^{2}H enrichment of 0.58±0.08% during BASAL and 0.62±0.13% during the period of LF and HF resistance exercise (P=0.107).

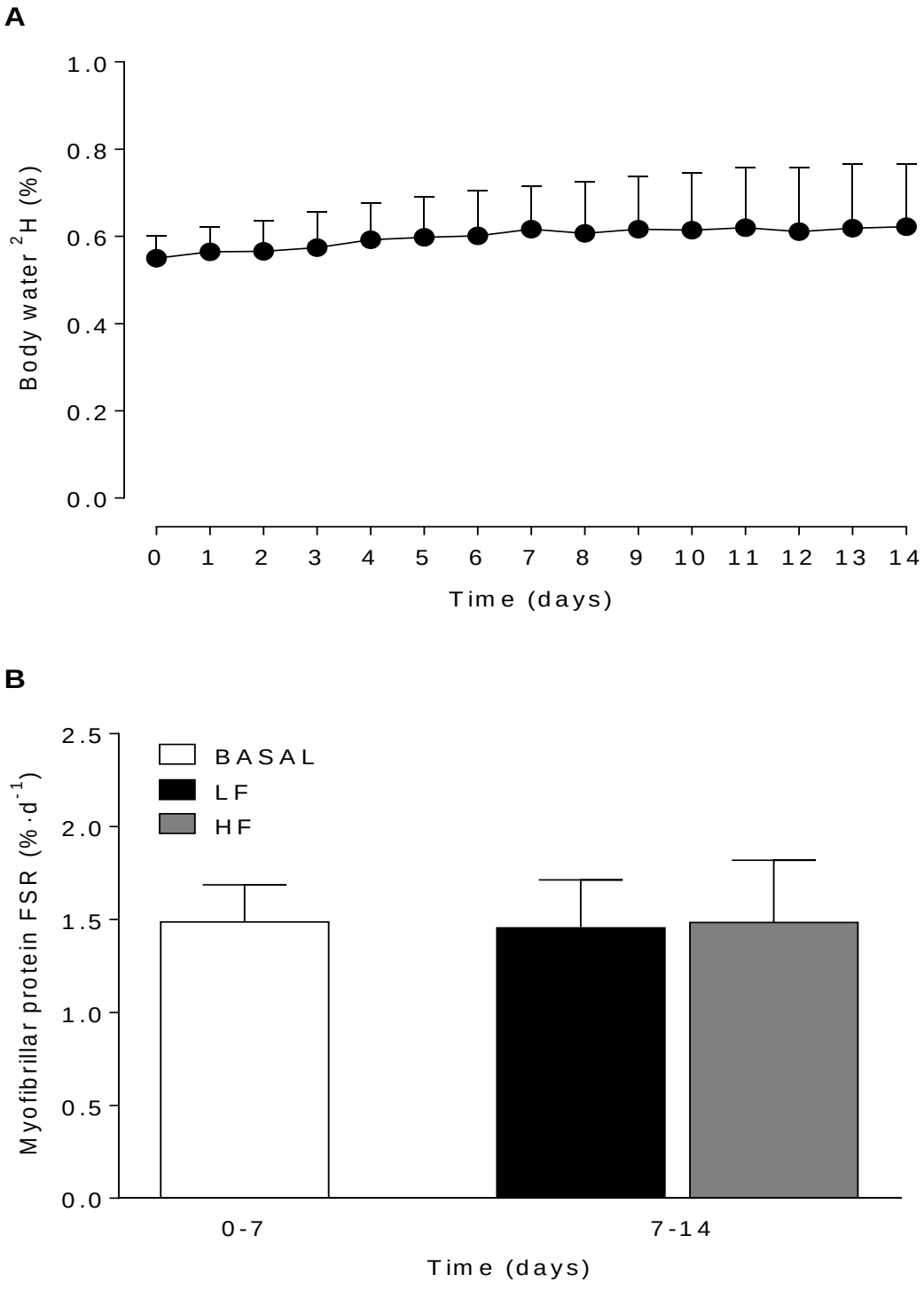

Figure 4.3 Body water ^{2}H enrichment and daily myofibrillar protein FSR during a seven-day period of habitual physical activity (BASAL) and a seven-day period of LF and HF resistance exercise (n=9). Data are mean±SD.

4.4.4 Myofibrillar protein synthesis

Daily myofibrillar protein synthesis rates were not different between LF (1.46±0.26 %·d^{-1}) and HF (1.48±0.33 %·d^{-1}) conditions over the entire seven-day (days 7-14) exercise period (**Figure 4.3B**; P=0.801). Moreover, there were no significant differences between LF and HF conditions over the first two days (days 7-9) of the exercise period (1.45±0.41 vs. 1.25±0.46 %·d^{-1}; **Figure 4.4**; P=0.342) or over the last five days (days 9-14) of the exercise period (1.47±0.30 vs. 1.50±0.41 %·d^{-1}; **Figure 4.4**; P=0.342). Daily myofibrillar protein synthesis rates were not different from BASAL at any time point during LF or HF (**Figures 4.3B and 4.4**; P=0.591).

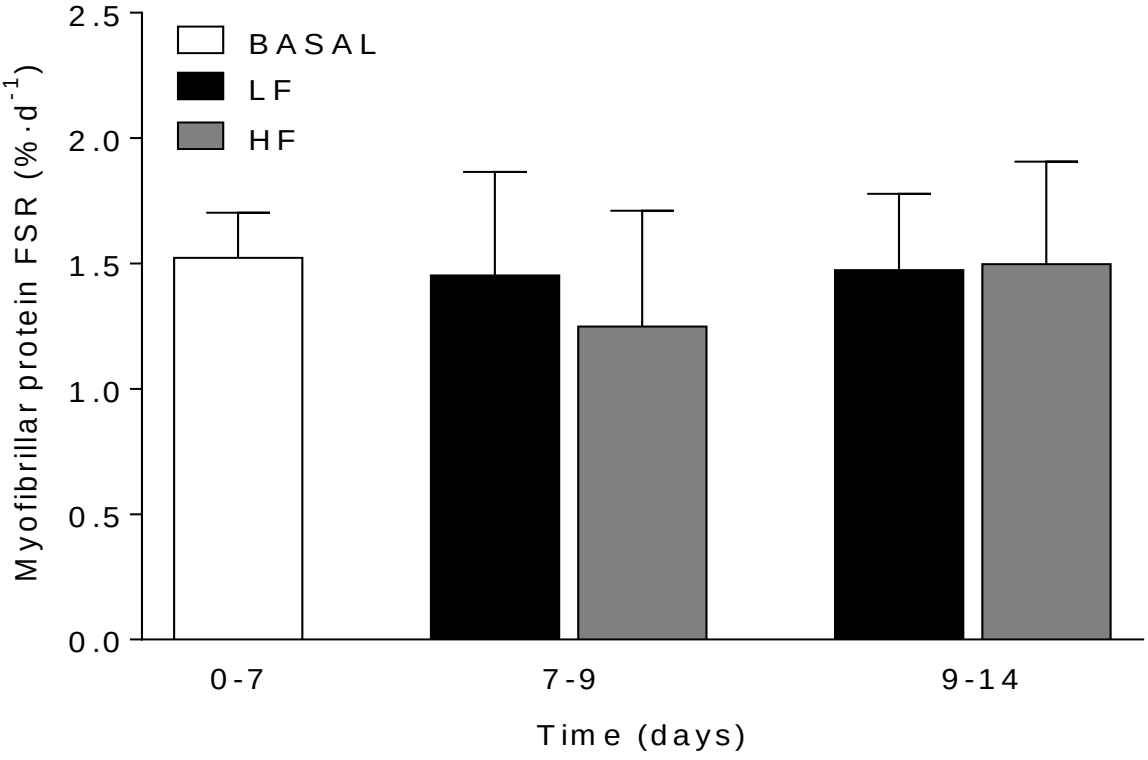

Figure 4.4 Daily myofibrillar protein FSR during a seven-day period of habitual physical activity (BASAL) and a seven-day period of LF and HF resistance exercise (n=8). Data are mean±SD.

4.4.5 Intracellular signaling

The phosphorylation status and total protein content of selected proteins implicated in skeletal muscle ribosomal biogenesis are presented in **Figure 4.5**. A main effect of time was observed for eIF4E total protein content (**Figure 4.5A**; P=0.029). Following correction for multiple comparisons, pairwise comparisons showed a tendency (P=0.056) for greater total protein content 48 hours (i.e., day 9) following the initial LF and HF resistance exercise bouts compared to day 7. A main effect of time was also observed for cyclin D1 total protein content (**Figure 4.5C**; P=0.046). However, following correction for multiple comparisons, pairwise comparisons showed no significant difference between time points. There were no significant changes over time (P=0.407) or differences between LF and HF conditions (P=0.345) for phosphorylation of eIF4E at Ser209 (**Figure 4.5B**). There were no significant changes over time (P=0.217) or differences between LF and HF conditions (P=0.891) for UBF total protein content (**Figure 4.5D**).

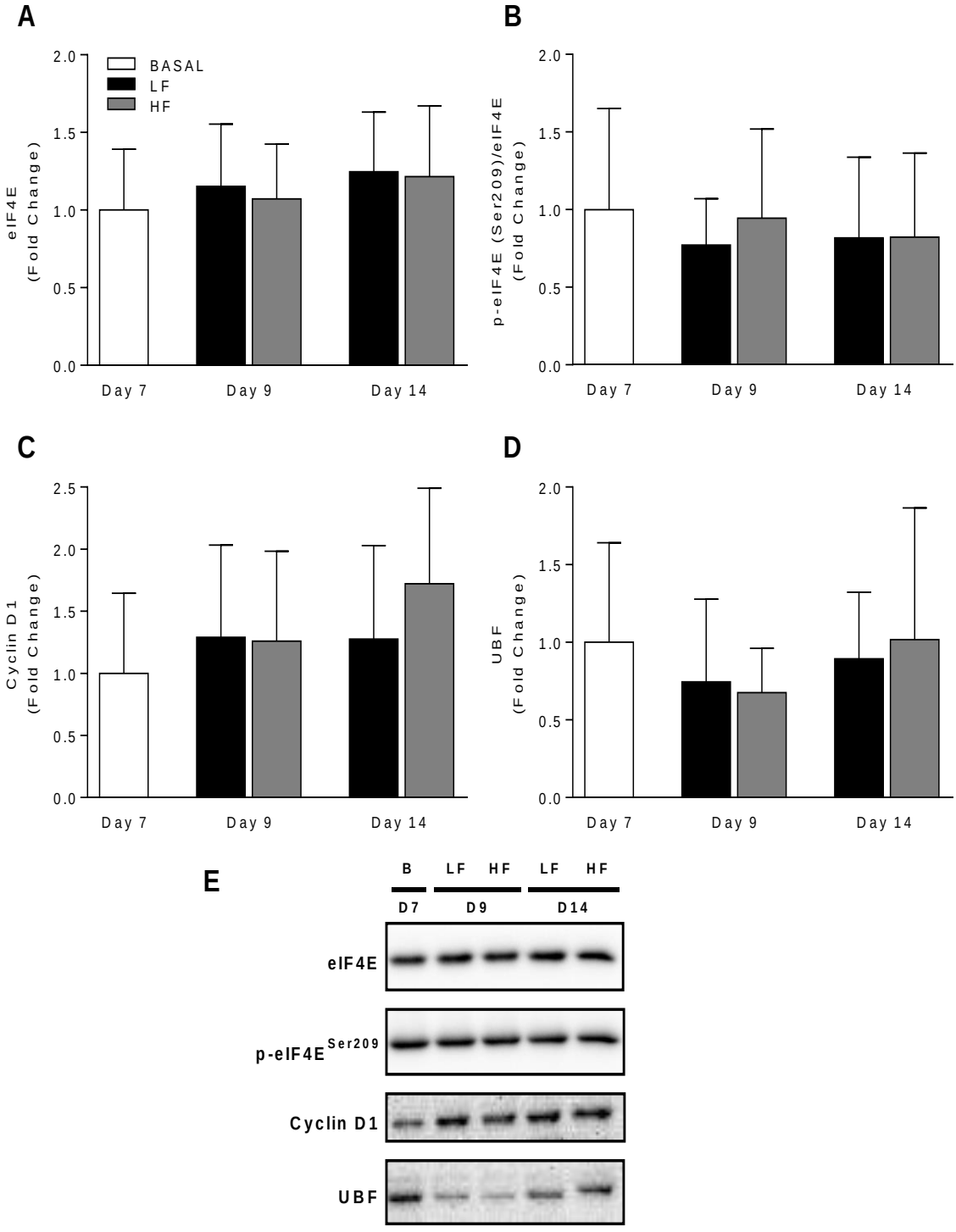

Figure 4.5 Impact of LF and HF resistance exercise on total protein content of eIF4E (A), phosphorylation of eIF4E at Ser209 (B), total protein content of cyclin D1 (C) and total protein content of UBF (D) (n=8). Data are mean±SD.

4.5 Discussion

The present study is the first to examine any impact that resistance exercise frequency may have on myofibrillar protein synthesis rates. The major finding was that daily myofibrillar protein synthesis rates did not differ between volume-matched low and high frequency resistance exercise over a seven-day period in young untrained men. In line with these findings, resistance exercise frequency did not clearly modulate the phosphorylation status and total protein content of selected proteins implicated in skeletal muscle ribosomal biogenesis.

Manipulation of resistance exercise frequency (i.e., the number of times a muscle group is exercised over a given period of time) has been proposed as an effective method to maximise muscle hypertrophy (Dankel *et al.*, 2017; Schoenfeld *et al.*, 2018). This is based on the premise that high resistance exercise frequencies could induce greater overall myofibrillar protein synthesis rates and thus result in a greater amount of time spent in a net positive protein balance (Dankel *et al.*, 2017). In the present study, a unilateral exercise model was utilised where each participant had one leg assigned to complete resistance exercise once per week (i.e., low frequency; LF) and the other leg to complete resistance exercise five times per week (i.e., high frequency; HF). This experimental design ensured that factors known to influence day-to-day MPS rates (e.g., sleep (Holwerda *et al.*, 2016), protein intake (Witard *et al.*, 2014), dietary composition (van Vliet *et al.*, 2017) and habitual physical activity (Shad *et al.*, 2019)) were identical between conditions and thus allowed the impact of different resistance exercise frequencies on myofibrillar protein synthesis rates to be assessed in isolation. In contrast to the aforementioned hypothesis, the findings of the present study demonstrate that under volume-matched conditions, higher resistance exercise frequencies do not result in greater daily myofibrillar protein synthesis rates **(Figures 4.3B and 4.4)**. These

findings lend support to the preponderance of evidence showing that resistance exercise frequency has no impact on muscle hypertrophy (Gentil *et al.*, 2015; Thomas & Burns, 2016; Barcelos *et al.*, 2018; Gomes *et al.*, 2018; Ochi *et al.*, 2018).

Only two studies to date have compared the impact of resistance training muscle groups once per week versus training muscle groups five times per week (Gomes *et al.*, 2018; Zaroni *et al.*, 2018). The present data are in line with evidence showing no difference in muscle hypertrophy with a resistance exercise frequency of one versus five times per week (Gomes *et al.*, 2018) but are inconsistent with findings of Zaroni and colleagues demonstrating greater muscle hypertrophy under similar conditions (Zaroni *et al.*, 2018). It is important to note that the total exercise volume completed in the study by Zaroni *et al.* (2018) was significantly higher in the group resistance training muscle groups five times per week compared to those training muscle groups once per week. In contrast, total exercise volume was intentionally matched between LF and HF conditions in the present study **(Figure 4.2)** which likely explains the lack of agreement in these findings. Indeed, exercise volume has an established role in determining the magnitude of the myofibrillar protein synthetic response to resistance exercise (Burd *et al.*, 2010a) and a recent meta-analysis, published whilst the present study was being undertaken, suggests that resistance exercise frequency does not significantly impact muscle hypertrophy when conducted under volume-matched conditions (Schoenfeld *et al.*, 2018). Taken together, it would appear that resistance exercise frequency *per se* (i.e., under volume matched conditions) does not impact daily myofibrillar protein synthesis rates or subsequent muscle hypertrophy in young individuals.

In contrast to previous studies (Wilkinson *et al.*, 2014; Damas *et al.*, 2016), acute resistance exercise in young adults failed to induce a detectable increase in daily myofibrillar protein synthesis rates throughout the exercise period compared to BASAL **(Figure 4.4)**. Previous

studies showing increased MPS rates and subsequent muscle hypertrophy in young individuals following resistance exercise bouts of a similar volume and relative intensity to that utilised in the present study suggests that the stimulus provided was sufficient (Holm *et al.*, 2010; Snijders *et al.*, 2015; Brook *et al.*, 2016). It must be acknowledged that the inability to detect an increase in daily myofibrillar protein synthesis rates following acute resistance exercise may also have precluded differences from being detected between LF and HF conditions. Whilst the present findings are difficult to reconcile, there are some possible explanations worthy of discussion.

One possibility is that the impact of resistance exercise was 'diluted' over the measurement period. $^{2}H_2O$ measures myofibrillar protein synthesis rates continuously, capturing all free-living activity including sleep and inactivity (Robinson *et al.*, 2011; Brook *et al.*, 2016; McGlory *et al.*, 2018). Whilst more representative of long-term muscle hypertrophy and remodelling (Damas *et al.*, 2016), the free-living nature of the $^{2}H_2O$ measurement may have masked the well-established increase in myofibrillar protein synthesis in the immediate hours following resistance exercise (Moore *et al.*, 2009; Burd *et al.*, 2010a; Kumar *et al.*, 2012). Indeed, Brook *et al.* (2016) recently showed that six weeks of unilateral resistance training induced skeletal muscle hypertrophy and was accompanied by an increase in cumulative myofibrillar protein synthesis rates relative to the untrained leg when measured over the first three weeks (Brook *et al.*, 2016). However, when myofibrillar protein synthesis rates were calculated over the entire six-week period of resistance training, no difference was observed between the untrained and resistance trained conditions (Brook *et al.*, 2016). In this regard, a shorter measurement period (i.e., $\leq$24 hours) following the resistance exercise stimulus may have been required to detect an increase in myofibrillar protein synthesis rates.

An alternative explanation could be related to familiarising participants with resistance exercise prior to the study. During the screening visit, participants completed a high volume familiarisation bout in order to avoid the muscle damage response associated with an unfamiliarised bout of resistance exercise. Given that Damas and colleagues (Damas *et al.*, 2016) recently demonstrated that the 48-hour myofibrillar protein synthetic response following resistance exercise is no longer different from resting values once participants have been familiarised with resistance exercise (and muscle damage has subsided), this may explain the undetectable increase in daily myofibrillar protein synthesis rates following resistance exercise.

A final possibility relates to the synthesis rates of individual myofibrillar proteins, which have been shown to be highly variable (Camera *et al.*, 2017). Indeed, recent evidence using dynamic proteomics found reduced synthesis rates of numerous individual myofibrillar proteins following resistance exercise in young individuals (Camera *et al.*, 2017). In relation to this, prolonged $^{2}H_2O$ labelling periods may also result in gradual lowering of measured MPS rates over time as the contribution of more slowly synthesised and less abundant proteins to the overall measurement becomes greater (Miller *et al.*, 2015). Thus, the undetectable increase in daily myofibrillar protein synthesis rates following resistance exercise could be related to the contribution of certain slowly synthesised or unresponsive individual proteins to overall myofibrillar protein synthesis rates. However, this supposition requires direct testing in future studies.

MPS and hypertrophy are partly regulated by translational capacity (i.e., ribosomal biogenesis), with recent studies demonstrating a significant positive correlation between RNA synthesis (i.e., ribosomal biogenesis) and MPS in response to prolonged resistance training and also markers of ribosomal biogenesis with skeletal muscle hypertrophy (Reidy *et*

al., 2017; Brook *et al.*, 2019; Figueiredo & McCarthy, 2019; Sieljacks *et al.*, 2019). As such, a secondary aim was to assess whether resistance exercise frequency impacts the phosphorylation status and total protein content of selected proteins implicated in skeletal muscle ribosomal biogenesis **(Figure 4.5)**. Transcription of rDNA requires the activation of eIF4E and cyclin D1 which can subsequently activate a number of transcription factors including UBF which forms part of the pre-initiation complex (Figueiredo & McCarthy, 2019). In line with previous findings (Figueiredo *et al.*, 2016), there was a tendency (P=0.056) for total eIF4E protein content **(Figure 4.5A)** to increase 48 hours following the initial bouts of LF and HF resistance exercise. Consistent with the finding that resistance exercise frequency had no impact on daily myofibrillar protein synthesis rates, no differences were observed at any time point for any marker of skeletal ribosomal biogenesis between LF and HF resistance exercise **(Figure 4.5)**. However, it should be acknowledged that skeletal muscle ribosomal biogenesis is activated at multiple time points following resistance exercise (Figueiredo *et al.*, 2016), and thus it is possible that biopsy timing, primarily intended to assess myofibrillar protein synthesis rates, missed differences that may have occurred at earlier time points.

Although total exercise volume was intentionally matched to isolate the impact of resistance exercise frequency *per se* on daily myofibrillar protein synthesis rates, it should be considered that higher resistance exercise frequencies can be used effectively to increase overall exercise volume for a given muscle group (Barcelos *et al.*, 2018; Zaroni *et al.*, 2018). Indeed, under non-volume equated conditions, higher resistance exercise frequencies are associated with greater muscle hypertrophy (Schoenfeld *et al.*, 2018) and also muscular strength (Grgic *et al.*, 2018) and thus the potential benefit of higher resistance exercise frequencies cannot be completely discounted.

It is also important to note that any change in muscle mass is ultimately determined by the overall NBAL between MPS and MPB. Whilst the absence of a measure of MPB may be considered a limitation of the present investigation, the myofibrillar protein synthesis measurements made in the present study align well with the general finding that volume-matched resistance exercise frequency has no impact on muscle hypertrophy (Gentil *et al.*, 2015; Barcelos *et al.*, 2018; Gomes *et al.*, 2018). Finally, this study was conducted in individuals unaccustomed to lower limb resistance exercise but it is possible that higher resistance exercise frequencies could be of greater benefit to resistance-trained individuals as has been previously suggested (Dankel *et al.*, 2017).

In conclusion, under the conditions of the present study, resistance exercise frequency does not modulate daily myofibrillar protein synthesis rates or the phosphorylation status and total protein content of selected proteins implicated in skeletal muscle ribosomal biogenesis in young men. These findings suggest that for a given exercise volume, resistance exercise frequency has minimal impact on skeletal muscle hypertrophy.

CHAPTER 5

GENERAL DISCUSSION

5.1 Summary of key findings

Sedentariness and physical inactivity are highly prevalent lifestyle behaviours linked to loss of muscle mass and strength across the lifespan (Gianoudis *et al.*, 2015). Prior to this thesis, the impact of reduced physical activity and increased time spent sedentary on muscle mass regulation had partially been addressed in older individuals but had yet to be investigated in young individuals. There were also no data available on the impact of these lifestyle behaviours on MPS rates over shorter periods of time typically associated with recovery from injury and/or acute illness (i.e., ≤ one week). Accordingly, the primary aim of the first experimental chapter **(Chapter 3)** of this thesis was to use a step reduction model to determine the impact of one week of reduced physical activity and increased sedentary time on daily myofibrillar protein synthesis rates in a healthy young population. The major novel finding was that just one week of reduced physical activity and increased sedentary time led to a substantial (~27%) decline in daily myofibrillar protein synthesis rates. An additional new finding of this investigation was that one week of step reduction altered the expression of several genes within skeletal muscle related to muscle mass regulation and oxidative metabolism. Collectively, these findings provide new insights into the consequences of relatively brief and benign changes in physical activity and sedentary behaviour on muscle mass regulation.

Considering the findings presented in **Chapter 3**, it is alarming that the majority of individuals in highly developed nations are highly inactive and sedentary (Loyen *et al.*, 2017). Resistance exercise is essential to the preservation of muscle mass across the lifespan, but participation and adherence is poor. As such, there remains a requirement to design resistance exercise-based strategies that are attainable for physically inactive populations. Although there are many barriers to physical activity participation, time is often cited as a

major barrier (Justine *et al.*, 2013; Ashton *et al.*, 2017). It had recently been proposed that low volume, high frequency resistance exercise could be used as a strategy to maximise the muscle protein synthetic and hypertrophic response to resistance exercise (Dankel *et al.*, 2017). If true, low volume, high frequency resistance exercise could potentially be used as a novel strategy to increase physical activity levels and ultimately slow the gradual decline in muscle mass and physical function across the lifespan. Whilst plausible, this hypothesis had yet to be directly tested.

Therefore, the primary aim of the second experimental chapter **(Chapter 4)** of this thesis was to compare daily myofibrillar protein synthesis rates over a seven-day period of volume-matched, low frequency (once per week) and high frequency (five times per week) resistance exercise in young individuals. The major finding was that daily myofibrillar protein synthesis rates did not differ between volume-matched low and high frequency resistance exercise over a seven-day period in young men. This study also provided new data demonstrating that resistance exercise frequency had no impact on the activation or abundance of selected proteins implicated in skeletal muscle ribosomal biogenesis.

The aim of the present chapter is to provide a discussion and critical overview of the findings from these studies in the context of the existing literature. Avenues for future research and the practical implications of these findings will also presented and discussed.

5.2 One week of step reduction lowers myofibrillar protein synthesis rates in young men

Much of our current understanding of the negative effects of reduced physical activity and increased sedentary behaviour has been inferred from extreme experimental models (i.e., muscle disuse). These studies have consistently shown that extreme muscle disuse (i.e., limb

immobilisation or bed rest) results in substantial loss of skeletal muscle mass and strength (Drummond *et al.*, 2012; Dirks *et al.*, 2016b; Wall *et al.*, 2016b). Whilst these studies provide information on the impact of severe muscle unloading, they do not necessarily provide an accurate representation of a typical physically inactive and sedentary lifestyle which requires some level of ambulation and thus muscular contraction (Perkin *et al.*, 2016). An alternative and less extreme model to study the consequences of physical inactivity and high sedentariness is the step reduction model. Prior to this thesis, few studies had evaluated the impact of step reduction on muscle mass regulation. The limited data available demonstrated that two weeks of step reduction in older, overweight individuals lowered postprandial MPS rates measured acutely using acute stable isotope infusions and integrated MPS rates measured using $^{2}H_2O$ (Breen *et al.*, 2013; McGlory *et al.*, 2018). These findings provided the first evidence that physical inactivity and sedentariness directly impact muscle mass regulatory processes which, in the long-term, could accelerate muscle loss.

The results presented in **Chapter 3** are consistent with these data and extend on these findings showing that just one week of step reduction led to significant (~27%) declines in daily myofibrillar protein synthesis rates in young healthy individuals. Some (Suetta *et al.*, 2009; Wall *et al.*, 2015a), but not all (Tanner *et al.*, 2015), studies have shown that younger individuals lose a greater amount of muscle mass following disuse. While a direct older comparator group was not studied in **Chapter 3**, the finding that daily myofibrillar protein synthesis rates declined to a greater extent in young (~27%) compared to older (~12%) adults (McGlory *et al.*, 2018) may support the idea that younger individuals are more susceptible to changes in physical activity status than older individuals. Alternatively, these differences could be related to the length of step reduction (i.e., one vs two weeks). Indeed, the greater relative decline in daily myofibrillar protein synthesis rates observed over one week of step

reduction is entirely consistent with the greater rate of muscle loss typically seen in the initial phase of disuse (LeBlanc *et al.*, 1992; Wall & van Loon, 2013). What is clear is that step reduction induces substantial declines in MPS in young and older adults and thus individuals of all ages are susceptible during brief periods of reduced activity. These findings are highly relevant given that the accumulation of short periods of reduced activity and/or disuse throughout the lifecycle may accelerate muscle loss over the lifespan (English & Paddon-Jones, 2010; Wall *et al.*, 2013a).

5.3 Implications and countermeasures

The findings presented in **Chapter 3** are likely to have significant implications for individuals undergoing injury, illness and/or other significant life events that lead to enforced inactivity. The findings are also of great clinical relevance given that inactivity (Ekelund *et al.*, 2016), low muscle mass (Srikanthan & Karlamangla, 2014) and insulin resistance are intrinsically linked (Srikanthan & Karlamangla, 2011) and associated with the development of chronic diseases and early mortality. This begs the question: what can be done to counteract the negative consequences of physical inactivity and increased sedentary behaviour? Muscular contraction would arguably be the most effective countermeasure to prevent inactivity-induced declines in MPS given its ability to attenuate or even prevent disuse-induce muscle atrophy (Bamman *et al.*, 1998; Alkner & Tesch, 2004; Oates *et al.*, 2010). Indeed, Devries and colleagues demonstrated that low-load resistance exercise protects against loss of muscle mass during a prolonged period of reduced physical activity in the older population (Devries *et al.*, 2015). However, muscular contraction is likely to be unfeasible for the majority of those undergoing periods of enforced physical inactivity. In the absence of an external muscle contraction mimetic (e.g., neuromuscular electrical stimulation (Dirks *et al.*, 2014b)), alternative countermeasures are required.

From a nutritional perspective, dietary protein, in particular the essential amino acid leucine, and its metabolite β-hydroxy-β-methylbutyrate (HMB), robustly stimulates MPS (Volpi *et al.*, 2003; Wall *et al.*, 2013b; Wilkinson *et al.*, 2013; Churchward-Venne *et al.*, 2014; Witard *et al.*, 2014). As such, protein, leucine and/or HMB supplementation could be suggested as strategies to offset inactivity-induced declines in MPS. However, evidence suggests this may be an ineffective countermeasure, with some (Ferrando *et al.*, 2010; Dirks *et al.*, 2014a; Backx *et al.*, 2018), but not all (Paddon-Jones *et al.*, 2004; Deutz *et al.*, 2013; English *et al.*, 2016) studies showing that protein, leucine and/or HMB supplementation fails to attenuate muscle loss during disuse in both young and older individuals. In agreement, a recent study by Oikawa *et al.* (2018) demonstrated that both whey and collagen-based protein supplements failed to prevent declines in daily myofibrillar protein synthesis rates and muscle loss during two weeks of step reduction and energy restriction in older adults; whether this would also be the case in younger individuals is unclear.

Given the mixed findings on the efficacy of dietary protein supplementation to offset inactivity-induced muscle loss, alternative nutritional interventions may be required. Creatine has an established role in augmenting muscle mass both with and without resistance training (Becque *et al.*, 2000; van Loon *et al.*, 2003) and thus, creatine supplementation has been proposed as a potential countermeasure to disuse-induced muscle atrophy. Whereas two studies have shown that creatine supplementation fails to attenuate muscle loss during limb immobilisation (Hespel *et al.*, 2001; Backx *et al.*, 2017), one study demonstrated creatine to be effective at preventing disuse-induced muscle loss (Johnston *et al.*, 2009). As such, the efficacy of creatine supplementation as a potential countermeasure to inactivity-induced declines in MPS and muscle mass remains unclear.

More promisingly, a recent study by McGlory *et al.* (2019) demonstrated that omega-3 fatty acid supplementation attenuated loss of muscle volume during two weeks of limb immobilisation in young women. This may be related to the ability of omega-3 fatty acid supplementation to enhance the muscle protein synthetic response to amino acids (Smith *et al.*, 2011). Indeed, daily myofibrillar protein synthesis rates were elevated during limb immobilisation in those supplemented with omega-3 fatty acids compared to those in the control group (McGlory *et al.*, 2019). Given these data, supplementing omega-3 fatty acids could prove to be an effective countermeasure to prevent inactivity-induced declines in MPS. However, future studies are required to support this supposition.

5.4 Are acute changes in muscle mass regulation following step reduction representative of long-term muscle mass regulation in chronically inactive and sedentary individuals?

The findings presented in **Chapter 3** could be interpreted to suggest that lifelong physical inactivity and sedentariness leads to a chronic imbalance between MPS and MPB and thus an accelerated loss of muscle mass over time. However, it should be considered whether the changes observed are representative of muscle mass regulation in chronically inactive and sedentary individuals.

Muscle loss is thought to be accelerated over the lifespan by an inactive and sedentary lifestyle and thus, the observed ~27% decline in daily myofibrillar protein synthesis rates following one week of step reduction provides insight into the physiological processes by which this may occur. Declines in MPS (with little change in MPB (Symons *et al.*, 2009a)) are thought to be the predominant mechanism by which muscle loss occurs in response to human muscle disuse and inactivity (Phillips & McGlory, 2014). However, if this is the case,

the substantial decline in myofibrillar protein synthesis observed in **Chapter 3,** and by others following muscle disuse (Wall *et al.*, 2016b; McGlory *et al.*, 2019), would result in a sizeable and implausible loss of muscle mass if sustained over months or years. In fact, the finding of increased MAFbx mRNA expression in **Chapter 3** following one week of step reduction and by others (Jones *et al.*, 2004; Wall *et al.*, 2014; McGlory *et al.*, 2019) at the onset of disuse suggests that MPB may even increase initially. This suggests that one of two adaptive processes must occur with prolonged (i.e., years of) physical inactivity and sedentariness.

The first is that MPB eventually adapts and returns to near basal levels. In other words, muscle protein turnover is reduced and NBAL is eventually brought back to a less negative or equilibrated state and a more gradual loss of muscle mass occurs over time. This hypothetical concept is depicted in **Figure 5.1**. Relatively crude estimates of MPB following prolonged limb immobilisation support this proposition (Gibson *et al.*, 1987). Although MPB has been shown to be unaffected by bed rest (Symons *et al.*, 2009a), this was after only three weeks of bed rest and it is well acknowledged that accurate methods for measuring MPB are currently lacking; thus the possibility that MPB adapts with chronic changes in physical activity and sedentary lifestyle patterns cannot be ruled out. The second possibility, although unlikely, is that MPS eventually returns to habitual levels once the initial loss of muscle mass occurs following muscle disuse or step reduction. Evidence that myofibrillar protein synthesis rates are reduced following 10 days of limb immobilisation and then stabilise after an additional 10 days of muscle disuse (de Boer *et al.*, 2007) suggests that this is unlikely to be the case, but time course studies over longer periods of time are needed to fully address this possibility.

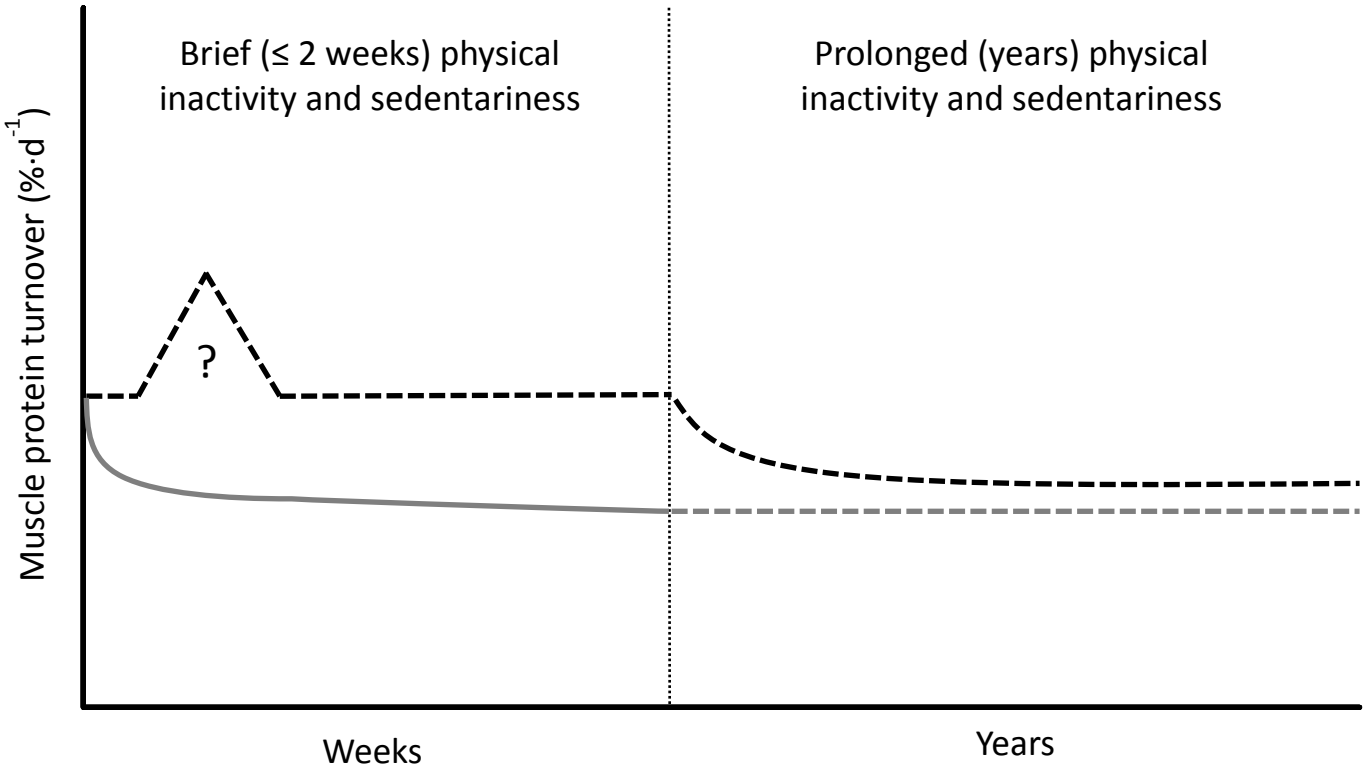

Figure 5.1 Hypothetical depiction of the muscle protein turnover response to brief and prolonged physical inactivity and sedentariness. In response to brief physical inactivity and sedentariness (i.e., step reduction), MPS (grey line) rates decline 10-30% and MPB (black dashed line) likely remains unchanged or may increase transiently. With prolonged physical inactivity and sedentariness, it is hypothesised that MPS remains chronically low but MPB adapts and returns to near basal levels; resulting in a reduced muscle protein turnover and more gradual loss of muscle mass over the lifespan. Solid line; scientific evidence available. Dashed line; lacks scientific evidence. Adapted from (Wall *et al.*, 2013a).

5.5 Future directions

Given that step reduction clearly lowers myofibrillar protein synthesis rates, it would be of interest to investigate whether increasing daily step count could be used as an intervention to improve muscle mass regulation in habitually inactive and sedentary individuals. Evidence that moderate intensity walking can improve the MPS response to EAA ingestion in older adults (Timmerman *et al.*, 2012) is encouraging and lends support to this proposition. Future research to assess how quickly the decrease in daily myofibrillar protein synthesis rates occurs following the onset of step reduction would also be valuable. Finally, understanding whether, and to what extent, young individuals recover from short periods of reduced physical activity is an important area for investigation. McGlory and colleagues recently showed that two weeks of recovery was insufficient for older adults to fully recover from a period of step reduction (McGlory *et al.*, 2018). Younger individuals appear to recover more effectively from muscle disuse than older individuals (Suetta *et al.*, 2013) and thus, it would be interesting to assess whether this is also the case following more benign periods of physical inactivity (i.e., step reduction).

5.6 Daily myofibrillar protein synthesis rates in response to low and high frequency resistance exercise in young men

The impact that resistance exercise frequency (i.e., the number of times a muscle group is exercised over a given period of time) has on muscle hypertrophy has garnered increasing attention in recent years. Most studies have found no difference in muscle hypertrophy between high and low resistance exercise frequencies (Gentil *et al.*, 2015; Thomas & Burns, 2016; Ochi *et al.*, 2018). However, these studies largely examined the impact of resistance exercise frequencies in the range of one-to-three times per week. Prior to this thesis, far less

research had been conducted on the impact of higher resistance exercise frequencies (i.e., five times per week). Indeed, it had recently been posited that relatively high resistance exercise frequencies could maximise muscle hypertrophy by inducing greater overall myofibrillar protein synthesis rates (Dankel *et al.*, 2017). Whilst plausible, this hypothesis had yet to be directly tested.

In contrast to the aforementioned hypothesis, the results presented in **Chapter 4** show that low (once per week) and high (five times per week) frequency resistance exercise resulted in indistinguishable daily myofibrillar protein synthesis rates over a seven-day period. Although these data fail to support the hypothesis of Dankel *et al.* (2017), they lend support to the preponderance of evidence showing that resistance exercise frequency has no impact on muscle hypertrophy (Gentil *et al.*, 2015; Thomas & Burns, 2016; Barcelos *et al.*, 2018; Ochi *et al.*, 2018). It is important to point out that total exercise volume completed in the low and high frequency conditions was intentionally matched when designing the study presented in **Chapter 4**. A number of studies that have assessed the impact of resistance exercise frequency on muscle hypertrophy failed to match exercise volume between conditions, which precludes conclusions being drawn on the impact of resistance exercise frequency *per se* on muscle hypertrophy. From the findings presented in **Chapter 4**, and by others, it would appear that under volume-matched conditions, resistance exercise frequency *per se* does not impact daily myofibrillar protein synthesis rates or subsequent muscle hypertrophy in young individuals.

5.7 Explaining the lack of an exercise effect on myofibrillar protein synthesis

Based on previous evidence, it was expected that an increase in daily myofibrillar protein synthesis rates would be detected over the 48-hour post-exercise period, at least in the low

frequency condition which consisted of five sets of leg press and five sets of leg extensions at ~70% 1RM. However, in contrast to two previous studies (Wilkinson *et al.*, 2014; Damas *et al.*, 2016), acute resistance exercise did not result in a detectable increase in daily myofibrillar protein synthesis rates compared to basal in **Chapter 4**. It must be acknowledged that the inability to detect an increase in daily myofibrillar protein synthesis rates following acute resistance exercise may also have precluded differences from being detected between low frequency and high frequency conditions over the seven-day exercise period.

The question that remains is what could explain this unexpected observation? A number of potential explanations were put forth in **Chapter 4** but other possibilities remain. A key assumption when measuring MPS using stable isotope tracers is that tracer recycling, and subsequent reincorporation of the labelled amino acid derived from MPB into the tissue of interest during the measurement period, is minimal (Wolfe & Chinkes, 2004). This assumption is likely to be acceptable when measuring MPS rates over relatively short (i.e., hours) periods. However, it has previously been shown that prolonged (i.e., days) stable isotope infusions result in significant recycling of the labelled amino acid (Schwenk *et al.*, 1985; Carraro *et al.*, 1991). Given that the primary purpose of $^{2}H_{2}O$ is to measure MPS rates over days to weeks, the prospect that significant recycling of ^{2}H-alanine occurs over these prolonged measurement periods cannot be dismissed (Fluckey *et al.*, 2015).

In theory, reincorporation of ^{2}H-alanine released from MPB, back into skeletal muscle, would result in an underestimation of MPS. Indeed, this may explain the findings of a previous study which showed that daily myofibrillar protein synthesis rates decreased over time under both exercised and basal, resting conditions (Wilkinson *et al.*, 2014). Placed in the context of the findings presented in **Chapter 4**, it is possible that tracer recycling was greater

during the exercise period as compared to the basal measurement period; resulting in underestimation of the muscle protein synthetic response to acute resistance exercise. This is pertinent given that resistance exercise increases MPB (Phillips *et al.*, 1997; Phillips *et al.*, 1999) and thus, may increase the potential for recycling and subsequent reincorporation of ^{2}H-alanine into skeletal muscle.

Considering the potential for tracer recycling when using $^{2}H_2O$, it may be advisable for future studies to counterbalance the order of the interventions/treatments being investigated. For example, studies assessing the impact of resistance exercise on MPS in comparison to basal MPS rates may wish to design the experiment so that half of the participants complete resistance exercise first followed by the basal measurement and the other half complete the interventions in the reverse order. Where possible, it would be most preferable to conduct measurements of MPS alongside each other (e.g., using a unilateral resistance exercise model) as has previously been done (Wilkinson *et al.*, 2014; Holwerda *et al.*, 2018). This would also control for the increasing contribution of more slowly synthesised and less abundant proteins to overall MPS measurements over time (Miller *et al.*, 2015).

5.8 Alternative applications and future directions for deuterated water

$^{2}H_2O$ was exclusively used to assess daily myofibrillar protein synthesis rates in **Chapters 3** and **4**. However, $^{2}H_2O$ has many other applications due to its non-substrate specific nature which allows multiple metabolic processes to be measured simultaneously (Hellerstein, 2004; Brook *et al.*, 2017a). Indeed, recent development of the $^{2}H_2O$ methodology has allowed new insight into factors regulating MPS and muscle mass; namely RNA synthesis (i.e., ribosomal biogenesis) and DNA synthesis (Robinson *et al.*, 2011; Brook *et al.*, 2017b; Sieljacks *et al.*, 2019). These studies have provided novel insight into skeletal muscle adaptive processes,

with a significant positive correlation observed between RNA synthesis and MPS in response to prolonged resistance training (Brook *et al.*, 2017b; Sieljacks *et al.*, 2019). Combined with other findings (Stec *et al.*, 2016), it is becoming increasingly evident that ribosomal biogenesis plays a key role in the muscle hypertrophic response to resistance training.

Equally promising, $^{2}H_2O$ has recently been used to determine the synthesis rates of individual skeletal muscle proteins using proteomic profiling (Camera *et al.*, 2017; Murphy *et al.*, 2018). These studies have established the wide range of synthesis rates that exist on a protein-by-protein basis in skeletal muscle and also highlight the unique response that each individual protein has to anabolic (i.e., resistance exercise (Camera *et al.*, 2017)) and catabolic (i.e., energy restriction (Murphy *et al.*, 2018)) stimuli. Considering the difficulties associated with measuring MPB, it is also encouraging that estimates of the breakdown rates of individual skeletal muscle proteins have been made using this approach (Camera *et al.*, 2017). This is based on the difference between the measured change in relative abundance and the rate of synthesis of each individual protein; although the accuracy of this approach is uncertain and requires further validation. Regardless, continued application of $^{2}H_2O$ to simultaneously measure MPS, RNA and DNA synthesis, combined with other approaches (i.e., proteomics), will no doubt provide unparalleled insight into the adaptive processes that regulate skeletal muscle remodelling.

5.9 Final conclusions and practical applications

The work contained within this thesis has provided new knowledge of longer-term muscle protein synthetic responses across the physical activity spectrum. For the first time, it has been shown that just one week of step reduction, resulting in reduced physical activity and increased sedentary behaviour, leads to substantial declines in daily myofibrillar protein

synthesis rates in a young healthy population. This was accompanied by a clinically relevant decline in whole-body insulin sensitivity. Indeed, the Matsuda index values (4.5) observed in young habitually active men following one week of step reduction are identical to those reported in older individuals with normal glucose tolerance (Fiorentino *et al.*, 2019) and only slightly higher than values reported in older adults with prediabetes (Fabbri *et al.*, 2017). Whilst clinical reference values have not been agreed upon, a Matsuda index value of <4.3 has been suggested as a lower reference limit indicative of insulin resistance (Takahara *et al.*, 2013) and thus the similar values seen following one week of step reduction strongly suggests that even relatively brief periods of reduced physical activity should be avoided where possible. However, this researcher acknowledges that brief periods of reduced activity are inevitable and often unavoidable (e.g., injury or illness). In this scenario, effective countermeasures will be required and it is hoped that the findings from this thesis stimulate future research in this area. It is also expected that findings within thesis will encourage future research aimed at elucidating the long-term impact of lifelong physical inactivity and sedentariness on muscle mass regulation.

The findings contained within this thesis also offer new insight into the impact of different resistance exercise frequencies on daily myofibrillar protein synthesis rates and thus, long-term muscle mass regulation. In line with long-term training studies (Barcelos *et al.*, 2018; Ochi *et al.*, 2018), and recent meta-analyses (Schoenfeld *et al.*, 2018; Grgic *et al.*, 2019), it would appear that exercise frequency has little bearing on the muscle hypertrophic response to resistance exercise under volume-matched conditions. This is encouraging from a practical perspective and suggests that individuals should exercise at a frequency that they most enjoy, and most importantly, that encourages long-term adherence which is likely to be the strongest predictor of positive health outcomes.

Finally, the unexpected finding within this thesis that acute resistance exercise failed to induce a detectable increase in daily myofibrillar protein synthesis rates has been insightful and the discussion within this thesis provides important considerations for researchers designing and implementing studies using the $^{2}H_2O$ methodology. This researcher has no doubt that the use of $^{2}H_2O$ will continue to provide valuable insight into long-term muscle mass regulation and the development of new approaches using this method will provide exciting research avenues for the future.

CHAPTER 6

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