Development and Application of Novel Stable Isotopic Approaches to Study Human Skeletal Muscle in Response to Exercise and Ageing

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Author Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Nottingham. The following collaborations were undertaken for this thesis

In Vivo – All animal procedures were performed at the University of Jyvaskyla under the supervision of Prof. Heikki Kainulainen, Dept of Biology of Physical Activity.

The human resistance exercise study was implemented and overseen by the author, with medical screening, tissue and blood collection performed with the help of Dr Kyle Mitchell, Dr Syed Bukhari, Ms Catherine Boereboom, Amanda Gates and Margaret Baker

The author undertook all subsequent analysis.

Signed: 

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Development and Application of Novel Stable Isotopic Approaches to Study Human Skeletal Muscle in Response to Exercise and Ageing

Chapter 1- Introduction: Skeletal Muscle in Health, Disease and Ageing 1
1.1 Skeletal muscle 2
1.2 Protein synthesis and breakdown: an overview 4
1.3 Control of skeletal muscle protein turnover by environmental stimuli 10
1.3.1 Regulation by nutrition 10
1.3.2 Regulation by exercise 12
1.4 Regulation of muscle mass by exercise training 15
1.4.1 Resistance exercise training and skeletal muscle hypertrophy 15
1.4.2 The role of muscle protein turnover in skeletal muscle hypertrophy 18
1.4.4 The role of ribosomal biogenesis in skeletal muscle hypertrophy 20
1.4.3 The role of satellite cells in skeletal muscle hypertrophy 22
1.4.5 The role of hormones in skeletal muscle hypertrophy 24
1.5 Ageing, skeletal muscle and sarcopenia 28
1.6 Ageing and the control of skeletal muscle protein turnover 34
1.6.1 Regulation by nutrition 34
1.6.2 Regulation by exercise 38
1.7 Ageing, exercise and skeletal muscle hypertrophy 42
1.7.1 Resistance exercise as a sarcopenia countermeasure 42
1.7.2 Effects of ageing upon hypertrophic responses to resistance exercise 43
1.7.3 Mechanisms of impaired muscle hypertrophy 44
1.8 Methods to quantify in vivo muscle metabolism 51
1.8.1 Established methodologies 51
1.8.1 D_2O as a novel tracer in the measurement of muscle metabolism 55
1.8.1 D_2O as a method for measuring muscle protein turnover 58
1.8.2 D_2O in the measurement of muscle DNA and RNA turnover 63
1.9 Summary 65

Chapter 2- General Methods 68
2.1 Subject recruitment and screening 69
2.2 Subject characteristics and ethics 69
2.3 Conduct of the study 70
2.4 Measure of habitual physical activity and diet 72
2.5 Muscle functional tests 73
2.6 Muscle architecture and DXA derived mass 73
2.7 Body water and protein bound alanine muscle fraction enrichment 74
2.8 Calculation of fractional synthetic rate (FSR), absolute synthetic rate (ASR) and absolute breakdown rate (ABR) 76
2.9 Immunoblotting 77
2.10 Hormone concentrations 78
2.11 Gene expression analysis 79
2.11 Primer Sequences 80
2.12 Skeletal muscle protein, DNA and RNA concentrations 81

Chapter 3 - Development of Methods Using D_2O in the Measurement of Protein, RNA and DNA Synthesis 82
3.1 Introduction 83
3.2 Methods 87

3.2.1 Determination of D_2O body water and plasma alanine kinetics 87
3.2.2 Cell culture 87
3.2.3 Animals 88
3.2.4 Plasma body water, alanine and muscle bound enrichment. 90
3.2.5 Peripheral blood mononuclear cell extraction 91
3.2.6 DNA extraction, digestion and derivatisation 91
3.2.7 GC-MS/MS instrument conditions 93
3.2.8 Statistical Analyses 93
3.3 Results: Development of methods using D_2O in the measurement of protein synthesis *94*
3.3.1 Pilot to determine D_2O dosing for MPS detection 94
3.4.2 D_2O equilibration in saliva, plasma water and plasma alanine 96
3.4.3 Validation of saliva enrichment in the representation of plasma alanine 97
3.4.4 Long term relationship between plasma water and plasma alanine 99
3.4.3 Non linear calculations in the determining long term MPS 100
3.5 Results: Development of a method using D_2O in the measurement of DNA and RNA synthesis *104*
3.5.1 Extraction, digestion and derivatisation 104
3.5.2 Calculation of deuterium amplification into DNA and RNA 107
3.5.3 Validation of D_2O incorporation in determining cell division 109
3.5.4 Stimulation of DNA and RNA synthesis 111
3.5.5 Measurement of DNA and RNA synthesis in muscle *in vivo* 113
3.5.6 Determination of DNA and RNA synthesis rates in humans 115
3.6 Discussion *117*
3.6.1 Application of D_2O in the measurement of MPS 117
3.6.2 Measurement of Muscle DNA RNA synthesis 119

Chapter 4 - Application of Novel D_2O Methods to Investigate Skeletal Muscle Hypertrophy at the Onset of Resistance Exercise Training 128
4.1 Introduction *129*
4.2 Methods *132*
Statistical Analyses 132
4.3 Results *133*
4.3.1 1-RM and MVC in UT and T legs 133
4.3.2 Muscle Mass and Architecture in UT and T legs 134
4.3.3 Body water enrichment and D_2O-Derived MPS (MPS) in UT and T legs 137
4.3.5 Muscle Protein, RNA and DNA concentrations 139
4.3.5 Muscle DNA and RNA synthesis 141
4.5.6 Intramuscular Anabolic Signaling 142
4.5.7 Correlations 144
4.4 Discussion *145*

Chapter 5 - The Effects of Age on Chronic MPS and Hypertrophic Mechanisms in Response to Resistance Exercise Training 154
5.1 Introduction *155*
5.2 Methods *158*
5.2.8 Statistical Analyses 158
5.3 Results *159*
5.3.1 Subject characteristics 159
5.3.2 Activity 159
5.3.3 Diet 161
5.3.4 Muscle strength 162
5.3.5 Muscle mass and architecture 164

5.3.6 Muscle RNA, DNA and protein concentrations 166
5.3.7 Muscle Protein Synthesis (MPS), absolute (ASR) and fractional growth rates (FGR) 168
5.3.8 Hormones 171
5.3.8 Intramuscular signaling 173
Intramuscular mRNA expression 177
Correlations 180
Discussion *183*
Chapter 6 – Overview **193**
References **199**

List of Tables

1.1 The acute effects of nutritional provision on muscle protein turnover in young and old individuals

1.2 The acute response of muscle protein turnover to exercise in young and old individuals

1.3 Adaptations to resistance exercise in young and old individuals

3.1 APE of deuterium in *vastus lateralis* muscle biopsies after either bolus or continuous labeling.

4.1 Skeletal muscle protein RNA and DNA concentrations

5.1 Physical characteristics of young and older subjects.

5.2 The amount of time spent at levels of physical activity in young and old individuals over one week of the training study define by the metabolic equivalent of task

5.3 Muscle mRNA expression of genes involved in ribosomal biogenesis.

5.4 Muscle mRNA expression of targets involved in MPB

5.5 Muscle mRNA expression of androgen receptor and myostatin

List of Figures

1.1 An overview of signaling pathways involved in MPS

1.2 An overview of mechanisms involved in MPB

1.3 Best practice life expectancy in selected countries

1.4 Typical applications of isotope tracers for measuring metabolism

1.5 A summary of how D_2O can be used as a stable isotope tracer for measuring metabolism of multiple substrates *in vivo*

2.1 Schematic representation of the 6 week training study

3.1 APE of deuterium in the body water after either continuous or bolus administration of 70-atom% D_2O

3.2 Time course of deuterium APE measured in saliva, plasma water and plasma alanine after consumption of a 150 ml bolus of 70-atom% D_2O

3.3 Time course of deuterium APE in saliva, plasma water and plasma alanine (x 3.7/11) after 150 ml bolus 70-atom % D_2O

3.4 Natural log of the time course of deuterium APE in saliva, plasma water and plasma alanine (x 3.7/11) after 150 ml bolus 70-atom % D_2O

3.5 Mole percent excess of plasma body water and plasma alanine in rats at 3 weeks 6 weeks

3.6 Time course of theoretical protein enrichment

3.7 Fraction new of protein bound alanine from rat quadriceps maintained at constant body water and myofibrillar muscle protein synthetic rate calculated using linear and non linear precursor product calculations

3.8 The percentage of deoxyribose and ribose released from each nucleoside upon digestion and derivatisation.

3.9 Typical GC-MS/MS chromatogram of the BHTA derivative of deoxyribose and ribose on a DB-17 column

3.10 The APE of the M+5 isotopomer of deoxyribose and ribose of DNA and RNA extracted from C2C12's in U-13C6 glucose enriched media

3.11 MPE of deuterium in deoxyribose and ribose at increasing concentrations of media D_2O enrichment. MPE of deuterium in deoxyribose vs. deoxyadenosine and ribose vs. adenosine at increasing concentrations of media enrichment

3.12 Time course of cell number and MPE of DNA and RNA in proliferating C2C12's. The fraction of new DNA vs. the fraction of new DNA calculated by replication number

3.13 The time course in concentrations of DNA and RNA and the MPE in DNA and RNA in non-treated condition (Control) and in responses to IGF1, AraC, CX5461, IGF1 + AraC and IGF1 + CX5461 treatments

3.14 The MPE of DNA and RNA, fraction new of DNA and RNA, FSR $\%.d^{-1}$ of DNA and RNA in quadriceps of control and HIIT of rats.

3.15 The FSR $\%.d^{-1}$ of myofibrillar, sarcoplasmic and mitochondrial protein fractions from rat quadriceps in control and HIIT trained rats

3.16 Time course of MPE in peripheral blood mononuclear cells after 3 and 6 weeks of continuous body water labeling

3.17 Time course of MPE in DNA and RNA from human VL muscle

3.18 Fractional synthesis rates of DNA and RNA 0-6 weeks from human VL muscle

4.1 Time course of changes in of T leg 1-RM and % change in T leg 1-RM from baseline.

4.2 Time course of change in MVC in T and UT legs over the 6 weeks of RET at 60^{o} and 70^{o}

4.3 Thigh lean mass over the 6-week training period.

4.4 Time course of VL, MT, L*f* and θ in UT and T legs at 0, 3 and 6 weeks

4.5 Time course of body water enrichment over 6 weeks and one phase association of the change in myofibrillar APE over 6 weeks

4.6 Temporal pattern of myofibrillar FSR in UT and T legs over 0-3 and 3-6 weeks and myofibrillar FSR over the 0-6 week training period.

4.7 ASR and ABR in UT and T legs 0-6 weeks

4.8 Change in μg ASP/μg DNA and μg RNA/μg DNA over the 6 weeks RET in T and UT legs

4.9 FSR $\%d^{-1}$ of DNA and RNA in T and UT legs over the 6 weeks

4.10 Relative change compared to UT in mTORc1 ^{Ser2448}, total mTOR, p70S6k1 ^{Thr389}, total p70S6K1, rps6$^{ser240/244}$, total rps6**,** 4EBP1$^{Thr37/46}$, total 4EBP1, Akt^{Ser473}, total AKT, eEF2^{Thr56}, total eEF2

4.11 Correlation of MT at 3 weeks in UT vs. myofibrillar MPS 0-3-weeks in UT and correlation of MT at 3 weeks in T vs. myofibrillar MPS 0-3weeks in T

5.1 Average activity counts measured over one week of the RET program in young and old subjects.

5.2 The average amount of Kcal, protein, fat and carbohydrate consumed per Kg of FFM per day in young and old subjects.

5.3 Time course of changes of 1RM and % change in 1RM in trained legs over 6 weeks in young and old subjects.

5.4 Time course of changes in average MVC and % change in average MVC 1RM in T legs over 6 weeks in young and old subjects

5.5 TFFM at baseline and after 6 weeks RET in young and old subjects

5.6 Time course of changes in MT, L*f* and θ over 6 weeks in young and old subjects.

5.7 Time course of the changes in $\mu g.mg^{-1}$ dry weight muscle of ASP, RNA, DNA and the ratios of ASP:DNA, RNA:DNA and RNA:ASP in young and old subjects.

5.8 Myofibrillar FSR $\%.d^{-1}$ at 0-3 weeks, 0-6 weeks and 3-6 weeks in young and old subjects.

5.9 Thigh myofibrillar ASR over 0-6 weeks and thigh myofibrillar ABR over 0-6 weeks in young and old subjects.

5.10 FGR in TFFM.d^{-1} over 0-6 weeks in young and old subjects.

5.11 Serum concentrations of testosterone, IGF-1 and myostatin at baseline and 60-90min after the first bout of RET in young and old subjects.

5.12 Relative change compared with UT in $mTOR^{Ser2448}$, $p70S6K1^{Thr389}$, $rps6^{ser240/244}$, $ERK1/2^{Thr202Tyr204}$, $eEF2^{Thr56}$, Akt^{Ser473}, $4EBP1^{Thr37/46}$, $AMPK^{Thr172}$ in young and old subjects.

5.13 Relative change compared with UT in Beclin 1, Cathepsin L, Calpain 1 in young and old subjects.

5.14 Relative change compared with UT in c-MYC, pRB^{Ser780}, $UBF1^{Ser484}$, UBF1, $TIF1a^{Ser649}$, TIF1a in young and old subjects.

5.15 Representative blots of signaling targets used in Fig 5.12, Fig 5.13 and Fig 5.14 for young and old subjects

5.16 Correlations between RNA:DNA at 3 weeks and MT at 3 weeks RNA:DNA at 6 weeks and TFFM at 6 weeks in young and old subjects.

5.17 Correlations between the increase in P70S6K1^{T389} after first RET bout and the % change in MT at 3 weeks and the increase in P70S6K1^{T389} after first RET bout and the % change in TFFM at 6 weeks in young and old subjects.

5.18 Correlations between testosterone after first RET bout and the % change in MT at 3 weeks and % change in testosterone after first RET bout and the % change in TFFM at 6 weeks in young and old subjects.

5.19 Correlations between protein intake g.kgFFM^{-1}.d^{-1} and % change in MT at 3 weeks H) Protein intake g.kgFFM^{-1}.d^{-1} and % change in TFFM at 6 weeks in young and old subjects.

Abbreviations

1 RM	one repetition maximum
4EBP	eukaryotic translation initiation factor 4E-binding protein 1
AA	amino acids
ABR	absolute breakdown rate
AKT	protein kinase B
ALM	appendicular lean mass
APE	atom percent excess
AraC	cytosine arabinoside
ASP	alkali soluble protein
ASR	absolute synthetic rate
BMI	body mass index
CSA	cross sectional area
CT	computed tomography
D2O	deuterium oxide
EAA	essential amino acids
eEF	eukaryotic elongation factor
eEF2	eukaryotic elongation factor 2
eIF	eukaryotic initiation factor
ERK	extracellular signal-regulated kinase
FBR	fractional breakdown rate
FFM	fat free mass
FGR	fractional growth rate
FSR	fractional synthetic rate
GC-MS/MS	Gas chromatography tandem mass spectrometry

GH	growth hormone
GnRH	gonadotropin-releasing hormone
HIIT	high intensity interval training
IGF-1	Insulin-like growth factor 1
IRMS	isotope ratio mass spectrometer
LC-MS/MS	liquid chromatography tandem mass spectrometry
Lf	fibre length
MAFbx	muscle atrophy F-box
MCME	n-methoxycarbonyl methyl esters
MET	metabolic equivalent
MPB	muscle protein breakdown
MPE	mole percent excess
MPS	muscle protein synthesis
MRI	magnetic resonance imaging
mRNA	messenger RNA
MT	muscle thickness
mTORC1	mechanistic target of rapamycin complex 1
MuRF1	muscle RING finger 1
MVC	maximum voluntary contraction
P70S6k1	ribosomal protein S6 kinase 1
PA	phosphatidic acid
PBMC	peripheral blood mononuclear cells
PCA	perchloric acid
PLD	phospholipase D
RB	retinoblastoma

rDNA	ribosomal DNA
RET	resistance exercise training
RPE	rating of perceived exertion
RPL	ribosomal protein large
RPS	ribosomal protein small
rps6	ribosomal protein S6
SMI	skeletal muscle index
T	trained
TAF1A	TBP-associated factor 1A
TC/EA	high temperature conversion elemental analyzer
TFFM	thigh fat free mass
TIF1a	transcription initiation factor 1 a
tRNA	transfer RNA
UBF	upstream binding factor 1
UPS	ubiquitin proteasome system
UT	untrained
VL	vastus lateralis
θ	pennation angle

Chapter 1- Introduction: Skeletal Muscle in Health, Disease and Ageing

1.1 Skeletal muscle

Skeletal muscle is indispensable in the production of force, upholding skeletal structure and enabling movement in the completion of simple but essential everyday activities (Reid & Fielding 2012). To meet the demands of these varied requirements, skeletal muscle can produce rapid powerful contractions, controlled precise movements and sustain or repeat contractions for considerable lengths of time. This range of controlled movements is achieved by many complex cooperating factors including neural control, varied contractile fiber types, metabolic composition and architectural arrangements (Fragala et al. 2015). Further to this, muscle shows immense adaptability in exposure to chronic contraction types, recognized by a complex network of signaling pathways that alter gene expression and drive physiological adaptation to meet external demands (Egan & Zierath 2013). Additionally skeletal muscle has a remarkable ability to recover and regenerative in response to injury (Lepper et al. 2011), further demonstrating muscle as a highly plastic and adaptable organ.

In constituting ~40% of body weight, skeletal muscle is the largest organ in the body and therefore has significant effects on whole body metabolism (Zurlo et al. 1990). The solid components of muscle are 80% protein; therefore skeletal muscle represents the bodies largest store of amino acids (AA)(Snyder *et al.,* 1979). AA are essential precursors constantly required by organs around the body for protein renewal and can be released from skeletal muscle into the blood stream during

nutritional absence. Further these AA can be used as glucogenic substrates by the liver and therefore muscle plays and important role in the maintenance of blood glucose concentrations (Pozefsky et al. 1976; Cahill et al. 1976; Owen et al. 1998; Felig 1972). Beyond an AA store, skeletal muscle is the largest site for glucose disposal and further contains significant stores of glycogen and fatty acids that can also be liberated in nutritional absence (DeFronzo & Tripathy 2009; Shulman et al. 1990). The capacity of muscle to uptake and process these metabolites is key in whole body homeostasis and in the prevention of metabolic conditions (Srikanthan & Karlamangla 2011).

Throughout life, skeletal muscle may be placed under many stressful and demanding situations. For instance, substantial loss of muscle mass occurs with trauma (Long et al. 1981) and critical illness (Reid et al. 2004; Helliwell et al. 1998) leading to prolonged recovery and increased mortality (Ali et al. 2008). Similarly, muscle loss is associated with many underlying diseases such as cancer, COPD, AIDS, chronic infections and organ failure (known as cachexia), leading to functional impairment and poor disease outcomes (Bachmann et al. 2008; Evans 2010). Not limited to disease, muscle loss occurs in situations of malnutrition and bed rest (Keys et al. 1945; P Kortebein et al. 2007), which may also be a result of lifestyle choices such as poor nutrition and inactivity (Houston et al. 2008a; Foong et al. 2015). Undoubtedly the best counterbalance in restoring muscle mass loss is physical activity (Liu & Latham 2009). Regular exercise can increase muscle mass, strength and quality with

further benefits on whole body metabolism (Speakman & Selman 2003; Fragala et al. 2014; Peterson et al. 2011), insulin sensitivity (Koopman et al. 2005; Holten et al. 2004) fatty acid oxidation (Goodpaster et al. 2003) and body composition (Phillips et al. 2012). With such important physical and metabolic roles, maintaining sufficient skeletal muscle is important in preserving quality of life (Verlaan et al. 2015). This is evidently important in old age where muscle mass is progressively compromised and losses beyond a certain limit lead to physical disability loss of independence and increased mortality (Rizzoli et al. 2013).

1.2 Protein synthesis and breakdown: an overview

Protein synthesis involves the translation of mRNA by ribosomes. The rate of this process can be rapidly modulated in response to a range of external stimuli and varied physiological conditions (Kimball 2013). It is important that this regulation is tightly controlled as protein synthesis is essential but also energy demanding.

Translation initiation involves the assembly of a mature ribosome at the start codon of an mRNA. This assembly initiates with the formation of an initiation complex, involving many additional proteins known as eukaryotic initiation factors (eIF's) (Reviewed in (Jackson et al. 2010)). Translation initiation requires eIF2, a protein that binds the initiator methionyl-tRNA and associates with the 40s ribosomal subunit. This complex then uses a 'scanning mechanism' along the 5' untranslated region (UTR) in order to recognize the initiation codon (Pestova &

Kolupaeva 2002). However, many mRNAs contain 5' UTRs with a secondary structure. To enable scanning the secondary structure needs to be unwound and is achieved through the cooperative actions of eIF4E, eIF4G, eIF4A and eIF4B. eIF4E binds the 5'-cap structure of mRNA along with eIF4G that itself acts as a scaffold protein for complex formation, eIF4A is an RNA helicase that can process secondary structure with eIF4B enhancing eIF4A helicase activity. eIF4E, G and A are referred to as eIF4F and together promote translation (Sonenberg & Hinnebusch 2009). Subsequent base pairing of the start codon-anticodon results in commitment of the ribosome and assembly of a competent 80s ribosome. The efficient translation of mRNA is further mediated by the eukaryotic elongation factors (eEF1 and eEF2) that promote translocation of the ribosome and peptide chain elongation(Proud 2007b).

Initiation complex formation and rate of mRNA translation are primarily controlled through the regulation of eIF's and eEF's (Fig 1.2). The signals that regulate protein synthesis originate from various sources such as mechanical loading, nutrients and hormones, with many converging upon the kinase the mammalian target of rapamycin complex 1 (mTORc1) (Kimball 2013). Upon activation, mTORc1 exerts many of its effects through phosphorylation of downstream proteins. Phosphorylation of p70S6K1 by mTORc1 leads to subsequent enhancement in the activity of eIF4B and eEF2 thereby increasing translation (Proud 2007a). Further p70S6K1 phosphorylates the ribosomal protein s6 (RPS6), part of the ribosomal subunit. The effect RPS6 phosphorylation has on protein

synthesis is poorly understood but is elevated in many situations of heightened protein synthesis (Meyuhas 2015). Additionally mTORc1 phosphorylates multiple residues on the eukaryotic initiation factor 4E-binding protein (4EBP1), a protein that is usually bound to eIF4E preventing interaction with eIF4G. Phosphorylation of 4EBP1 by mTORc1 releases eIF4E from 4EBP1 enabling formation of the initiation complex. Together, mTORc1 activity controls many aspects of cellular metabolism and enhances protein translation by both initiation and elongation. However increased protein synthesis does not exclusively occur through mTORc1, with other pathways such as MAPK, IGF1-AKT affecting translation activity (Proud 2007b).

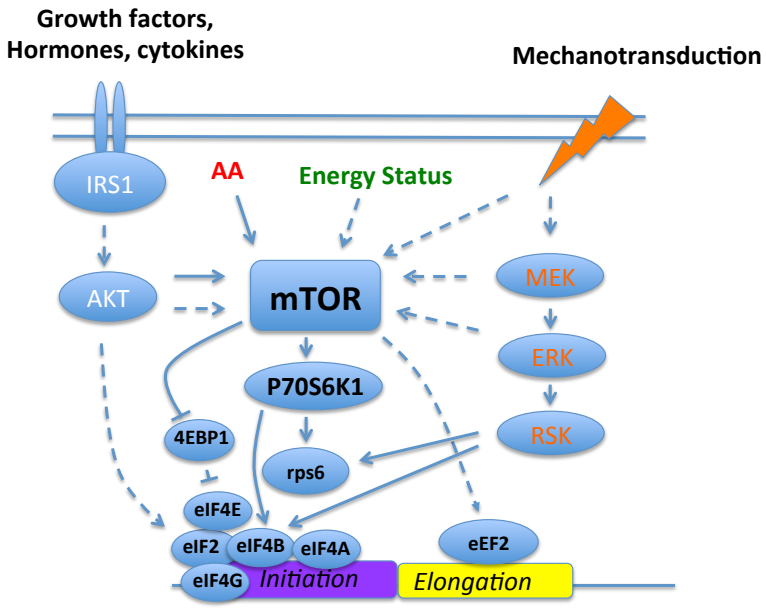


Figure 1.1 An overview of signaling pathways that influence initiation and elongation of mRNA translation. Solid arrow direct stimulation, dashed arrow indirect simulation.

Muscle protein breakdown is essential in muscle homeostasis to remove damaged proteins (Schiaffino et al. 2013). In contrast to MPS, there are four main proteolytic pathways in which protein breakdown can occur; the ubiquitin-proteasome pathway, autophagy-lysosomal pathway, cysteine protease caspase enzymes and calcium dependent enzymes (Fig 1.3) (Pasiakos 2012). The contribution of each pathway to muscle protein breakdown is dependent on a range of factors, including energy status, nutritional supply and mechanical force (Reid 2005; Béchet et al. 2005; Mordier et al. 2000).

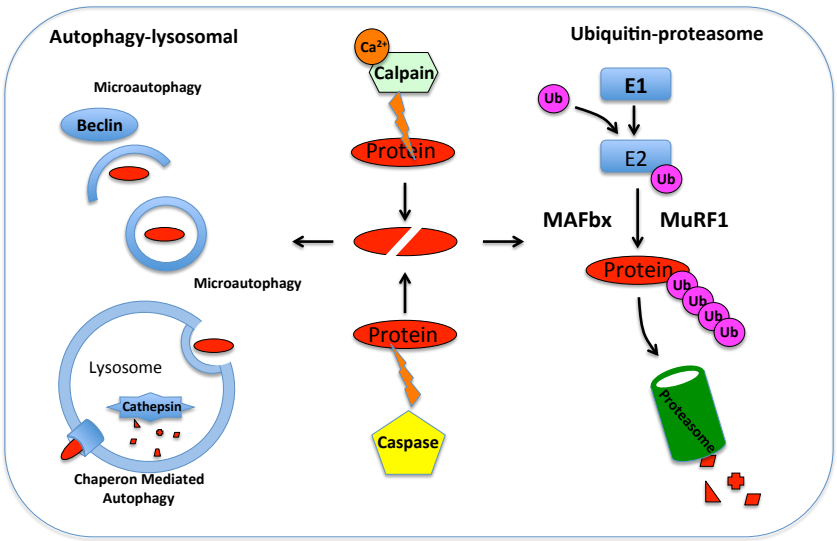


Figure 1.3 An overview of mechanisms involved in muscle protein breakdown autophagy-lysosomal, ubiquitin-proteasome, calpain and caspase mediated

The ubiquitin-proteasome system is believed to be predominantly responsible for the breakdown of contractile elements (Solomon & Goldberg 1996). This is achieved by the addition of ubiquitin to the target proteins that are then directed to the proteasome for digestion. The attachment of ubiquitin requires multiple steps. First activation of ubiquitin by the ubiquitin-activating enzyme E1 is required with subsequent conjugation to the ubiquitin-conjugating enzyme E2. Ubiquitin is then transferred to the target protein by a ubiquitin ligase and the protein directed to the proteasome for breakdown (Bodine & Baehr 2014). There are many ubiquitin ligases within the human genome, however the most commonly up-regulated during atrophic situations are MuRF1 and MAFbx that not only direct the breakdown of muscle structural proteins but also those involved in synthesis (Foletta et al. 2011). It is most likely many other E3 ligases are involved in the removal of proteins, however knockout of MuRF1 and MAFbx have shown to prevent atrophy highlighting their role in muscle protein breakdown (Bodine et al. 2007).

Autophagy is a highly conserved proteolytic pathway that is primarily responsible for the degradation of long-lived cytoplasmic proteins and organelles (Mizushima & Komatsu 2011). Engulfment of the proteins into lysosomes can occur by three mechanisms; micro, macro or chaperon mediated autophagy. The most prevalent is macroautophagy and involves the formation of a phagophore, which matures into a double membrane bound vesicle known as an autophagosome that fuses with a lysosome (Feng et al. 2014). Formation of the phagaphore requires the coordination

of multiple proteins to form the class III PI3K core complex, requiring beclin for nucleation. Degradation within lysosomes relies on low pH and numerous acid hydrolases within cathepsins being the most abundant (Bechet et al. 2005).

In addition to these two pathways proteins maybe be broken down by caspases and calpains. Caspases are proteases commonly known for their role in cellular apoptosis and are activated in response to cellular damage. Calpains are calcium dependent proteases that are activated when calcium levels are perturbed (Costelli et al. 2005). Both have been shown to cleave myofibrillar and structural proteins, however compared to autophagy and UPS pathways that can completely degrade proteins, calpains and caspases have limited proteolytic ability (Du et al. 2004; Williams et al. 1999). It is proposed therefore that by initially cleaving myofibrillar proteins into smaller fragments these can then acts as substrate for UPS and autophagy breakdown (Goll et al. 2003)

The balance between rates of MPS and MPB ultimately control muscle mass, with hypertrophy occurring when MPS>MPB and muscle atrophy when MPB>MPS (Atherton & Smith 2012). The activity of MPS and MPB can be inferred by measuring the phosphorylation state of proteins involved in these pathways. However it is important that we have methods in which we can determine dynamic rates of MPS and MPB. This not only allows us to link 'activation' of key intracellular pathways to an effect, but

also allows determination of potential regulators these processes that will be instrumental in developing strategies against sarcopenia.

1.3 Control of skeletal muscle protein turnover by environmental stimuli

1.3.1 Regulation by nutrition

In the post absorptive state rates of MPS have been reported ~0.03-0.07%/h (Cuthbertson et al. 2005a; Welle et al. 1995; Kumar, Selby, et al. 2009; Mittendorfer et al. 2005) and MPB 0.08-0.11%/h (Phillips et al. 1999; S M Phillips et al. 1997) creating an overall negative net balance of -0.01 to -0.08%/h (Tipton et al. 2003). This negative protein balance represents loss of muscle protein and if this balance were to remain for a considerable time muscle atrophy would occur (Keys et al. 1945).

One of the most potent stimulators of MPS is food intake- particularly the protein content of a meal (Rennie et al. 1982; Bennet et al. 1989). After food consumption, MPS transiently increases and reverses negative balance for ~2hrs before returning back to baseline (Bohé et al. 2001; Atherton, Etheridge, et al. 2010c). This replaces protein lost in times of fasting such that day-to-day feeding habits generally ensure muscle mass remains stable in healthy adults. The potent increase in MPS is driven almost entirely by essential amino acids (Smith et al. 1992; Volpi et al. 2003), with the branched chain AA's isoleucine, valine and in particular leucine central to these effects (Wilkinson et al. 2013). Although the

mechanisms underlying the unique anabolic properties of leucine, are incompletely defined recent work in yeast and cultured mammalians cells have demonstrated that leucyl tRNA synthetase is upstream of activating mTORc1 in response to leucine (Han et al. 2012; Bonfils et al. 2012). This was reaffirmed by experiments showing that of all the EAA, leucine is the most effective EAA at increasing the activity (i.e. phosphorylation) of mTORc1 (Atherton, Smith, et al. 2010) and its substrates. Indeed, mTORC1 is known to be involved in coordinating MPS responses to nutrition, since anabolic responses to nutrition are ablated when the mTORc1 inhibitor is provided alongside EAA (Dickinson et al. 2011)

The anabolic responses to food-intake is dose-dependent with maximal increases in MPS achieved upon provision of 10g EAA equivalent to 20g protein (Witard et al. 2014; Moore, Robinson, et al. 2009; Cuthbertson et al. 2005a). Further, MPS begins to decline ~ 1.5h after consumption even in the face of continued substrate availability (Atherton, Etheridge, et al. 2010c). This represents the "muscle full" state (Bohé et al. 2001; Atherton, Etheridge, et al. 2010a) whereby skeletal muscles become anabolically refractory to enduring nutrient availability. While the regulation of this phenomenon is poorly understood, it is speculated that the sensory mechanisms are in some way related to the ensuring complete replacement of muscle protein stores lost during fasted periods; clearly this refilling "set point" is influenced by physical activity (Bukhari et al. 2015).

The nutritional regulation of MPB has been studied to a lesser extent with the temporal profile being undefined. Nonetheless, it has been clearly shown that increases in plasma insulin associated with food-intake (i.e. nutrients with pancreatic beta-cell secretagogue properties (carbohydrates, AA's (Atherton, Etheridge, et al. 2010a; Juntunen et al. 2002)), are both necessary and sufficient to suppress MPB (Wilkes et al. 2009); while raised plasma concentrations of EAA alone are not (Greenhaff et al. 2008). Thus, it is both an increase in MPS and suppression in MPB that govern the characteristic shift from negative-to-positive protein balance in postprandial periods.

1.3.2 Regulation by exercise

Similar to the consumption of high-quality protein, RE has been shown to induce a 2-3 fold increase in mixed and myofibrillar MPS following a single bout of RE (Phillips et al. 1997; Kumar et al. 2009; Holm et al. 2010). Moreover, the anabolic effect of RE is augmented by consumption of protein in proximity to RE (West et al. 2011) such that with adequate nutrition, increases can be sustained for >24 h (Phillips et al. 1997; Miller et al. 2005; Cuthbertson et al. 2006). This is driven by intake of EAA resulting in an extension in the duration, rather than amplitude, of muscle anabolic responses to RE i.e. extending the "anabolic window"(Bukhari et al. 2015). Inline with the anabolic response in muscle to RE, muscle and tendon collagen appear to be coordinately regulated, following a similar pattern of response as myofibrillar protein, peaking at 24 h then returning to baseline by 72 h with supportive nutrition (Miller et al. 2005). With

significant collagen encompassing the contractile material, it seems logical that for adaptation considerable turnover would occur. Interestingly muscle collagen turnover is not acutely regulated by feeding, nor does feeding further enhance the response to RE, highlighting the importance of load in driving these adaptations (Miller et al. 2005; Holm et al. 2010). As a result of the potentiation of MPS by EAA-intake following RE, net protein balance remains positive despite concordant increases in MPB (Phillips et al. 1997; Phillips et al. 1999). It is the cumulative effect of repeated bouts of exercise and feeding combinations which drives RE-induced hypertrophy (Volek et al. 2013).

As one might expect, there is also a dose response relationship between RE and MPS. MPS peaks at ~1-2 h after RE in the fasted state and returns to baseline by 4 h in the absence of EAA-intake; (Kumar et al. 2009; Kumar et al. 2012)), and follows a dose dependent increase in MPS being near maximal at 60-90% of an individuals 1RM, when external work is matched between loads for total volume (Kumar et al. 2009). Nonetheless, it is not always the case that heavier weights promote greater protein accretion. This was highlighted by Burd et al.(Burd et al. 2010), who employing a unilateral exercise model, exposed volunteers to performing RE at 90% 1RM to fatigue, 30% 1RM work-matched to 90% 1RM group and at 30% 1RM to fatigue. They found MPS was similarly increased in both 90% 1RM and 30% 1RM groups 4 h post RE despite major discrepancies in absolute load (Burd et al. 2010). However this effect was only observed when RE was performed to volitional failure at 30%, and

not when work matched (Burd et al. 2010). This is likely due to increased type II muscle fibre recruitment through fatiguing contractions resulting in maximal fibre recruitment (Burd et al. 2010).

The cellular processes regulating anabolic responses to exercise are more complex than those with nutrition alone as RE triggers multiple intramuscular signaling networks associated with cellular biochemical, mechanical and metabolic stress. Nonetheless, as with MPS responses to nutrition, mTORc1 plays a key role in coordinating these responses (Drummond et al. 2009), consistently up regulated in the hrs after RE (Glover et al. 2008; Fernandez-Gonzalo et al. 2013; Lundberg et al. 2012; Burd et al. 2010; Kumar et al. 2009; Cuthbertson et al. 2005a). The precise regulation of mTORC1 by mechanotransduction is yet to be determined, whilst initially a canonical pathway of regulation for mTORC1 via IGF1-PI3K- Akt/PKB-mTOR was suggested (Bodine et al. 2001; Rommel et al. 2001), recent evidence has pointed to the existence of muscle intrinsic mechano-sensitive signaling pathway e.g. through production of the lipid second messenger, phosphatidic acid (PA)/phospholipase D (PLD) (Hornberger et al. 2006; O'Neil et al. 2009) and adhesome proteins such as focal adhesion kinase (FAK) (Klossner et al. 2009) as signaling to activate mTORC1 post-RE .

The increase in protein breakdown after exerciser occurs primarily through the UPS (Raue et al. 2007) and autophagy pathways(Fry et al. 2013), with UPS primarily degrading myofibrillar proteins (Solomon &

Goldberg 1996). Rates of MPB using dilution tracer techniques have shown that MPB is similarly increased after a resistance exercise bout and can remain elevated ~24h (Phillips et al. 1997). As discussed above, in the fasted state MPB>MPS and both simultaneous increases after RE maintaining negative balance. The addition of protein and or carbohydrate after RE does little in the way of attenuating increases in MPB (Staples et al. 2011) with positive balance primarily due to enhanced MPS upon consumption of protein.

1.4 Regulation of muscle mass by exercise training

1.4.1 Resistance exercise training and skeletal muscle hypertrophy

Skeletal muscle hypertrophy in response to RE is the result of chronic increases in MPS (Mccall et al. 1996; Narici et al. 1996; Wilkinson et al. 2014). Overtime this results in the accumulation of contractile material, increasing fibre dimensions (and unlikely myocellular hyperplasia) and whole muscle CSA (Ahtiainen et al. 2003; Narici et al. 1996; Hulmi et al. 2009). Changes may become apparent after just a few weeks of training with ~5% increase regularly reported in leg muscles (Seynnes et al. 2007; Norrbrand et al. 2008; Blazevich et al. 2007). The rate of muscle hypertrophy gradually declines after this point where muscle becomes refractory to the stimulus of external loading (Wernbom et al. 2007; Ogasawara et al. 2013). Perhaps surprisingly, the time course of muscle hypertrophy still remains poorly defined, likely due to a range of different

methods with varied analytical sensitivity in detecting changes (Heymsfield et al. 2014).

As expected, hypertrophy produced through muscular loading is associated with concomitant improvements in strength, as greater amounts of contractile material are able to produce greater force (Narici et al. 1996; Garfinkel & Cafarelli 1992). Nonetheless, increases in strength are often reported to be much greater than that of mass (Jones & Rutherford 1987), which may be in part due to increased neural recruitment activating a larger proportion of the muscle (Häkkinen & Komi 1982). Changes in muscle architecture such as pennation angle and fascicle length (Seynnes et al. 2007; Franchi et al. 2014)) also contribute to muscle hypertrophy and functional improvements (Aagaard et al. 2001).

As discussed above, positive net balance is achieved upon the consumption of protein and therefore sufficient protein intake is required for hypertrophy. However the effect additional supplementation of protein has during RET programs is rather small (Cermak et al. 2012), suggesting habitual protein intake , usually ~0.8-1g/kg, is adequate to support hypertrophic adaptations. What has become apparent is that a major influence in prevailing muscle hypertrophy is one out-with of an individual's control. RE does not result in uniform growth responses between individuals, with a vast range of values reported i.e. many showing zero hypertrophy responses to RET (Bamman et al. 2007). Therefore, hypertrophic adaptation is impacted by individual genetics

(Phillips et al. 2013); intriguingly, the molecular governance of this remains completely undefined.

Hypertrophy at the cellular level can be detected by an increase in fibre CSA. However, muscle hypertrophy involves a multitude of architectural adaptations which fiber CSA cannot solely account for (D'Antona et al. 2006). Indeed, changes in fibre CSA in response to training have been reported to be greater or less than changes in whole muscle CSA (Narici et al. 1996; Aagaard et al. 2001). These differences maybe a result of variability in fibre (and fiber-type) hypertrophy and reproducibility of fiber CSA estimates (Viitasalo et al. 1980). Further, myofibrillar packing in parallel can increase physiological CSA (CSA area perpendicular to muscle fibres) greater than anatomical CSA, while muscle hypertrophy is non uniform along the muscle belly, and influenced by contraction type (Aagaard et al. 2001; Narici et al. 1996; Franchi et al. 2014). Finally, the point at which addition of new contractile protein produces a detectable increase in CSA of a single fiber is unclear and will likely involve significant rearrangement of the surrounding extracellular matrix (Miller et al. 2005).

At the whole muscle level MRI and CT scans are considered the gold standard for providing accurate muscle measures (Mitsiopoulos et al. 1998). However repeated measures using these techniques are costly, potentially limiting frequent practice. Although initial costs for all these instruments are expensive, Dual-energy X-ray absorptiometry (DXA)

provides measures of muscle mass at relatively low running costs and minimal radiation exposure (Heymsfield et al. 1990). When compared to MRI and CT, DXA may overestimate muscle mass (Maden-Wilkinson et al. 2013) although shows good agreement in representing training induced changes in body composition (Nindl et al. 2000). The use of ultrasound techniques to quantify hypertrophy, i.e. muscle thickness, is gaining popularity primarily as it offers a cheap and minimally invasive technique that also provides information about architectural adaptations (i.e. fascicle length and pennation angle (Franchi et al. 2014)). The choice of method depends on the endpoint of the study, for example, whether regional or whole body measures of mass, volume or architectural adaptations are required. Nonetheless, it is important to highlight that data in the literature on "changes" in muscle mass in response to RET depends heavily upon the method chosen to detect muscle growth (Heymsfield et al. 1997).

1.4.2 The role of protein turnover in skeletal muscle hypertrophy

With continued RET the acute anabolic responses will change to reflect the overall rates of hypertrophy. As such it has been shown that responses to RE are different depending upon training status (Damas et al. 2015; Wilkinson et al. 2008). Indeed, part of the initial elevation in MPS to an unaccustomed bout of RE is likely to represent repair of damaged proteins and is largely determined by an individuals previous exposure to external loading (Murton et al. 2014; Constantin et al. 2013), whereas later changes will more likely reflect specific adaptive, or maintenance processes

(Wilkinson et al. 2006). In studies comparing how the fractional synthetic rates of muscle proteins change with training, it was shown that sedentary individuals exhibit greater MPS responses to a bout of RE when compared with strength-trained athletes (Phillips et al. 1999). This was further demonstrated in longitudinal studies whereupon completion of a unilateral RET program, reduced protein turnover was evident in the RET leg whether RE was performed at the same absolute or relative intensity (Phillips et al. 2002; Kim et al. 2005). Similarly, MPS responses (0-28 h post RE) to successive bouts of RE are attenuated with ongoing training (Tang et al. 2008). Furthermore the specific pools of proteins affected become more specific to the adaptive response; for example, after 10 weeks of RET non-myofibrillar proteins (mitochondrial) are no longer stimulated, whereas myofibrillar proteins are i.e. to facilitate maintenance of increased muscle mass after RET (Wilkinson et al. 2008).

As discussed above, repeated acute increases in translational efficiency and MPS after bouts of RE drive muscle hypertrophy. Increased translational efficiency is the result of activating anabolic pathways by mechanical signaling and nutrient availability (Kimball 2013). However the magnitude of this response and the ability to chronically sustain elevated MPS is influenced by multiple factors. For instance, translational efficiency describes the activity of ribosomes. Increasing the number of ribosomes, known as translational capacity, can theoretically therefore produce more protein. Further DNA represents the foundation in which to produce mRNA and subsequently protein. With a growing muscle the

ability to add additional nuclei to sustain increased protein synthesis may be crucial. Further RE acutely increases anabolic hormones known to have potent effects on muscle growth and could therefore potentially augment RE adaptations (West & Phillips 2010). The necessity and interplay of these factors in sustaining chronic MPS and hypertrophy are unclear and need to be taken into consideration

1.4.4 The role of ribosomal biogenesis in skeletal muscle hypertrophy

The synthesis of new ribosomes is complex, constructed of RNA subunits along with many additional cooperating proteins. This processes refers to ribosomal biogenesis and begins with the transcription of ribosomal DNA (rDNA) located in the nucleolus as many tandem repeats (Moss et al. 2007). Transcription of rDNA is performed by RNA polymerase I (Pol1), producing the polycistronic 47S pre-rRNA that is subsequently cleaved into 28S, 18S and 5.8S. The assembly of ribosomal short proteins and 18s forms the ribosomal 40S subunit, whilst 28S assembled with ribosomal long proteins forms the 60S subunit. Both are exported into the cytoplasm where they form the mature 80S ribosome (Panse & Johnson 2010).

Pol1 is the rate-limiting step of ribosomal biogenesis and its activity is controlled by multiple regulatory factors. The three transcription factors RRN3 (TIF-1A), selectivity factor 1 (TIF-1B) and the upstream binding factor (UBF) come together to form the pre initiation complex with Pol1 at the rDNA promoter. The regulation of TIF-1A and UBF activity is achieved

through multiple proteins including ERK, AMPK, mTORc1 and P70S6K1 (reviewed in (Kusnadi et al. 2015)). This enables the control of ribosomal biogenesis to be influenced by multiple pathways such as hormones, nutrients and activity. Another key regulator of ribosomal biogenesis is c-MYC, an oncoprotein involved in regulating cell growth and virtually all aspects of ribosomal biogenesis. c-MYC directly up regulates many of the proteins involved in rDNA transcriptional control including UBF, TIF-1A, TIF-1B, Pol1 and many other proteins involved in the formation, processing and export of mature ribosomes. Further c-MYC enhances Pol1 transcription by remodeling rDNA chromatin structure and directly interacting with the SL1 complex, stabilizing Pol1 recruitment at the promoter (van Riggelen et al. 2010).

The regulation and rate of ribosomal biogenesis in muscle during habitual activity is relatively unknown. Ribosomes contribute up 80% of total muscle RNA and thus renewal of this pool is necessary to maintain protein synthesis. However, the half-life of ribosomes within muscle has not been described and therefore daily synthesis rates are unknown.

The rate of protein synthesis is determined by the total number ribosomes (ribosomal capacity) and the translational activity (translational efficiency) of these ribosomes. As such the rate of protein synthesis is related to its RNA content (Goldberg 1967; Millward et al. 1973). Acute changes in MPS are primarily propagated by changes in translational efficiency with no change in RNA content (Chesley et al. 1992). However it

has been shown that ribosomal biogenesis is stimulated in response to a bout of RE (Stec et al. 2015; Figueiredo et al. 2015; Figueiredo et al. 2016). Further RNA content increase with muscle hypertrophy (Nakada et al. 2016; Kirby et al. 2015) and has additionally been suggested to determine the extent of muscle hypertrophy (Stec et al. 2016).

1.4.3 The role of satellite cells in skeletal muscle hypertrophy

Skeletal muscle consists of post mitotic multinucleated fibres, as such any additional DNA requires the fusion of undifferentiated myogenic precursor cells known as satellite cells. Satellite cells are located within the basal lamina of muscle and under sedentary conditions mostly remain mitotically quiescent (Zammit 2008). A key attribute of satellite cells is their ability to proliferate and differentiate to repair myofibres whilst some cells remain undifferentiated to replenish the satellite cell pool (Troy et al. 2012; Moss & Leblond 1971). This enables skeletal muscle to have marked regenerative capacity in response to crush injury or myotoxin exposure (Lepper et al. 2011; Lefaucheur & Sébille 1995; Carlson 1968).

In adult fully grown muscles the rate of nuclear turnover is thought to be minimal in everyday life (Schultz et al. 1978), whilst the role satellite cells have in mediating hypertrophic adaptations to exercise remains a more contentious issue (McCarthy & Esser 2007). When a muscle cell grows it is argued that the nuclear content will become diluted as each nucleus can manage only a certain volume of cytoplasm (Allen et al. 1999; Petrella et

al. 2006; Petrella et al. 2008). For this ratio to be maintained addition of new nuclei will be necessary and thus creates the potential importance of satellite cells in mediating muscle hypertrophy. Satellite cells are activated by numerous external signals (Kadi et al. 2005), particularly in response to muscle trauma (Lepper et al. 2011) but also in response to muscle loading through RET (Snijders et al. 2012; Kadi et al. 2004). Once activated satellite cells are referred to as myogenic precursor cells and up regulate the myogenic regulatory factors (MRF's) MyoD and Myf5 that are essential for satellite cell proliferation and differentiation (Ciciliot & Schiaffino 2010).

During RET, the recruitment of new nuclei from satellite cells by fusing with pre-existing muscle fibers has been noted as a feature of hypertrophy in humans (Kadi et al. 1999). Similarly, altered regulation of MRF's is observed in the hrs following a bout of RE (McKay et al. 2008). Further supporting a role for satellite cells in muscle adaptation are reports describing the stimulatory effects of short and long-term resistance training increasing satellite cell content (O'Reilly et al. 2008; Crameri et al. 2004; Kadi et al. 2004; Kadi et al. 1999). Using a novel approach, Petrella et al. (Petrella et al. 2008) applied cluster analysis to investigate the relationship between the degree of hypertrophic responsiveness to resistance exercise and satellite cell activity. They showed that high-responders for hypertrophy exhibited increased satellite cell number pre-training, and greater myonuclei numbers following resistance training. In contrast, data from others have pointed to a poor correlation between

fiber CSA and myonuclei number (Bruusgaard et al. 2012). Further, depletion of satellite cells in animal models does not prevent overload induced hypertrophy (McCarthy et al. 2011) and therefore the requirement of satellite cells during hypertrophy has been of much debate.

1.4.5 The role of hormones in skeletal muscle hypertrophy

Muscle mass is highly influenced by loading and dietary factors, with the hypertrophic/atrophic effects suggested to be brought about mainly by intrinsic mechanisms (West et al. 2010). However many muscle responses are initiated by interactions with proteins in the circulatory system. Hormones in particular are known to have substantial effects on whole body lean mass, such as the noticeable difference between male and females caused primarily by testosterone. Anabolic hormones such as testosterone and growth hormone rise during puberty and have been the focus of ample research due to their considerable effects on growth and development (Stárka et al. 2009). For instance GH deficiency leads to blunted growth and development, whilst excessive growth hormone can lead to acromegaly (Ayuk & Sheppard 2006). Similarly testosterone deficiency causes incomplete development whilst increasing testosterone increase muscle mass (Bhasin et al. 2001). Yet it is unclear the exact roles these hormones have on hypertrophic responses and muscle mass declines with ageing.

Testosterone is a steroid hormone that plays a key role in the development of male reproductive tissues and characteristics.

Testosterone exerts its effects through the androgen receptor (AR). Upon binding AR goes through a series of conformational changes before trans locating to the nucleus where it binds to androgen response elements located in promoter or enhancer region of AR gene targets (Gao et al. 2007). Array analysis has shown that AR binding leads to the up regulation of many genes involved in insulin signaling, transcription factors involved in tissue remodeling, MAPK activation, inhibition of adipogenesis, myogenic differentiation and chromatin remodelling (Montano et al. 2007). AR binding therefore up regulates pathways in promoting protein synthesis and indeed testosterone treatment does lead to increased MPS (Ferrando et al. 1998). Further, testosterone supplementation increases muscle mass and strength, with the extent of adaptation behaving in a positive dose dependent manner (Bhasin et al. 2001).

Growth hormone is a peptide hormone mainly secreted by the pituitary gland in a pulsatile manner and has a direct effect on most tissues; generally to stimulate growth. GH stimulates the release of IGF1, forming what's known as the GH/IGF1 axis. GH stimulated release of IGF1 from the liver produces the majority circulatory IGF1, although IGF1 can be locally produced in tissues such as bone fat and muscle (Ho 2011). GH can therefore act directly through its own receptor and indirectly through IGF1 with both having independent effects on growth (Lupu et al. 2001). Binding of GH to the GH receptor activates the tyrosine kinase JAK2,

activating downstream targets including STAT transcription factor family, MAPK and PI3K. Binding of IGF1 to the IGF1 receptor activates the insulin receptor substrate 1 and is primarily how IGF1 exerts its effects. IRS1 positively regulates the downstream target pathways of AKT and mTORc1 inducing protein synthesis.

GH treatment in hypopituatary subjects stimulates the increase in gene expression of IGF1, collagens and fuel metabolism with limited effect on genes involved in protein synthesis. Clinical measures however have shown increased metabolic rate, lipid oxidation and protein synthesis (Sjögren et al. 2007). The stimulation of protein synthesis is generally seen when GH is administered to GH deficient individuals (Russell-Jones et al. 1993) and has also shown increased lean body mass in these subjects (Götherström et al. 2001). Together this highlights that GH and IGF1 have pro anabolic roles in muscle. However in healthy adults administration of GH and IGF1 has shown mixed results on whole body protein synthesis of either increases or no change (Fryburg et al. 1991; Doessing et al. 2010; Russell-Jones et al. 1994). In younger healthy individuals GH administration increases local and circulating IGF1 although does not increase myofibrillar MPS (Doessing et al. 2010). Additionally, increases in lean body mass associated with GH treatment can almost be entirely attributed to increases in extracellular water (Meinhardt et al. 2013). GH administration however does improve basal metabolic rate and reduces fat mass increasing overall body composition.

Resistance exercise is known to acutely effect the levels of many hormones such as testosterone, GH, IGF1 and cortisol (West et al. 2010). The magnitude of change depends on the intensity of exercise and is most commonly seen when recruiting larger muscle groups during RE e.g legs (West et al. 2009). With the aforementioned hormones having powerful anabolic effects on muscle, this acute elevation in hormones has been proposed to be a significant contributor in hypertrophic RE adaptions. However, acute increases in GH, testosterone and IGF1 have a lack of, or weak association with muscle hypertrophy (West & Phillips 2012). Additionally, post exercise increases in hormones have shown no additional effect on acute MPS (West et al. 2009), albeit shown in the fed state that may mask acute MPS differences. The combined hormonal response of Cortisol, GH, IGF-1 and testosterone has shown to be responsible for 73% of the variance in muscle size after 8 weeks RET indicating hormonal responses may have a synergistic effects (Mangine et al. 2016). Further, studies administering testosterone by itself are directly correlated to muscle growth, whilst suppression of testosterone reduces resistance exercise induced hypertrophy and suggests that testosterone does effect adaptation (Kvorning et al. 2006; Bhasin et al. 2001).

As discussed in the sections above, a wealth of information is now available on acute RE responses and the potential role they play in muscle hypertrophy. However despite this considerable amount of research there are still large gaps in our understanding of the temporal and mechanistic basis of muscle hypertrophy. There is substantial evidence to support that

RE induced muscle hypertrophy is primarily the results of increased MPS leading to chronic periods of MPS>MPB. Yet, the time courses of these responses are unclear. Further how acute MPS and intramuscular signaling relate to chronic outcomes and adapt with continued RET are not defined. In addition to the acute activations of translation initiation, other factors such as satellite cell activity, ribosomal biogenesis and anabolic hormones are likely to affect RE adaptation. The coordinated actions of these factors are yet to be resolved and so the continued progression of methodologies and studies investigating these mechanisms are of great importance. This will be pivotal in determining how muscle homeostasis is altered during atrophic conditions and with advanced ageing.

1.5 Ageing, skeletal muscle and sarcopenia

It has long been recognized that one of the most noticeable changes in body composition with ageing is loss of muscle mass (Frontera et al. 2000; Frontera et al. 2008). However, it was not until the use and development of techniques in accurate muscle quantification that the onset and severity of this process was revealed (Delmonico et al. 2009; Janssen et al. 2000; Heymsfield et al. 1997). During early life, and bearing good health, muscle mass is maintained up to the age of 45 at which point declines of 0.5-1% occur and by the 7th decade can accumulate to ~15% (Kyle et al. 2001; Mitchell et al. 2012). The loss of muscle is primarily of the contractile material; observed by a progressive reduction in size and number of muscle fibers, primarily of 'fast' type II fibers (Lexell et al. 1988; Nilwik et al. 2013). Not only does low muscle mass compromise whole body health, but also significantly contributes to the progressive and rapid loss of

strength in later life (Lauretani et al. 2003; Srikanthan & Karlamangla 2011; Visser et al. 2005). Declines in strength are generally faster than that of muscle mass; occurring up to 2-5 times greater and can happen even if muscle mass remains (Goodpaster et al. 2006; Delmonico et al. 2009). Accelerated strength losses are a product of declines in muscle quality (the amount of strength produced per CSA or muscle mass) a product of increased fat and connective tissue infiltration in-between muscle fibers reducing overall contractile CSA (Delmonico et al. 2009; Csapo et al. 2014) and potentially loss of alpha motor neurons with age reducing muscle activation (Tomlinson & Irving 1977; Campbell et al. 1973).

Low muscle mass and strength are closely linked to increased weakness, frailty, mobility limitations and the capability to perform everyday tasks, (Visser et al. 2005; Hairi et al. 2010; Janssen et al. 2002). With the continual advances in health care, life expectancy shows no signs of slowing (Fig 1.3)(Christensen et al. 2009) and by 2050 those >60 y are expected to represent 25% of the population. Although life expectancy is increasing, the progressive and unrelenting loss of muscle mass will undoubtedly affect quality of life in older age (Rizzoli et al. 2013) placing substantially strains on the healthcare system (Janssen et al. 2004). Preservation of muscle mass and strength with advancing age should therefore be of high clinical significance. However research in the area was lacking until Rosenberg in 1989 proposed the term sarcopenia; from the Greek ‘sarco’ meaning ‘flesh’ and ‘penia’ meaning ‘loss’ (Rosenberg 1997),

in order to strengthen the concept that muscle mass loss with age is its own phenomenon. Since then, the number of publications containing the word sarcopenia has risen from 4 in 1993 to over 500 a year since 2014 (http://www.ncbi.nlm.nih.gov/pubmed). Even with such a rapid rise in interest in the research area and frequent use of the word sarcopenia, an agreed clinical definition has not been achieved(Cruz-Jentoft et al. 2010; Newman et al. 2003; Fielding et al. 2011).

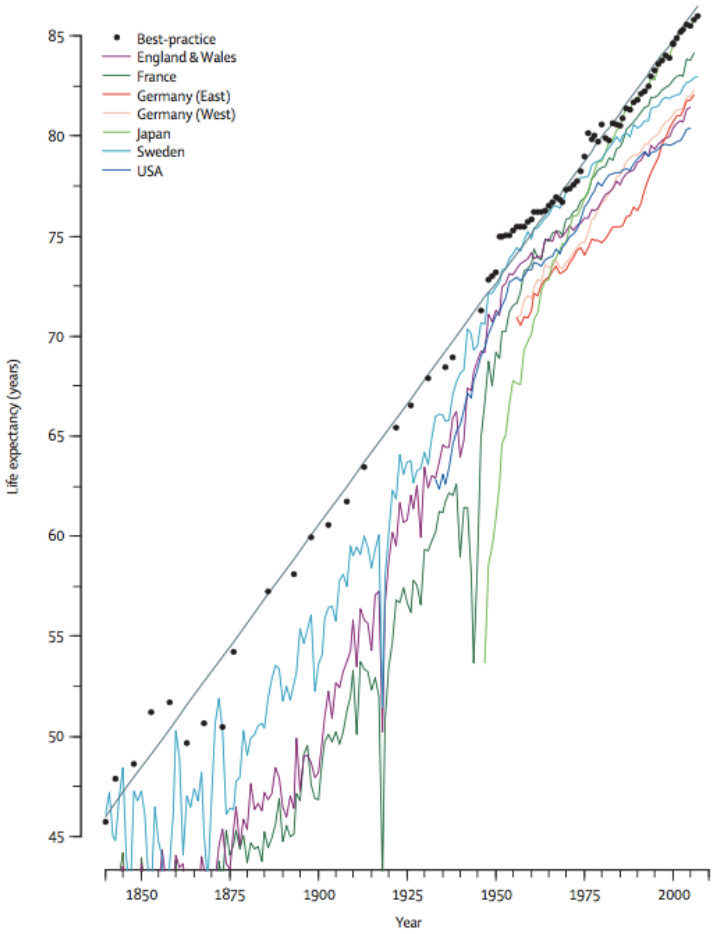


Figure 1.3 Adapted from Christensen *et al.*, 2009. Life expectancy in selected countries and best practice life expectancy for women from 1840 to 2007

Many studies have proposed criteria to establish 'cut off' values in assessing the prevalence of sarcopenia and identifying those at risk of disability (Baumgartner et al. 1998; Janssen et al. 2002; Newman et al. 2003). However great variability occurs between studies, due to criteria used, measurement techniques and different study populations. Cut off values are often based around appendicular skeletal muscle mass (ASM) being <2 SD below young controls and has been used either alone (Levine & Crimmin, 2012), corrected for height (Baumgartner et al. 1998; Janssen, Baumgartner, et al. 2004) body weight (Janssen et al. 2002) or height and fat mass (Newman et al. 2003). However, depending on which criteria are used the prevalence of reported sarcopenia may vary from 14% to >50%. An important factor in using these cut off values is do they identify or predict those at risk of functional impairment, which has shown to be true (Baumgartner et al. 1998; Janssen et al. 2004). However, the use of just muscle mass alone has come under much criticism (Clark & Manini 2008), due to the findings of strength being a greater predictor of disability and mortality outcomes (Newman et al. 2006; Visser et al. 2005). Definitions of sarcopenia have therefore further developed to include measures of strength and function.

The European working group on sarcopenia in older people (EWGSOP) aimed to develop an operational definition and diagnostic criteria for sarcopenia to be used in clinical practice as well as in research studies. The EWGSOP defined sarcopenia as '*a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a*

risk of adverse outcomes such as physical disability, poor quality of life and death'. For identifying sarcopenia they recommend a measure of low muscle mass and strength or performance; with appropriate cut off values provided within (Cruz-Jentoft et al. 2010). However, they also recommend that initial assessments should be based on a slow gate speed followed by measures of muscle mass. In the same year the international working group on sarcopenia defined sarcopenia (IWGS) as '*the age-associated loss of skeletal muscle mass and function. Sarcopenia is a complex syndrome that is associated with muscle mass loss alone or in conjunction with increased fat mass. The causes of sarcopenia are multi-factorial and can include disuse, changing endocrine function, chronic diseases, inflammation, insulin resistance, and nutritional deficiencies. While cachexia may be a component of sarcopenia, the two conditions are not the same'*. The IWGS suggested that sarcopenia should be assessed in older people who have observed declines in physical functioning; strength or health status and patients who are at risk i.e. bed ridden or non-ambulatory. Similarly to the EWGSOP, the recommended assessment starts with that of gait speed or poor functional capacity, with those below the cut off having subsequent measures of mass (Fielding et al. 2011).

It would appear that the identification of sarcopenia is currently in favor of strength and physical outcomes, with definitions of sarcopenia evolving from just muscle mass to also strength and function. Even further it has been suggested that muscle mass is a poor predictor in functional outcomes and that muscle weakness assessments should be preferentially

used in sarcopenia cut offs (Menant et al. 2016). Yet, although weakness is strongly associated with disability (Visser et al. 2005) low muscle mass is associated with weakness (Newman et al. 2003). Further a recent longitudinal study using community dwelling older adults study has shown that ASM alone is associated with greater incident disability and mortality; with the association stronger when weakness or weakness and slowness are combined (Hirani et al. 2015). Although mass may not always be the strongest predictor of function, a portion of the population will have poor function caused by an underlying lack of muscle mass. With low muscle mass being one of the contributors to strength, it is therefore important to not only preserve strength, but also identify interventions that maintain or increase muscle mass in those potentially at risk (Clark & Manini 2008).

Sarcopenia is part of the natural ageing process and imbalances in protein metabolism are hard to identify as these accumulate over years rather than days or weeks. The development of sarcopenia is also multifactorial, and may arise from combinations of hormone imbalance (Feldman et al. 2002; Morley et al. 1997) chronic inflammation (Schaap et al. 2009; Visser et al. 2002), neural changes (Campbell et al. 1973) and increased ectopic fat (Zoico et al. 2010). The regenerative capacity in aged muscle is further compromised due to a decrease in satellite cell number an activation (Kadi et al. 2004). On a molecular level, there is a blunted response to the anabolic stimuli of nutrition and exercise (Cuthbertson et al. 2005a; Kumar et al. 2009) and so development of sarcopenia can be further

enhanced by lifestyle changes such as physical activity (Breen et al. 2013), bed rest (Kortebein et al. 2007) and nutritional status (Houston et al. 2008b; Paddon-Jones et al. 2008)

Pharmacological strategies against sarcopenia have shown limited efficiency and applicability (Borst 2004; Burton & Sumukadas 2010), with exercise shown to be effective at increasing muscle mass, strength and quality in the elderly (Fiatarone & O'Neill 1994; Ivey et al. 2000). The mechanisms resulting in loss of muscle mass with disease and age still remain unclear, although essentially arise from chronic imbalances of MPS (MPS) and muscle protein breakdown (MPB). Thus our ability to understand and regulate these processes is of great importance

1.6 Ageing and the control of skeletal muscle protein turnover

1.6.1 Regulation by nutrition

Despite these tightly regulated metabolic responses to nutrient-intake in healthy younger adults, declines in muscle mass are commonly observed beyond >50y, even in individuals otherwise considered healthy (Mitchell et al. 2012). This suggests that an alteration in the delicate balance between MPS and MPB is likely to occur in older age. It was initially believed that age-related reductions in muscle mass were due to significant attenuation in rates of postabsorptive MPS (Welle et al. 1995; Yarasheski et al. 1993; Welle & Thornton 1993; Balagopal et al. 1997). This notion has since been largely disproven, with consistent findings

revealing equal rates of postabsorptive MPS between healthy younger and older adults (Babraj et al. 2005; Cuthbertson et al. 2005a; Kumar et al. 2009; V. Kumar et al. 2012; Symons et al. 2009; Symons et al. 2011; E Volpi et al. 2001). This has led to seeking alternative avenues of metabolic investigation that can explain the mechanisms underlying sarcopenia.

These avenues led to the theory of anabolic resistance, in which increases in postprandial MPS are less in older compared to young adults (Guillet et al. 2004; Cuthbertson et al. 2005a; Smith et al. 2012; Volpi & Mittendorfer 2000) in some instances older individuals yielding ~40% lower increases in MPS following 20g of EAA (Cuthbertson et al. 2005a). The thinking behind this is that it is the repeated inability to recoup postabsorptive muscle losses during fed periods that is driving sarcopenia (Pennings et al. 2012; Volpi et al. 2000; Cuthbertson et al. 2005a; Guillet et al. 2004). Nonetheless, until recently, direct comparison studies (young vs. old-same nutrition; Table 1) have provided mixed results with some reporting equal responses between young and older participants and others revealing a deficit in the responses of older individuals (Symons et al. 2007; Symons et al. 2011; Paddon-Jones et al. 2004). A recent study rather definitively showed that higher doses of protein are indeed required for older individuals to generate equivalent anabolic response to younger individuals. This new data complied from a substantial number of people demonstrates the existence of anabolic resistance (Moore et al. 2015)

.

Table 1.1:The acute effects of nutritional provision on muscle protein turnover in young (Y) and old (O) individuals

Author	Subjects		Feed	FSR Period	Muscle Fraction	MPS (FSR)	MPB (Ra)	Comment
					Blunted			
Volpi 2000	4M 1F (Y) 4M 1F(O)	Oral	40g CHO + 40g AA (over 3h)	0-3h	Mixed	↑ Y > O (↔)	↓Y = O	MPS increase is impaired in O
Guillet 2004	6(Y) 8(O)	IV	Hyperinsulinemia + Hyperaminoacidemia	0-4h	Mixed Mito Sarc	↑ Y > O ↑ Y > O ↑ Y = O		MPS increase is impaired in O
Cuthbertson 2005	20M (Y) 24M (O)	Oral	0, 2.5, 5, 10, 20, 40g EAA	0-3h	Myo	↑ Y > O		MPS increases in a dose-dependent manner saturated at 20g EAA, O have lower maximal response
Babraj 2005	8M (Y) 8M (O)	Oral	0 or 20g EAA (4 Y+O/group)	0-3h	Myo	↑ Y > O		MPS increase is impaired in O
Katsanos 2005	4M 4F (Y) 7M 4F (O)	Oral	6.7g EAA	0-1, 3.5h	Phe Kinetics	↑ Y > O (NB)	↔	Phe Net uptake 40% greater in young
Katsanos 2006	8M 8F (Y) 12M 8F (O)	Oral	(1) 6.7g EAA 26% Leu (2) 6.7g EAA 41% Leu	0-1, 3.5h	Mixed	(1) ↑ Y > O (↔) (2) ↑ Y > O	(1) ↔ (2) ↔	MPS increases only in Y with 27% Leu, Y+O increase MPS with 41% Leu
Smith 2012	8M 10W (Y) 10M 10W (O)	IV	Hyperinsulinemia Hyperaminoacidemia	0-3h	Mixed	↑ YM > OM ↑ YW > OW (↔)		MPS increase is impaired in O
					Not Blunted			
Volpi 1999	4M 3F (Y) 6M 2F (O)	Oral	40g AA (over 3h)	0-3h	Mixed	↑ Y = O	↔	Equal increase in MPS
Paddon-Jones 2004	2M 4F (Y) 3M 4F (O)	Oral	15g EAA	0-3.5/4h	Mixed	↑ Y = O	↔	Equal increase in MPS, slower but more sustained positive net balance in O
Symons 2007	5M 5F (Y) 5M 5F (O)	Oral	113g Beef	0-5h	Mixed	↑ Y = O		Equal increase in MPS
Symons 2009	8M 9F (Y) 10M 7F (O)	Oral	113g Beef 340g Beef	0-5h	Mixed	↑ Y = O ↑ Y = O		Equal increase in MPS , not further enhanced by greater protein
Chevalier 2011	8W (Y) 8W (O)	IV	Hyperinsulinemia + Hyperaminoacidemia	0-2h	Mixed	↑ Y = O		Equal increase in MPS

Summary: Provision of sufficient AA induces a robust increase in MPS in young individuals. Equivalent increases in mixed MPS can be reproduced in old with greater amounts of AA/Leu.
Male (M), Female (F), Net Balance (NB), Essential amino acids (AA), amino acids (AA), Carbohydrate (CHO), MPS (MPS),

If this is to be an important mechanism underlying sarcopenia, then identifying regulatory mechanisms and providing means to mitigate it are clearly central. Attempts have been made to bolster MPS responses to nutrition in older adults, for instance, by both increasing the amount of protein consumed (Symons et al. 2009; Pennings et al. 2012) or the leucine content (Katsanos et al. 2006; Casperson et al. 2012; Rieu et al. 2006). For example, it has been reported that increasing protein intake from 10 to 35 g in a single bolus leads to a ~50% enhancement of MPS in older men (Pennings et al. 2012), while supplementation of the RDA (0.8 g/kg body weight) of protein with additional leucine over a 2-week period led to increased rates of MPS in older adults (Casperson et al. 2012). Furthermore, 6.7 g of EAAs enriched with leucine enhanced MPS in a group of older adults compared with there being no increase in the absence of added leucine (Katsanos et al. 2006); similarly, there was a ~50% increase in MPS in older men who consumed a mixed meal enriched with leucine (Rieu et al. 2006). These data highlight that increasing quantities of protein or leucine availability can provide efficacy in bolstering the anabolic effects of nutrition in older age.

Few studies have investigated the age related effects of MPB in response to feeding. One study has shown that low physiological concentrations of insulin revealed a blunted inhibition of MPB (Wilkes et al. 2009) whilst AA were kept at postabsorptive concentrations. The effects modest increases in insulin have on muscle protein breakdown are therefore diminished in older age. These increases in insulin are similar to what may be seen after

a small meal and could progressively lead to a chronic lack of suppression of MPB. This combined with a desensitization of MPS to protein intake will further promote sarcopenia. However whether this effect remains upon larger stimulations of insulin with ageing is unknown (Wilkes et al. 2009).

1.6.2 Regulation by exercise

Despite RE being a potent anabolic stimulus, as observed with nutrition, ageing is also associated with blunted MPS responses to RE across a range of exercise 'doses' (e.g. 30-90% 1-RM) (Kumar et al. 2012; Volpi et al. 2000; Kumar et al. 2009), and the combination of RE plus nutrition (Fry et al. 2011a). This age-related blunting of acute anabolism is accompanied by concomitant depressions in mTORc1 activation and its associated down stream proteins (Fry et al. 2011a; Kumar et al. 2009) with blunted activation of P70S6K1 and 4EBP1 being demonstrated from 1 h after exercise (Kumar et al. 2009) and up to 24hrs (Fry et al. 2011a) in older individuals. Similarly, only young individuals have shown correlations between the extent of mTORc1 and P70S6k1 phosphorylation with MPS (Kumar et al. 2009; Fry et al. 2011a); highlighting their roles in driving MPS and dysregulation with age. Nonetheless, blunted anabolic signaling is not shown by all (Drummond et al. 2008; Mayhew et al. 2009), and may be a product of varied sampling times and exercise regimes.

Indeed, not all studies show such a blunting although few studies have directly compared young and old subjects concurrently (Yang et al. 2012; Pennings et al. 2012; Moore et al. 2009) and in those which have (Table 2),

differing results may be reflective of different muscle fractions beings measured, i.e. mixed muscle or myofibrillar (Drummond et al. 2008; Symons et al. 2011; Kumar et al. 2009; Moore et al. 2015). For instance, despite 60% of mixed muscle consisting of myofibrillar protein, the remaining fraction is a mix of protein turning over at varied rates, which may compound the data showing no difference when this is measured. Irrespectively, there is a clear need to define optimal ways to promote anabolism in older age, using exercise and exercise/nutritional-(pharmacological) combinations. Finally, given the potent effects of nutrition upon promoting sustainment of anabolic responses to RE, it has been tested whether this blunting may be "overcome" by dietary manipulation. In this context, similar to findings reported at rest (Katsanos et al. 2006; Wall et al. 2013), enrichment of low dose-EAA with the addition of leucine (Churchward-Venne et al. 2012) after RE has shown anabolic efficacy.

Table 1.2: The acute response of muscle protein turnover to exercise in young (Y) and old (O) individuals

Author	Subjects	Exercise	Feed	FSR Period	Muscle Fraction	MPS (FSR)	MPB (Ra)	Comment
					Blunted			
Sheffield-Moore 2004	6M (Y) 6M (O)	45min Walking 40% Vo_{2peak}	_	0-10 min, 1h, 3h	Mixed	↑Y > O	↑Y = O (0-10 min)	Y + O increase MPS 0-10min but only remains elevated 10-60min in Y
Sheffield-Moore 2005	6M (Y) 6M (O)	KE 6 X 8, 80% 1RM	_	0-10 min, 1h, 3h	Mixed	↑Y > O	↑O (0-10 min)	O increase MPS early 0-10min declining thereafter. Y show late increase in MPS although sustained for longer, 1h-3h .
Kumar 2009	25M (Y) 25M (O)	KE 20-90% 1RM	_	0-1, 2, 4h	Myo	↑Y > O		Sigmoidal dose-related effect of RE, maximised 60-90%. Overall (AUC) Y show 30% > MPS
Mayhew 2009	8M (Y) 6M (O)	3 X 8-12RM KE + LP + Squat	_	~21-24h	Mixed	↑Y > O		MPS is only increased in Y, although does not impact eventual muscle gains in O
Fry 2011	8M 8W (Y) 8M 8W (O)	KE 8 X 10 70% 1RM	_	0-3, 6h 22-24h	Mixed	↑Y > O		MPS increase is impaired in O
					Not Blunted			
Drummond 2008	7M (Y) 6M (O)	8 X 10 KE 70% 1RM	20g EAA at 60min	0-1, 3, 6h	Mixed	↑Y = O		MPS increase is delayed in O, although equal over 5h (AUC)
Durham 2010	9M (Y) 8M (O)	45min Walking 40% Vo_{2peak}	AA infusion	0-10min, 3h	Mixed	↑Y = O	↓Y = O	Equal increase in MPS, although O Show blunted synthetic efficiency
Symons 2011	7M (Y) 7M (O)	6 X 8 80% 1RM KE (60 Min Post Feed)	340g Beef	0-5h	Mixed	↑Y = O		Equal increase in MPS
Kumar 2012	12M (Y) 12M (O)	(1) KE 3 or 6 X 14 40% 1RM (2) KE 3 or 6 X 8 75% 1RM	_	0-1, 2, 4h	Myo	(1) 3 sets ↑Y > O 6 sets ↑Y = O (2) 3 sets ↑Y = O 6 sets ↑Y = O		Increasing volume enhances post exercise response in O (AUC)

Summary: MPS is robustly stimulated in response to resistance exercise in young, however at the same volume and load MPS is blunted in older muscle. MPS is seemingly stimulated to similar levels as in young, when a greater volume of exercise is performed in older adults

Knee Extension (KE), Leg Press (LP), Isokinetoc (Iso), Area Under Curve (AUC), Male (M), Myofibrillar (Myo)

Similar to other regulatory aspects of MPB, the effect of exercise and ageing is understudied. Probably the most complete study investigating MPB in young and old individuals after RE was that of Fry et al 2013 who measured fractional breakdown rates over 24h after (Fry et al. 2013). Although there no significant increase in MPB, both groups displayed an increase of 16% similar to what has been previously reported in young (Stuart M Phillips et al. 1997). This was not different between young or old subjects and was further reflected in similar UPS gene expression. The temporal nature of age related differences in MPB therefore requires further study, although is currently limited by methodological practices.

As discussed above, studies investigating the acute responses to RE and nutrition are beginning to inform on the potential mechanisms of altered muscle regulation with age. However, there is still much discrepancy in the literature possibly the result of variations in analytical procedures, sample collection timing and exercise/nutritional combinations. Nonetheless, a true test as to whether short term blunted anabolic responses are reflective of adaptive outcomes is to directly compare young and old individual in the long term. This is crucially important in response RET, that currently represents one of the most effective strategies and improving muscle mass and strength.

1.7 Ageing, exercise and skeletal muscle hypertrophy

1.7.1 Resistance exercise as a sarcopenia countermeasure

Despite extensive investment in pharmaceutical interventions (Onder et al. 2009) and the discovery of a number of potential novel targeted pharmaconutrients (Ursolic Acid (UA), HMB, PA etc (Kunkel et al. 2011; Vukovich et al. 2001; Hoffman et al. 2012), RE with appropriate supportive nutrition remains the current most effective and safe means by which to maintain or increase muscle mass in older adults (Parise & Yarasheski 2000; Häkkinen et al. 2001; Ivey et al. 2000; Kumar et al. 2012; Fiatarone & O'Neill 1994). Further to the improvements in strength and mass benefits in resting metabolic rate (Lemmer et al. 2001), glucose tolerance (Cuthbertson et al. 2006), reduced blood pressure (Hagberg et al. 1989) and improved lipid profile (Kelley et al. 2005) may be acquired.

Meta-analysis of studies involving RET in older adults have revealed that RE is effective at increasing strength when exercises of a higher intensity are performed (Peterson et al. 2010). Similar analysis also concluded that RE is effective in eliciting gains in lean body mass among older people, particularly if they performed higher volume programs (Peterson et al. 2011). Further a review of studies assessing the effects of progressive RET on physical function showed that when training was performed 2–3 times a week with high intensity, there was large positive effect on muscle strength and a significant improvement in physical ability. This was accompanied by a large improvement in getting out of a chair and modest improvement sin gait speed (Liu & Latham 2009). Progressive resistance

training with aging is therefore an effective intervention for improving strength, physical functioning and the ability to perform everyday tasks.

Despite RE being effective at increasing muscle mass and strength in older age, individuals that continue to train into their 7th decade of life still show decreases in muscle mass, strength and power (Mikkelsen et al. 2013; Faulkner et al. 2008). RE alone is therefore certainty not enough to restore deficits in muscle mass and strength and at best acts to slow the inevitable onset of mobility limitations. As such, it appears there are significant alterations with age in the capacity to build and sustain muscle

1.7.2 Effects of ageing upon hypertrophic responses to resistance exercise

The most effective strategy to determine why there are impairments in exercise responses with age is by comparing young and old individuals undertaking the same RE regime (Table 1.3). In agreement with this, some studies have indicated that whole body RET induces greater gains in FFM (Phillips et al. 2012; Lemmer et al. 2000) and CSA (Welle et al. 1996) in younger than older individuals. Yet other studies focusing on quadriceps muscles are of mixed consensus with some reporting greater increases in young (Raue et al. 2009; Greig et al. 2011), and others equal responses (Häkkinen et al. 2001; Ivey, Roth, et al. 2000; Mayhew et al. 2009). At the level of fibre type CSA, the available data generally show young subjects exhibit greater increases in type I fibre CSA (Kosek et al. 2006; Martel et al. 2006; Mero et al. 2013) with increases in type II CSA also being greater

(Raue et al. 2009; Mero et al. 2013; Kosek et al. 2006) or equal (Mayhew et al. 2009; Häkkinen, Newton, et al. 1998; Martel et al. 2006). These discrepancies are likely to arise from variances in training regimes, nutritional support and analytical measurement techniques. Progression of training loads are often similar between young and old individuals (Mayhew et al. 2009; Kosek et al. 2006; Ivey, Roth, et al. 2000) although in part may be due to increased confidence and learning the training movement with synergistic cooperation. Indeed, increased strength can be the result of neural contributions (Häkkinen, Newton, et al. 1998) since equal strength gains have been produced with limited gains in contractile mass in older age (Mero et al. 2013; Kosek et al. 2006; Moritani & deVries 1980). Resistance exercise certainly improves muscle health in older age (Peterson et al. 2011; Macaluso & De Vito 2004), yet the gains in mass and strength appear diminished compared with those of young subjects.

1.7.3 Mechanisms of impaired muscle hypertrophy

As discussed in sections 1.6.2 and 1.7.2 there is considerable evidence that age related impairments in RE induced skeletal muscle hypertrophy exist and that these are associated with blunted increases in MPS and activation of anabolic signaling pathways. Reduced activation of mTORc1 and MAPK signaling pathways with age have been identified acutely after a bout of RE (Kumar et al. 2009; Fry et al. 2011b). Additionally REDD1 has been suggested to contribute to the impairment of mTORc1 signaling after RE with ageing (Greig et al. 2011). However there is further little research linking blunted anabolic signaling with the contraction induced stimulus.

Further, anabolic resistance to RE is not reported by everyone, with evidence that anabolic resistance may be overcome according to the loading paradigm and nutritional status (Drummond et al. 2008; Symons et al. 2007). In addition to blunted acute increases in MPS, there are other factors that likely contribute sustained impairments in chronic MPS and therefore hypertrophy.

As discussed in section 1.4.3 the muscle ribosomal content has a significant effect on MPS and is highly responsive to RE. However, in response to synergist ablation in young and old rats significant reductions in hypertrophy with age were seen and mirrored by a blunted increase in total RNA content. This impaired ribosomal response was primarily a dysfunction of rDNA transcription in old animals during hypertrophy (Kirby et al. 2015). Further to this, 24h after an exercise bout in human's, only young subjects increase rDNA transcription, reflected by increased markers of ribosomal biogenesis (Stec et al. 2015). This suggests that there may be impairments in ribosomal biogenesis with age in response to RET and may contribute to the age related deficits in hypertrophy.

Muscle regeneration requires satellite cells and so throughout life numbers need to be maintained for growth and maintenance of muscle. A decrease in the overall numbers and activation of satellite cells with ageing could contribute to the gradual loss of muscle to regenerate and therefore development of sarcopenia. It has been shown that in later life there is a gradual decline in the number of satellite cells associated with

type II fibers (Verdijk et al. 2014). Yet, in response to RET elderly subjects have shown significant increases in satellite cell content and myonuclei suggesting that satellite cells remain active in response to external stimuli with ageing (Verdijk et al. 2014). However satellite cell proliferation in response to a bout of RE in both young and older subjects has shown to be reduced in older subjects (Dreyer et al. 2006; Snijders et al. 2014). Additionally, after 16 weeks of RET in young and old individuals undertaking the same RET program, only young subjects increased the number of satellite cells and the number of myonuclei (Petrella et al. 2006). This is may be the result of reduced activation of MRFs', indicative of impaired SC activation responses to RE in old age (Snijders et al. 2014). Thus, a de-sensitization to both myogenic and anabolic cues could underpin the apparent blunted responsiveness of older aged skeletal muscles to exercise-mediated hypertrophic stimuli; clearly the integrated molecular basis of this is a focus of pursuit.

Pro anabolic hormones such as testosterone, GH and IGF1 all peak early in life around the age of puberty, with a gradual decline thereafter (Harman *et al.*, 2001; Feldman *et al.*, 2002; Anderson *et al.*, 2008). In later life this may progress to 30% of men being GH deficient and 50% defined as having hypogynodism (Surampudi et al. 2012; Sattler 2013). The decline in GH/IGF1 and testosterone may contribute to negative effects of ageing and is associated with a loss of lean tissue, accumulation of fat, and loss of strength and mobility (Giannoulis *et al.*, 2012). Further higher levels of testosterone in older individuals may help reduce loss of lean mass and

physical function (Cawthon et al. 2009; LeBlanc et al. 2011). Administration of testosterone, GH or both combined has consistently shown increases in lean body mass with relatively little adverse outcomes (Giannoulis et al. 2012). Declines in body composition and function in ageing may therefore be associated with an unfavourable anabolic hormonal milieu, a potentially modifiable factor.

Table 1.3: Adaptations to resistance exercise in young (Y) and old (O) individuals

Reference	Subjects	Exercise	Regime		Strength		Mass Changes	
			Volume	Frequency	1RM	MVC		
				Blunted				
Moritani 1980	5M (Y) 5M (O)	Elbow Flexors	2 x 10 66% 1RM	3d.Wk^{-1} 12 wks.	↑ Y 29% = O 21%		CSA Skin Fold	↑ 5.4cm Y
Well 1996	5M 4F (Y) 4M 4F (O)	Whole Body	12 x 8 80% 1RM	3d.Wk^{-1} 12 wks.	↑ EF 21% Y **>** 19% O ↑ KF 38% Y **>** 32% O ↑ KE 28% Y = 64% O	-	CSA (MRI)	↑EF 22% Y **>** 9% O ↑KF 8% Y **>** 2% **O** ↑KE 4% Y = 6% O
Lemmer 2000	10M 8F (Y) 12M 11F (O)	Unilateral KE	150 Maximal contractions	3d.Wk^{-1}. 9 wks.	↑ 34% Y **>** 28% O	↑ 34% = Y (M)		
Lemmer 2001	10M 10F (Y) 11M 11F (O	Whole Body	~ 6-12 x 15 Upper Body ~ 6 x 15 Lower Body	3d.Wk^{-1}. 24 wks.	↑ LP 31% Y **>** 21% O ↑ CP 28% Y **>** 16% O		FFM (DXA)	↑ 2Kg Y > 1Kg O
Kosek 2005	13 M 11F (Y) 12 M 13F (O)	Lower body	9 x 8-12 80% 1RM	3d.Wk^{-1} 16 wks.	↑ 28-47% Y = 33-39% O	-	CSA (Fiber)	↑Type I CSA Y 18% ↑Type II Y 32% > 23 % O
Martel 2006	13 M 9F (Y) 11 M 7F (O)	Unilateral KE	150 Maximal contractions	3d.Wk^{-1}. 9 wks.	↑ 34% Y **>** 28% O	-	CSA (Fiber	↑ Type I Y 20% ↑ Type IIa Y 20% O **=.**(M) 24% ↑ Type IIX Y(M) 41% O(W) 49%
Raue 2009	9W (Y) 6W (O)	KE	3 x 10 - 75% 1RM	3d.Wk^{-1} 12 wks.	↑36%Y = 26% O	-	CSA (Fiber) CSA (MRI)	↑ Type IIa Y 28% ↑ **Y** 5%
Phillips 2011	9M 5F (Y) 10M 10F (Mid) 10M 7F (O)	Whole Body		3d.Wk^{-1} 20 wks.	↑36%Y = 35% = Mid 39% O	-	FFM (DXA)	↑ **Y** 5% Mid 1%
Greig 2011	16W (Y) 9 W (O)	Isometric KE	4 X 15	3d.Wk^{-1} 12 wks.		↑Y 27% > O 16%	Volume (MRI)	↑ 6.2% Y* 2.5% O
Mero 2013	21M (Y) 18M (O)	Whole Body	2 sets of Each	2d.Wk^{-1} 21 wks.	↑Y = O	↑Y = O	Fiber CSA	↑ Type I Y ↑ Type II Y
Mitchell 2014	16M (Y) 16M (O)	Whole Body	75%-85% 1RM	3d.Wk^{-1} 12 wks.	↑Y = O	↑Y = O	Fiber CSA	↑ Type I Y = O ↑ Type II Y > O

			Not Blunted					
Hakkinen 1998	8M (Y) 10M (O)	Lower Body	Mixed Rep Range	3d.Wk^{-1}10 wks.	-	↑ **Y** 15% **O** 16%	CSA (MRI) CSA (Fiber)	↑ QF 12.2 % Y 8.5% O ↑ Type I CSA 22% Y 23% O ↑ Type II CSA 25% Y 37% O
Ivey 2000	11M 9F (Y) 11M 11F (O)	Unilateral KE	150 Maximal contractions	3d.Wk^{-1} 9 wks.	↑ Y M 27% F 40% ↑ O M 26% F 28%	-	Volume (MRI)	↑ 12% Y M 5% F ↑ 11% O **M**12% F
Mayhew 2009	21 (Y) 15 (O)	Lower Body	9 x 8 - 12 RM	3d.Wk^{-1} 16 wks.	↑ **Y** 44% O 38%	-	CSA (Fiber) FFM (DXA)	↑ Type IIa Y 37% O 42% ↑ Y 5% O 6%

Summary: Resistance exercise has benefits on muscle mass and strength in both Y and O, Yet Y individuals show more consistently a greater hypertrophic response.
KE = Knee Extension

The mechanisms behind this anabolic resistance and therefor muscle hypertrophy with ageing are yet to be unraveled and are unlikely to be the result of a single factor. Results from acute metabolic and molecular studies have provided many of the assumptions behind the mechanisms of muscle hypertrophy in youth and aging. However many of these studies are after a single exercise bout and varied results are seen likely due to differences experimental conditions such as nutritional provision, length of measurement and exercise paradigms. Further acute anabolic responses have shown not to be related to chronic outcomes such as muscle hypertrophy (Mitchell et al. 2014). This has brought into question the value of acute measure in determining physiological outcomes (Mitchell et al. 2014). A large gap in our knowledge therefore remains concerning age related RE responses. Currently no studies have investigated the links between chronic MPS with age and hypertrophic adaptations. Similarly little is known on the time course of muscle hypertrophy with age and how acute signaling and MPS adapt with progressive RT

There are many aspects involved in muscle hypertrophy including ribosomal biogenesis, satellite cell activity and hormonal profiles. With ageing these processes have shown to be altered in response to RE and could all theoretically represent unfavorable attributes for hypertrophy. Although the role acute hormone increases with RE have been questioned, the effect chronic deficits with ageing have on hypertrophic responses is unknown. Further the role of satellite cells and ribosomal biogenesis in

permitting hypertrophy is unclear and is impart limited by current methodologies. The need for the development of new dynamic measures of DNA and RNA synthesis will be pivotal in unraveling the involvement and temporal nature of these processes.

1.8 Methods to quantify *in vivo* muscle metabolism

1.8.1 Established methodologies

The use of stable isotopes has been key in unraveling many muscle metabolic processes (Wilkinson 2016). Stable isotopes are elements that posses the same number of protons and electrons but differ in the number of neutrons. This means that they posses the same chemical characteristics, although differ in their atomic masses. Each element has an isotope with the highest natural abundance, for instance ^{12}C represents 98.9% of the earths carbon with ^{13}C accounting for the remaining 1.1%. The heavier, least abundant stable isotope can be incorporated into many compounds in varied positions to produce a range of isotope tracers (Wolfe & Chinkes 2005). These tracers are therefore functionally identical to the naturally occurring compound (tracee) but chemically and detectably different. Importantly the body treats them in the same way as the endogenous compound and so through regular sampling, tracers can be followed through tissues and compartments allowing turnover of the endogenous compounds to be measured (Rennie 2007).

Incorporation of a tracer into tissues increases the abundance of the heavier isotope, which can be detected through mass spectrometry (Muccio & Jackson 2009). The amount of incorporation over time can therefore gives rates of synthesis of that particular substrate (Rennie et al. 1982). A huge variety of stable isotope tracers are now produced and typically involve replacing carbons, hydrogen's or nitrogen's in a molecule with its naturally occurring stable isotope equivalent; ^{13}Carbon, Deuterium (^{2}H) or ^{15}Nitrogen. The use of isotope tracers have been part of metabolic research for over 70 years, and are now the 'gold standard' for *in vivo* assessment of protein, carbohydrate, lipid, DNA turnover (Brunengraber et al. 2003; Hellerstein 1999; Macallan et al. 1998; Smith et al. 1992). The insight provided by this tool into the understanding of perturbations in metabolism associated with disease (Kao et al. 2011), ageing (Cuthbertson et al. 2005b) and genetic disorders (Mew et al. 2014), has been extensive, and is growing ever rapidly with the advent of new and more sensitive analytical instruments

Over the years many methods using stable isotope tracers have been developed and fine-tuned for measuring muscle protein metabolism (Wolfe & Chinkes 2005). In order to measure the rate of a biological process the tracer of interest must be incorporated or metabolized by into those pathways. As such, skeletal MPS is measured by the use of labeled amino acids (Smith et al. 2011). The start of a stable isotope tracers study requires the introduction of the amino acid tracer to the system. In humans this has typically involves a primed constant infusion of the tracer

into the blood stream, rapidly equilibrating all the amino acid pools and maintaining tracer levels in the blood (Smith et al. 1992; Phillips et al. 1997). As muscle protein is synthesized, tracer is taken up into muscle over time and disappears from the arterial pool, with the rate of disappearance reflecting rates of synthesis MPS. Similarly tracee is released from muscle into the venous pool diluting the tracer with the rate of appearance reflects MPB (Fig1.4)(Biolo et al. 1994).

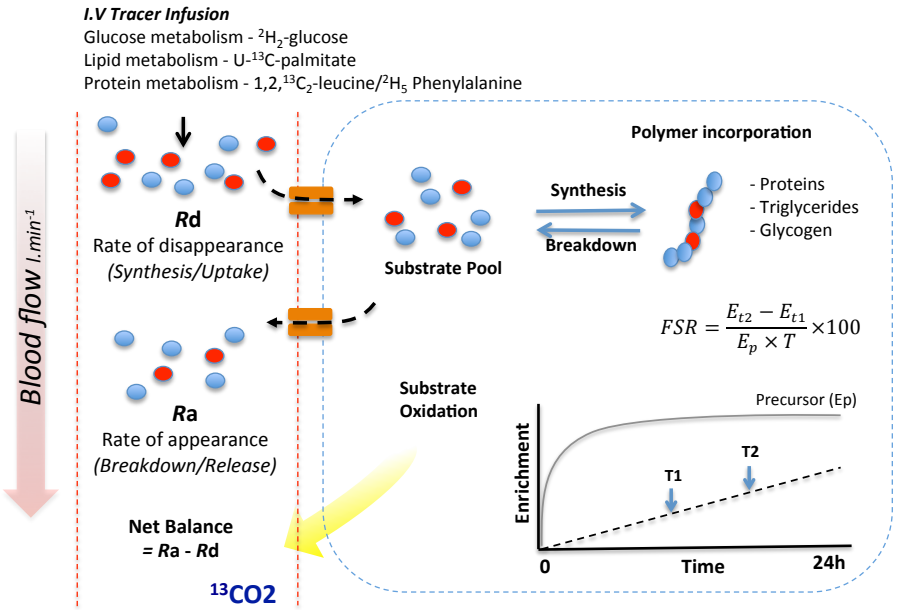


Figure 1.4 typical applications of isotope tracers for measuring metabolism, utilizing both precursor-product and arterial-venous (A-V) balance techniques. Measurement of the rate of disappearance (Rd) of the stable isotope tracer from the arterial pool provides a proxy for synthesis, while the rate of appearance (Ra) of the tracee in the venous pools provides that of breakdown. Furthermore, the rate of synthesis or

incorporation into tissue pools can be measured using the precursor-product approach.

The accuracy of arterial-venous techniques in determining rates of MPS is compromised by the uptake of tracer into other organs in the limb such as skin and bone, along with changes in blood flow (Biolo et al. 1994). Alternatively, rates of MPS can be determined by the direct incorporation of the tracer into the muscle. During the infusion, tracer that enters the muscle will get linked to its aminocayl-tRNA and incorporated into newly synthesized muscle proteins. Repeated sampling of the muscle enables the measurement of the amount of tracer incorporated. Combining this with a measurement of either intracellular or plasma tracer enrichment as the precursor pool allows a rate of incorporation to be calculated (Rennie et al. 1982). A fractional synthesis rate can be calculated using the following equation

$$\textit{Fractional Synthesis Rate (FSR) (\%•h}^{-1}) = \frac{E_{t2} - E_{t1}}{Ep \; x \; t} \text{ x } 100$$

Where $E_{t2} - E_{t1}$ is the change in muscle protein bound labeling between two biopsy samples, Ep is the mean labeling over time of the precursor i.e. intracellular tRNA or surrogate precursors such as plasma AA enrichment, and t is the time between biopsies in hrs.

The direct incorporation method is considered to be the 'gold standard' for protein metabolism and much of what is known about the responses in

muscle to nutrition, activity and exercise (Kumar et al. 2009; Cuthbertson et al. 2005b; Breen et al. 2013). Although these methods are routinely used and provide reproducible measurements of muscle protein turnover, they have numerous drawbacks. Plasma enrichment of the amino acid is often used for precursor enrichment, although this does not represent the true precursor of intracellular t-RNA bound amino acids (Watt et al. 1991). Labeled amino acids are often administered through intravenous infusion, limiting studies to acute time periods of <24h, require cannulation and multiple biopsies within this period making their representation of normal life limited. That being said, these methods have unbeatable resolution in determining subtle differences between subjects in response to anabolic and catabolic stimuli and thus will remain a key part of muscle metabolic research.

1.8.1 D_2O as a novel tracer in the measurement of muscle metabolism

Deuterium Oxide (D_2O) was one of the first stable isotopes to be used as a tracer in the study of dynamic metabolism. After being introduced to heavy water, Ussing noticed that in living animals some of the deuterium 'disappeared' into organic substances (Ussing 1980). Soon after it was suggested that by maintaining a constant level of D_2O in the body water, the kinetics of deuterium stably bound into new molecules could be observed and was subsequently used in the study of proteins, fatty acids and cholesterol (Schoenheimer & Rittenberg 1937; Ussing 1941). However the use of over this time period was minimal and required the further development of analytical tools.

The advantages of using D_2O to study metabolism resurged in the last ~20 years. D_2O is easily administered via oral consumption and has a long half-life in the body water (MacDonald et al. 2013). Additionally, hydrogen from body water is almost universally incorporated into biosynthetic processes and can measure multiple pathways in metabolism (Figure 1.5). As such, D_2O offers great flexibility in study design and overcomes many of the limitations mentioned in traditionally acute amino acid tracers. Utilizing these properties of D_2O Marc Hellerstein and Stephen Previs developed many methodologies in measurement of proteins, lipids, DNA and glucose (Previs et al. 2011; Hellerstein 1999; Neese et al. 2001; Belloto et al. 2007; Bederman et al. 2009).

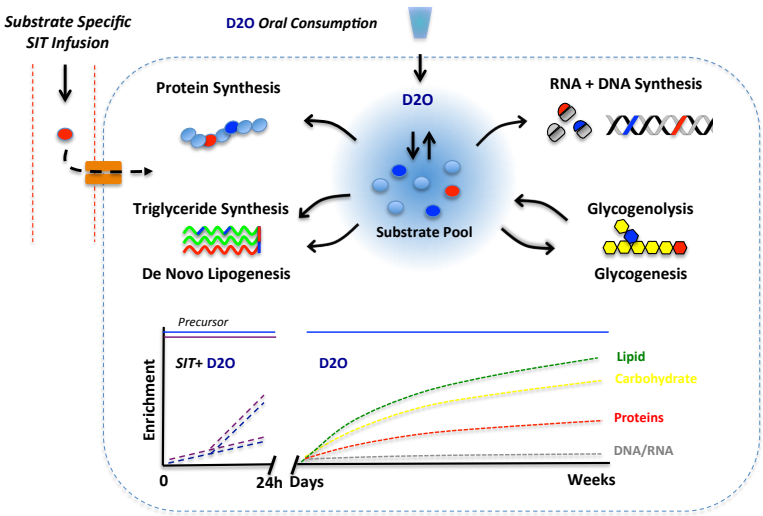


Figure 1.5 A summary of how D_2O can be used as a stable isotope tracer for measuring metabolism of multiple substrates *in vivo*. Upon oral administration D_2O rapidly equilibrates with the body water pool and distributes equally throughout all tissues and organs. Deuterium from the body water is subsequently incorporated into multiple biological substrates such as glucose, AA and fatty acids allowing for the measurement of turnover of multiple substrate pools simultaneously. Due to the slow turnover of the body water pool (half-life ~9-11 days) D2O can provide measurement over periods as short as a few hrs.

Typically in humans D_2O is orally consumed (Robinson et al. 2011; Gasier et al. 2012) provided as single bolus or continuously (MacDonald et al. 2015; Robinson et al. 2011), with rodent studies using intraperitoneal injections and enriched drinking water (Dufner et al. 2005; Busch et al. 2006). The amount administered and for how long ultimately depends on the substrate of interest and limits of detection. Once administered deuterium atoms rapidly distribute equally throughout the body water and organs, taking around ~20 min in rodents (Dufner et al. 2005) and 1-2h in humans (AEA Human Health Series 2011). Deuterium is incorporated into many biosynthetic pathways and therefore turnover of multiple tissues and organs can be simultaneously measured provided sufficient extraction and purification. As such, using these methods the turnover of heart, adipose tissue, muscle and many other tissues and substrates have been made (Strawford et al. 2004; Neese et al. 2002;

Turner et al. 2003; Belloto et al. 2007; Kasumov et al. 2013). However, these tissues or subfractions are made up of thousands of different proteins and can have considerable different rates of turnover (Decaris et al. 2015). By combining LC-MS/MS proteomic approaches with stable isotope tracers, tracking the dynamic flow of the tracer through a system provides powerful insight into the functionally important pathways driving a specific phenotype (Overmyer et al. 2015). By providing daily doses of D_2O measurement of the turnover of 114 individual plasma proteins was achieved (Price et al. 2012). This technique provides a platform in which to investigate individual's proteins or groups of proteins most effect by the stimulus. This has been successfully used in humans and rats to determine individual protein turnover in muscle in response to RE (Shankaran et al. 2015; Scalzo et al. 2014). With the ease of administration and the relative non-invasiveness of sample collection, the use of D_2O combined with proteo/fluxomic techniques will undoubtedly provide a powerful method in unraveling processes in muscle regulation

1.8.1 D_2O as a method for measuring muscle protein turnover

The use of D_2O as a tracer in the study of muscle protein metabolism is fundamentally the same as labeled amino acid tracer. A specific amount of tracer is given and by following incorporation into a final product a rate of synthesis can be made using precursor product calculations (Wolfe & Chinkes 2005). In this case, the tracer is the amino acid into which hydrogen atoms are replaced with deuterium and the subsequent incorporation of this amino acid into protein. The turnover of muscle

protein is relatively slow, and so the long half-life of D_2O within the body lends itself for MPS measures (Robinson et al. 2011; MacDonald et al. 2015).

Upon consumption D_2O enters the cell via diffusion and deuterium will be incorporated into free amino acids replacing those of hydrogen (Dufner & Previs 2003). All amino acids can become labeled and occurs through various enzymatic reactions including transamination and de novo synthesis (Kasumov et al. 2013). Although amino acids may vary considerably in hydrogen atoms, this does not ultimately determine the amount of deuterium that maybe incorporated. This is defined by number of theoretical exchangeable hydrogen's. For instance alanine and glutamine incorporate high levels of deuterium, 3.9 and 4.2 relative to the total bound hydrogen's 4 and 5, due to their extensive role in intermediary metabolism. On the other hand phenylalanine and isoleucine incorporate 0.8 and 0.9 out of a total 7 and 8 (Kasumov et al. 2013). As deuterium is incorporated into all amino acids, in theory any of these could be used to measure MPS as long as, the number of hydrogen's labeled remains constant and the deuterium incorporated into stable positions is not removed

Alanine is rapidly and extensive labeled though transamination reactions, with almost all hydrogen's becoming completely labeled, ~ 3.7-4 (Oshima & Tamiya 1961; Previs et al. 2004). Additionally the equilibration between plasma alanine and body water in rodents is rapid ~20 min and in humans

has shown to quickly equilibrate in dialysis patients (Previs et al. 2004; Belloto et al. 2007). It is also important that the labeling between body water and alanine is consistent and not perturbed by habitual activities such as exercise and nutritional intake. A large supraphysiological bolus of alanine is briefly able to disrupt the labeling of alanine; however even after consumption of a large meal (a rats food for 24h in one meal) or regular food consumption ad libitum did not perturb the labeling relationship between body water and alanine (Dufner et al. 2005; Anderson et al. 2008). Additionally in humans alanine enrichments have shown not to be effected by glucose consumption (Belloto et al. 2007). As the relationship between body water and plasma alanine remains constant, body water can be used to represent the precursor enrichment through simple collections of saliva (MacDonald et al. 2013; Robinson et al. 2011).

A concern with the use of labeled amino acid tracers is the formation of gradients from the plasma to the intracellular space; creating discrepancies in what most accurately reflect the true precursor for protein synthesis i.e. the t-RNA (Watt et al. 1991). When using deuterium transamination occurs intracellularly and as such plasma alanine should represent the intracellular precursor, as this is its origin (Gasier et al. 2010). Further any sources of unlabeled alanine from protein breakdown or food ingestion should rapidly equilibrate with the body water and so not dilute the intracellular enrichment. In rodents these assumptions have shown to be correct, with the intratissullar alanine enrichment in heart,

skeletal muscle and liver reflecting that of plasma alanine and further these are not affecting by feeding or fasting (Belloto et al. 2007).

Alternatively, when using D_2O estimations of the true precursor, alanyl-tRNA, can be calculated by mass isotope distribution analysis (MIDA). MIDA enables calculations of the precursor pool enrichment even if that pool is not accessible. As detailed in (Hellerstein & Neese 2012; Busch et al. 2006) the pattern of mass isotopomer distributions after a labeling experiment contains information about the precursor pool. By extracting this information, combinatorial probabilities can be used to determine mass isotopomer patterns. The distribution pattern can be calculated by knowing 1) the amount of C-H bonds that label can be incorporated, *n* 2) the enrichment of the precursor pool, *p* and 3) the proportion of newly synthesized protein, *f*. Busch et al investigated this by labeling rats in utero, all the newly synthesized proteins are therefore made in the labeling period and so *f* will be 100% and the mass distribution of alanine will depend on *n* and *P.* By using the measured body water as *p,* a value of close to 4 hydrogen's labeled is calculated; and by assuming the theoretical labeling *n* as 4 a value close to the true body water is calculated (Busch et al. 2006). Together these strongly suggest that minimal dilution occurs with intracellular tRNA. However, that all said, the deuterium labeling of alanyl-tRNA has not been directly measured and so the exact scenario is unknown.

Using the developed D_2O methods described above, many measures of MPS, especially in rodents, have been made over a range of durations and conditions including in response to nutrition (Anderson et al. 2008; Dufner et al. 2005), chronic fasting (Yuan et al. 2008), immobilization (Bederman et al. 2015) and exercise (Gasier et al. 2011). By maintaining body water in rats through enriched drinking water MPS was measured over 14 weeks at a rate of 23%.wk^{-1} (Busch et al. 2006), with similar rates of protein synthesis achieved over 3 d of 19%.wk^{-1} (Belloto et al. 2007). These methods were beginning to provide the first long term and integrative measures of MPS at rest that also integrate activity and feeding regimes.

In humans, little information is available on long-term dynamic processes of MPS and even further the effects of interventions such exercise and nutrition. By adapting methods developed in rodents, D_2O has been used to measure MPS in humans. Habitual MPS has now been measured over weeks, giving values of ~1.2-1.5%.d^{-1} (Robinson et al. 2011; MacDonald et al. 2013) agreeing with those values obtained using acute stable isotope techniques of 0.05-0.06%.h^{-1} (Kumar et al. 2012; Smith et al. 2011). This provides evidence that D_2O can measure longer term MPS in a free-living situation. Further, using D_2O MPS has shown to be increased over 24 h, highlighting the ability to detect changes in the rates of synthesis (Gasier et al. 2012). The ease of administration and options for minimal invasiveness offers this technique to measure habitual MPS in a range of populations and conditions.

1.8.2 D_2O in the measurement of muscle DNA and RNA turnover

Fundamental processes in basic biology are DNA replication and RNA synthesis that are both key in cellular division. However if these processes become unregulated they can be the origin of many pathological disorders. Safe and dynamic methods in the measurement of DNA and RNA synthesis would therefore have a broad application in determining progression of many diseases. However measurement has bee restricted by methodological difficulties.

As discussed above the role satellite cells and ribosomal biogenesis have in muscle hypertrophy are incompletely defined. Current practices to assess these changes rely on histology or crude measurement of cellular concentrations (Kadi et al. 1999; Laurent & Sparrow 1977; Petrella et al. 2006; Figueiredo et al. 2015). However these measure don't represent dynamic rates of the changes in RNA and DNA synthesis. Development of new methods involving stable isotope tracers would offer a great potential in satellite cell activity.

DNA and RNA synthesis can be measured using labeled pyrimidine analogues that get incorporated via base salvage pathways (Waldman et al. 1991). However a major limitation using these is they are toxic to humans and can decrease cell proliferation in animals limiting their use to *in vivo* situations (Rocha et al. 1990; Asher et al. 1995). By using infusions of stable isotope glucose tracers, measurements of DNA and RNA synthesis have been made through de novo pathways (Macallan et al. 1998; Defoiche

et al. 2009). Although this method is safe and has shown utility in the measurement of human granulocyte turnover, this required a 48h infusion and rapid cellular proliferation rates. The measurement of slow turnover cell populations using this technique is therefore highly unlikely. Hellerstein and co- workers furthered the use of stable isotopes in DNA synthesis using D_2O. This involved the natural incorporation of D_2O into newly synthesized nucleotides. By maintaining a constant D_2O body water enrichment measurement of slow turnover tissue can be made (Neese et al. 2002; Neese et al. 2001). These methods have made measure of muscle DNA synthesis in mice (Drake et al. 2015; Drake et al. 2014; Miller et al. 2012; Drake et al. 2013)

With the vast potential applications of D_2O described above and the ability to make many different measures of metabolism simultaneously (i.e. MPS, multi substrate turnover and individual proteomics) D_2O will undoubtedly play a key role in unraveling the links of muscle regulation and chronic adaptations. However the use of D_2O in long-term intervention studies is relatively new and much of the work is still in transition from animals to humans. Additionally with a wide range of analytical tools used across facilities, standard procedures for measuring different endpoints using D_2O are still in development.

Currently there is a lack in methods enabling the dynamic measurement of some muscle fractions, particularly DNA and RNA synthesis. As such there is very little known in rates of human muscle DNA and RNA synthesis at

rest, in response to activity and the effects of ageing. To date, in humans, there has only been one study that has looked at the direct incorporation of D_2O into the DNA of human muscle. Further, there have been no long-term measures of muscle RNA synthesis and therefore further development of methods is needed in this area.

1.9 Summary

Skeletal muscle has many essential metabolic and physiological roles and therefore is essential throughout life. However with advanced ageing there is a progressive decline in muscle mass that will contribute to frailty, weakness increase morbidity and mortality (Cruz-Jentoft et al. 2010). The underlying mechanisms of this loss are incompletely defined, although is likely a manifestation of 'anabolic resistance'. This describes the inability to adequately replenish muscle protein lost due to blunted anabolic responses to both nutritional intake and physical activity, with the underlying mechanisms of anabolic resistance incompletely defined (Kumar et al. 2009; Cuthbertson et al. 2005b). Further, multiple factors that are involved in muscle hypertrophy have shown to be altered with ageing such as satellite cell activity, ribosomal biogenesis and unfavorable anabolic profiles (Snijders et al. 2012; Stec et al. 2015; Kraemer & Häkkinen 1998). As such impaired hypertrophy is likely the result of many factors.

MPS plays a pivotal role in the regulation of muscle mass in response to anabolic stimuli (Kumar et al. 2012). However, our current knowledge of

changes in MPS are limited by time (<24h), controlled environments and measures of a single metabolic substrate. They cannot therefore provide chronic measures of muscle metabolism or simultaneous measure of various metabolic substrates. Our knowledge on integrative habitual measures of MPS, over days to weeks is therefore severely lacking. Further to this, there is very little known about the habitual synthesis of muscle DNA and RNA. Using D_2O, we aim to develop novel isotope tracer techniques to permit chronic, weeks to months, measurement of muscle protein, DNA and RNA synthesis under free-living conditions.

Muscle hypertrophy is a well-known result of RE training (Volek et al. 2013), yet the time course and underlying metabolic adaptations with progress RET unclear. Further the association between acute measures of MPS and long-term physiological outcomes are unclear and is in part due to limitation of acute isotope measures (Mitchell et al. 2014). Thus we aim to apply our newly developed tracer techniques to investigate chronic temporal responses of MPS, muscle signaling and hypertrophy. Additionally for the first time we aim to investigate the role synthesis of new DNA and RNA has on muscle hypertrophy. We hypothesized that the temporal profile of RET-induced hypertrophy would be reflected in long-term MPS and anabolic signaling profiles.

Acute measures of MPS have identified notable differences in the anabolic responses to both nutrition and exercise with age (Moore et al. 2015; Fry et al. 2011a). However how these acute differences propagate into long term MPS has not been determined. As such we aim to use these newly

developed techniques to investigate the effects of age on chronic protein turnover in free-living humans. Further we will investigate how age affects MPS in response to exercise along with measure of intramuscular signaling, gene expression, hormonal profiles and RNA content. We hypothesized that following RET skeletal muscle hypertrophy would be blunted with age, primarily due to long term deficits in cumulative MPS responses with the underlying mechanisms multifactorial.

Chapter 2- General Methods

2.1 Subject recruitment and screening

Recruitment targeted healthy volunteers from the local Derbyshire area by poster advertisements and letters sent to individuals within the designated age categories. Full informed consent was taken from all volunteers and pre-screened by a clinical fellow including medical history, blood tests and full ECG. Subjects were excluded if they had evidence of active cardiovascular disease, cerebrovascular disease including previous stroke, aneurysm (large vessel or intracranial), respiratory disease including pulmonary hypertension or COPD, hyper/ hypo parathyroidism, hyper/ hypothyroidism, Cushing's disease, diabetes, active inflammatory bowel disease, renal disease, malignancy, recent steroid treatment (within 6 month), or hormone replacement therapy, clotting dysfunction, musculoskeletal or neurological disorders and any disease requiring long-term drug prescriptions, including statins. The university of Nottingham Medical School granted ethical approval for all aspects of this study

2.2 Subject characteristics and ethics

Ten healthy younger males (23±1y, BMI: 24±1) and older (69±1y, BMI 25.8 ±1) males were recruited. All volunteers were screened by medical questionnaire, physical examination, and resting electrocardiogram, with exclusions for metabolic, respiratory, and cardiovascular disorders or any other symptoms of ill health. Subjects had normal blood chemistry, were normotensive (<140/90), and were not prescribed any medications: all subjects performed activities of daily living and recreation but did not undertake in any RET other than that described in the study and had not

participated in any RET within the last 12 months. All subjects provided their written, informed consent to participate after all procedures and risks (in relation to muscle biopsies, blood sampling etc) were explained. This study was approved by The University of Nottingham Ethics Committee and complied with the Declaration of Helsinki.

2.3 Conduct of the study

This study involved a bilateral protocol whereby one leg was used as an untrained (UT) internal time control with the other leg acting as the RET (T) leg, with the dominant leg assigned as T. Following inclusion to the study, subjects were studied over a 6-week period. On the first day of study, subjects arrived at the laboratory at 0830 h following an overnight fast. Following assessment of *Vastus Lateralis* (VL) skeletal muscle architecture by ultrasound (Mylab 70; Esaote Biomedical) and body composition by dual-energy X-ray absorptiometry (DXA; Lunar Prodigy II, GE Medical Systems, Little Chalfont, Buckinghamshire, UK), subjects completed the 1st session of RET consisting of unilateral knee-extension exercise od 6×8 reps at 75% 1-RM. Bilateral biopsies were taken from the VL muscle 60-90 min (75±2 min) after unilateral exercise under sterile conditions, using the conchotome biopsy technique (Dietrichson et al. 1987) with 1% lidocaine (B. Braun Melsungen) as local anaesthetic. Muscle was rapidly dissected free of fat and connective tissue, washed in ice-cold PBS, and then frozen in liquid N_2 and stored at -80°C until further analysis. Immediately post-RET, subjects provided a saliva sample (collected in sterile plastic tubes) and consumed a 150 ml bolus of D_2O (70 atom%; Sigma-Aldrich, Poole, UK); with the aim to label the body water

pool to ~0.2% APE which was maintained in a pseudo-steady state with weekly top-up boluses (50 ml.wk^{-1}). In addition, venous blood samples were collected into lithium heparin coated tubes, immediately cold centrifuged at 1,750 g, with plasma fractions aliquoted and frozen at -80°C until analysis. Thereafter, subjects returned to the lab 3.wk^{-1} to undertake supervised unilateral RET with 1-RM assessments of the T leg every ~10 d to ensure progressive intensity. Further bilateral muscle biopsies (~90 min after RET in order to investigate the temporal nature of acute anabolic signaling responses to progressive RET), ultrasound measures of muscle architecture and venous blood samples were taken at 3 and 6 weeks and DXA at 6 weeks. For the temporal monitoring of body water enrichment, each participant provided a saliva sample on RET-visits >30 min after their last meal or drink, with extra samples taken ~3 h after weekly 50 ml boluses to ensure body water enrichment was accurately represented. These were collected in sterile plastic tubes and immediately cold centrifuged at 16,000 g to remove any debris that might be present; they were then aliquoted into 2-ml glass vials and frozen at -20°C until analysis. A detailed representative schematic of the study protocol in its entirety is depicted in Figure 2.1.

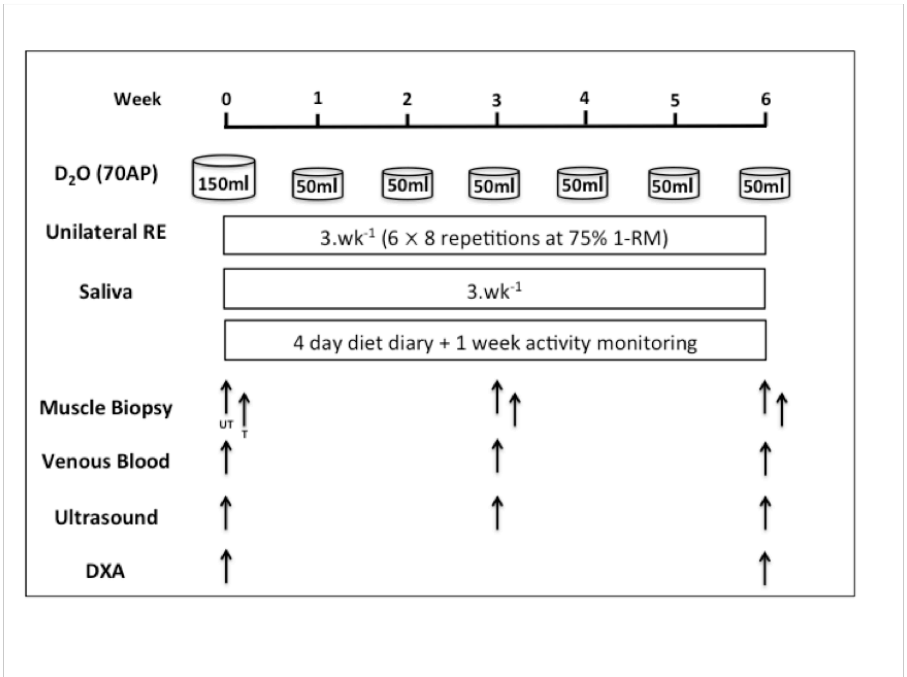


Figure 2.1 Schematic representation of the 6 week training study

2.4 Measure of habitual physical activity and diet

To monitor levels of habitual physical activity, subjects wore combined heart rate and activity monitors (Actiheart, CamMtech Limited, Cambridge, UK) for 1-week during the study. Actiheart data was checked for missing or extrapolated values; ≥80% of minute-by-minute physical activity data had to be available for a 24 h recording to be accepted, 85% of subjects had at least ≥4 days of data with the remaining having ≥1 day of data that was included in the analysis. Dietary intake was monitored by completion of 4-day diet diaries during the study. The 4-days consisted of two-week days and two weekend days in which subjects were instructed to record all food and drink intake. Dietary intake was analyzed using Microdiet (Downlee Systems Ltd, City, Country).

2.5 Muscle functional tests

Regular measures of MVC and 1-RM were performed to monitor strength gains and ensure training progression. Basal MVC and 1-RM were assessed in the week prior to the first study day. MVC was measured with isometric contractions conducted in a sitting position using an isokinetic dynamometer (Isocom; Isokinetic Technologies, Eurokinet-ics) over a range of four knee joint angles (90° to 60°), with full extension corresponding to 0°. Subjects were seated in the dynamometer chair and secured into position using a chest strap. Contractions lasted 4 s, with a 30s rest period between contractions and 90 s between knee joint angles. Unilateral leg extension 1-RM was assessed on the dominant leg (Technogym, Gambettola, Italy). After the procedure was explained, subjects performed a light warm up to avoid injury and ensure familiarity whilst avoiding fatigue; 1-RM was then achieved (20 on Borg's RPE scale) in as few repetitions as possible with a maximum of 5 repetitions. The first repetition was estimated at 50% 1-RM and then increased until subjects could not complete controlled contraction throughout the range of motion, with 3 min in-between attempts. Functional tests were repeated throughout (~ every 10 days), with each set of tests preformed on a non-biopsy day to avoid influencing subsequent measures.

2.6 Muscle architecture and DXA derived mass

Ultrasound was used to monitor temporal changes in VL muscle architecture over the 6-week training protocol. The architectural parameters muscle thickness (MT), fascicle length (L*f*) and pennation

angle (θ) were quantified by the same blinded (MB) investigator from ultrasound scans using Image J 1.42q (National Institutes of Health). The visible portion of the L*f* was assessed directly using this software with MT measured as the distance between the superficial and deep aponeuroses. Pennation angle was measured as the intersection between fascicles and the deep tendon aponeurosis. The coefficient of variation was 2.6% for MT, 3.7% for L*f* and 3.6% for θ. DXA derived thigh muscle mass was determined from the lowest point of the Ischium to the knee space.

2.7 Body water and protein bound alanine muscle fraction enrichment

The deuterium enrichment in saliva was measured over the 6-week period to monitor changes in body water enrichment. 100µl of saliva was inverted in a 2ml auto sampler vial for 4 hrs at 100°C. Vials were then placed upright on ice to condense extracted body water and transferred to a clean auto sampler vial ready for injection. 0.1µl of body water was injected into a high-temperature conversion elemental analyzer (TC/EA; Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an isotope ratio mass spectrometer (IRMS; Delta V Advantage, Thermo).

To measure the changes in muscle myofibrillar enrichment, 30–50 mg of muscle was homogenized in ice-cold homogenization buffer pH7.5 (Tris-HCL 50mM, EDTA 1mM, EGTA 1mM, B-glycerophosphate 10mM, NaF 50mM) containing a protease inhibitor tablet (Roche) and sodium orthavanadate 0.5mM, rotated for 10 min. The supernatant containing

sarcoplasmic proteins was collected after centrifugation at 13,000 *g* for 5 min at 4°C. After washing, myofibrillar proteins were extracted from the remaining pellet by solubilizing in 0.3M NaOH and separated from the insoluble collagen by centrifugation, with myofibrillar proteins precipitated using 1M of perchloric acid (PCA). Protein-bound AA were released using acid hydrolysis by incubating in 0.1M HCl in Dowex H^+ resin slurry overnight before being washed and eluted from the resin with 2M NH_4OH and evaporated to dryness.

To permit GC analysis AA were derivatized as their n-methoxycarbonyl methyl esters. Dried samples were suspended in 60 µl distilled water and 32µ l methanol, and following vortex, 10 µl of pyridine and 8 µl of methylchloroformate were added. Samples were vortexed for 30 s and left to react at room temperature for 5 min. The newly formed n-methoxycarbonyl methyl esters (MCME) AAs were then extracted into 100µl of chloroform. A molecular sieve was added to each sample for ~20 s before being transferred to a clean glass GC insert, removing any remaining water by size exclusion absorption. Incorporation of deuterium into protein bound alanine was determined by gas chromatography-pyrolysis-isotope ratio mass spectrometry (Delta V Advantage; Thermo Scientific), alongside a standard curve of known DL-Alanine-2,3,3,3-d4 enrichment to validate measurement accuracy of machine.

2.8 Calculation of fractional synthetic rate (FSR), absolute synthetic rate (ASR) and absolute breakdown rate (ABR)

Myofibrillar MPS was calculated from the incorporation of deuterium-labeled alanine into protein, using the enrichment of body water (corrected for the mean number of deuterium moieties incorporated per alanine, 3.7, and the dilution from the total number of hydrogen's in the derivative, i.e. 11) as the surrogate precursor labeling between subsequent biopsies. The equation used was:

$$FSR = -Ln\left(\frac{-1\left[\frac{(APEala)}{(APEp)}\right]}{t}\right)$$

where APE_{Ala} = deuterium enrichment of protein-bound alanine, APE_P = Mean precursor enrichment over the time period, and t is the time between biopsies. Additionally as protein synthesis tends to follow zero order kinetics the fractional synthesis rate was also determined based on a single compartmental model by fitting a time course of net labeling to an exponential rise curve equation $E(t) = Emax \times (1 - e^{-kt})$ where $Emax$ is the number of hydrogens labeled assuming 3.7 multiplied by the average body water labeling.

Thigh absolute synthetic rate was calculated as $ASR = \frac{FSR}{100} \times TFFM \times \frac{(ASP)}{100}$ where *TFFM* is thigh fat free mass derived by DXA and *ASP* (alkali soluble protein) is the average soluble protein over the 6 weeks. Absolute protein breakdown rates were calculated as: $ABR = \frac{FBR}{100} \times TFFM \times \frac{(ASP)}{100}$ where *FBR* represents the fractional breakdown rate, calculated as $FBR = FSR -$

FGR with the fractional growth rate assumed to be a linear increase in the DXA derived TFFM mass over 6 weeks. Using a linear growth rate limits the ability to investigate temporal changes in absolute rates, however this takes into account the average adaptation in growth over the 6 weeks.

2.9 Immunoblotting

Immunoblotting was performed on muscle sarcoplasmic fractions extracted as described in section 2.7. For nuclear proteins involved in ribosomal biogenesis muscle was initially homogenized in RIPA Buffer (Thermo Scientific) with 1mM EDTA, 1 mM activated Na_3 VO_4 (Sigma-Aldrich) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK). Protein concentrations were determined using a NanoDrop (NanoDrop Lite Spectrophotometer Thermo Scientific) after which samples were standardized to 1mg/ml by dilution with 3X Laemmli loading buffer and heated at 95^{o}C for 5 min. 15 µg of proteins were loaded onto Criterion XT Bis-Tris- 12% SDS-PAGE gels (Bio-Rad Hemel Hempstead, UK) for electrophoresis at 200 V for 1 h. Samples were transferred to polyvinylidene difluoride membranes for 45 min at 100 V. Membranes were subsequently blocked in 2.5% low-fat milk [diluted in Tris-buffered saline and 0.1% Tween-20 (TBS-T)] for 1h at ambient temperature and then incubated overnight at 4°C in the presence primary antibodies rocking overnight at 4°C. Membranes were washed 3×5 min in TBS-T, incubated in HRP-conjugated secondary antibodies; Mechanistic target of rapamycin (mTOR)^{Ser2448}, total mTOR, ribosomal protein S6 kinase 1 (p70S6k1)^{Thr389}, total p70S6K1, protein kinase B (Akt)^{Ser473}, total

AKT, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1)$^{Thr37/46}$, total 4EBP1, eukaryotic elongation factor 2 (eEF2)^{Thr56}, total eEF2, ribosomal protein S6 (rps6)$^{ser240/244}$ Beclin-1, ERK 1/2 $^{Thr202/Tyr204}$, (pRB)^{S780} , C-Myc (New England Biolabs, Hertfordshire, UK; 1:2000 in 2.5% BSA in TBS-T) Cathepsin L, Calpain 1, (UBF1)^{S484}, UBF1, total transcription initiation factor 1 TIF1a, RRN3^{S649} (Abcam, cambridge,UK) at room temperature for 1 h, before the last 3×5 min washes in TBS-T. Membranes were exposed to Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA-US) for 5 min and bands quantified by Chemidoc MP (BioRad, Hertfordshire, UK). Software measures were taken to prevent pixel saturation; protein-loading anomalies were corrected to total Coomassie protein (Welinder & Ekblad 2011).

2.10 Hormone concentrations

To determine circulating levels of testosterone, IGF1 and myostatin enzyme-linked immunosobent assays (ELISA) were performed on plasma samples of all old subjects and 9 young subjects. ELISA's were performed according to the manufacturers protocols using the following commercially available kits; GDF-8/Myostatin Quanitkine ELISA Kit (R&D Systems DGDF80, Abingdom, UK), Testosterone ELISA Kit (Abcam ab108666) and IGF1Quantikine Elisa Kit (R&D DG100)

2.11 Gene expression analysis

To investigate changes in muscle mRNA expression, total RNA was isolated 5-10 mg of muscle homogenized in 200 µl of TRizol (LifeTechnologies, ThermoFisher Scientific) using two stainless steel beads (Tissue Lyser II, Qiagen, UK) for 1 min at 30/s. Samples were placed at ambient temperature for 10 min before 80 µl of chloroform was added and samples vortexed and incubated at ambient temperature for 10 min. After centrifugation at 12000 g for 15 min at 4^{o}C the upper aqueous layer was removed and RNA precipitated with an equal volume of isopropanol, incubation at RT for 10 min and subsequent centrifugation at 7000 g for 10 min at 4^{o}C. The pellet was washed twice with 1 ml of 80% ethanol, dissolved in 22 µl of RNA-free water and quantified by spectrophotometry (NanoDrop Lite). For RT-qPCR 500 ng of total RNA was reversed-transcribed with the high-capacity cDNA reverse transcription kit (Life Technologies) according to manufacturer's protocol. Resulting cDNA was diluted 1:5 and 1 µl was added per well of the 384-optical well plates (Life Technologies). Exon specific primers were mixed with SYBR Select Master Mix (Life Technologies) and 11 µl of master-mix were added to each well, with samples run in triplicate. Thermal cycling conditions were 2 min at 50^{o}C followed by 2 min 95^{o}C and 40 cycles of 15 s at 95^{o}C and 60 s at 60^{o}C on a ViiATM 7 Real-Time PCR System (Life Technologies). To control for RNA input, PPIA levels were measured and target mRNA expression was quantified using the ΔΔCt method (Schmittgen & Livak 2008).

2.11 Primer Sequences

The following primer sequences were used in to measure mRNA expression as described above

C-MYC	*Fwd*	*5'-GGTAGTGGAAAACCAGCAGCC-3'*
	Rev	*5'-TCTCCTCCTCGTCGCAGTA-3'*
POL1RA	*Fwd*	*5'-CCTCAAGGTATCGCCCAGTC-3'*
	Rev	*5'-GGCAACTTCTGTTCTTGGGC-3'*
POL1RB	*Fwd*	*5'-TACTGTGCAACTTGGGGGTC-3'*
	Rev	*5'-GAGAATCTGCGATGCCTGGA-3'*
TAF1A	*Fwd*	*5'-AGGTTTAGCGCCTGCTCATA-3'*
	Rev	*5'-CTGAAATCACTCATACCCGCCT-3'*
TIF1A	*Fwd*	*5'-CATTTTGTGCCTCCCCGAGT*
	Rev	*5'-GTATTGGCATGAGAAACCACGG-3'*
UBF	*Fwd*	*5'-AAGAAGCCTCCCATGAACGG-3'*
	Rev	*5'-CGGCCAGCTTTTTGTAGTGC-3'*
MuRF1	*Fwd*	*5'-GTGTTTGGGGCTCACCAGGC-3'*
	Rev	*5'-ACCTGGTGGCTATTCTCCTTGGT-3'*
MAFbx	*Fwd*	*5'-CTTTCAACAGACTGGACTTCTCGA-3'*
	Rev	*5'-CAGCTCCAACAGCCTTACTACGT-3'*
20S proteasome	*Fwd*	*5'-CGTTTTCAACGGAGGTACTA-3'*
	Rev	*5'-TCAGCGTAAGACAGTCTCCA-3'*
Caspase 3	*Fwd*	*5'-TCCACAGCACCTGGTTATTATTC-3'*
	Rev	*5'-GCGTCAAAGGAAAAGGACTC-3'*
Calpain L	*Fwd*	*5'-GCCAAGCAGGTGAAC-3'*
	Rev	*5'-TGAAGTCTCGGAATGACATC-3'*
RPS5	*Fwd*	*5'-ATCATCAACAGTGGTCCCCG-3'*
	Rev	*5'-AGATGGCCTGGTTCACACG-3'*
RPSA	*Fwd*	*5'-CCCTACTGAAGACTGGAGCG-3'*
	Rev	*5'-AGAGCCTATGCAAGAACAGCTT-3'*
RPS26	*Fwd*	*5'-CGAGCGTCTTCGATGCCTAT-3'*
	Rev	*5'-CAGGTCTAAATCGGGGTGGG-3'*
RPS3	*Fwd*	*5'-GGAAGTTTGTCGCTGATGGC-3'*
	Rev	*5'-ACATTCTGTGTTCTGGTGGCT-3'*
RPL13A	*Fwd*	*5'-TAAACAGGTACTGCTGGGCCG*
	Rev	*5'-CTCGGGAAGGGTTGGTGTTC*
RPL13A	*Fwd*	*5'-TAAACAGGTACTGCTGGGCCG*
	Rev	*5'-CTCGGGAAGGGTTGGTGTTC*
RPL11	*Fwd*	*5'-AGGGTCTAAAGGTGCGGGA-3'*
	Rev	*5'-AGTCCAGGCCGTAGATACCA-3'*

Androgen Receptor	*Fwd*	*5'-TCAGCATTATTCCAGTGGATG-3'*
	Rev	*5'-GGAGCTTGGTGAGCTGGTAG-3'*
Myostatin	*Fwd*	*5'-GCTGCGCCTGGAAACAGCTC-3'*
	Rev	*5'-ATCAGTTCCCGGAGTGGAGGC-3'*

2.12 Skeletal muscle protein, DNA and RNA concentrations

To determine muscle protein, DNA and RNA concentrations μg.mg dry weight^{-1} ~15 mg muscle tissue was freeze dried. Dry tissue was then homogenized in 0.2M PCA. After centrifugation at 11,680 g and washing with 0.2 M PCA, the pellet was resuspended in 0.3 M NaOH and alkali soluble proteins quantified using NanoDrop (NanoDrop Lite Spectrophotometer Thermo Scientific). Next proteins were precipitated with 1 M PCA before centrifugation and removal of the supernatant for RNA quantification by spectrophotometry. The remaining pellet was resuspended in 2 M PCA and incubated at 70^{o}C for 1 h before centrifugation and removal of the supernatant for quantification of DNA by spectrophotometry.

Chapter 3 - Development of Methods Using D_2O in the Measurement of Protein, RNA and DNA Synthesis

3.1 Introduction

To understand changes in muscle mass, whether intentional through exercise and nutritional interventions (Volek et al. 2013), or unintentional such as the detrimental effects of disease, bed rest and malnutrition (Kortebein et al. 2007; Keys et al. 1945; Rutten et al. 2006), an array of methodologies are essential. Changes in muscle mass are held in the balance between MPS and MPB with great effort invested in understanding the control of these two processes (Atherton & Smith 2012). Many techniques such as immunoblotting, mRNA expression, biochemical composition and assays etc. are all routine used in the study of muscle physiology, however to some extent these methods only provide 'snapshots' of activity at any given time point and so are somewhat limited.

As discussed in section 1.4.1, muscle growth primarily occurs through sustained increases in MPS that increase contractile proteins, fiber CSA and therefore whole muscle volume (Wilkinson et al. 2006). However, in order for 'optimum' MPS and hypertrophy there is a consortium of contributing factors with the necessity and interplay of these unclear. For instance, muscle hypertrophy has been shown to occur both with and without the addition of myonuclei (McCarthy et al. 2011), questioning the role satellite cells play in muscle growth. Similarly, RNA content, representing the synthetic capacity of a muscle (Millward et al. 1973) substantially increases during overload-induced hypertrophy (Nakada et

al. 2016) and has suggested to be key in the extent of muscle hypertrophy (Stec et al. 2016). Yet, if increases in myonuclei and synthetic capacity are essential or limit muscle hypertrophy are incompletely defined, with current methodological practices limiting further advances.

In order to further shed light on these processes the continual development of dynamic measures of muscle metabolism is of great importance, as these not only provide information on the rates of biosynthetic processes, but also the flow of substrates through pathways (Wolfe & Chinkes 2005). MPS is routinely measured in acute settings using stable isotope labeled amino acid tracers, with much development and validation performed over the past 50 years (Wolfe & Chinkes 2005). Measures of DNA and RNA synthesis using isotope tracers are lagging; restricted due to significant problems in the interpretation of incorporation, in that the actual precursor pools for DNA and RNA synthesis are not fully understood (Grimble et al. 2000). Additionally, in adult skeletal muscle, synthesis of new DNA is thought to be little to none, whilst that of RNA pools relatively unknown (Schultz et al. 1978). The length of isotope infusion studies is limited to <24h and thus the ability to achieve significant detectable enrichment in such slow turnover fractions is a problem. As discussed in section 1.8.1, the re development of methods using deuterium oxide provides new avenues in the measurement of MPS and further that of other biosynthetic pathways (Belloto et al. 2007; Hellerstein 1999). The ability to easily maintain body water enrichment

over weeks particularly lends itself to the measurement of slow turnover fractions such as DNA and RNA.

There are multiple avenues in which stable isotope labels can be incorporated during the synthesis of DNA and RNA (Grimble et al. 2000). However caution must be taken as each subunit will have different precursor pool behaviors and follow different incorporation kinetics. For instance, nucleoside precursors are salvaged from degraded cellular material in which labeled nucleoside analogues can be incorporated. However, these analogues are often harmful and salvage pathways are unpredictable between tissues with pyrimidine nucleotides affected by extracellular concentrations (Cohens et al. 1983; Martini et al. 2004). Alternatively stable isotope labels can be incorporated into DNA and RNA by de novo synthesis. The most attractive route is particularly that of purine de novo synthesis, in which 80-90% of deoxyribose arises from extracellular glucose, is not effected by extracellular nucleotide concentrations and is consistent across a range of tissues (Neese et al. 2002; Macallan et al. 1998). During nucleotide synthesis deuterium is in incorporated into all nucleosides, and in any carbon bound hydrogen that exchanges with body water. Specifically, all hydrogen's in deoxyribose are labeled during de novo synthesis from glucose, with additional deuterium incorporated into newly synthesized nucleobases (Neese et al. 2001). Further, the plateau incorporation of a fully turned over cell population can be used to represent the precursor pool negating the need for direct measurement (Neese et al. 2002). Using these principles, measures of

cellular proliferation have been made with purine deoxyribose, however thus far these methods routinely rely on NCI GC-MS and therefore require high body water enrichments and cellular populations of fast turnover rates (Kim et al. 2005; Hayes et al. 2010; Chen et al. 2007).

The application of D_2O in humans to measure muscle metabolism is still relatively new. Measures of human MPS are becoming more frequent, whilst those of muscle DNA and RNA synthesis minimal. In planning such studies there are many methodological aspects to consider such as appropriate D_2O administration determined by analytical sensitivity and rate of substrate turnover. Initial studies used burdensome D_2O loading and GC-MS techniques that lack temporal resolution and are limited in detection of nucleotide synthesis (Gasier et al. 2011; Robinson et al. 2011). Combining studies with IRMS and GC-MS/MS techniques offers increased sensitivity up to 100-fold for measuring tissue isotopic enrichment compared with GC-MS. Thus, our objectives were to i) investigate D_2O dosing kinecits and the measurement of MPS ii) develop sensitive methods in the dual measurement in the synthesis of DNA and RNA.

3.2 Methods

3.2.1 Determination of D_2O body water and plasma alanine kinetics

To determine the acute equilibration of D2O with saliva, plasma water and alanine, subjects consumed 150 ml of 70% D_2O with regular saliva and blood samples were collected regularly thereafter. Subjects were informed not to have recently (>30 min) consumed any food or drink to avoid contamination of saliva samples. Blood was collected into lithium heparin tubes and the deuterium enrichment determined in saliva and plasma was measured as previously describe in section 2.7.

To determine the deuterium incorporation into plasma alanine, 5 mls of ice-cold ethanol was added to 1 ml of plasma and placed at 4^{o}C for 20 minutes to precipitate proteins. Proteins were pelleted by centrifugation at 1700g for 5 min and the supernatant containing AA decanted into a boiling tube and evaporated to dryness. 300 µl of 0.5 M HCL was added and vortex mixed to dissolve samples. Lipids were extracted by vortex mixing upon the addition of 2ml of ethyl acetate and removal of the upper layer. AA were the evaporated to dryness and derivatised to MCME's and measured using GC-*pyr*-IRMS as previously described in section 2.7.

3.2.2 Cell culture

Murine C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Lonza BioWhittaker, Ver- viers,

Belgium), penicillin (100 U/ml), streptomycin (100 ug/ml), and amphotericin B (250 mg/ml; all from Sigma-Aldrich, Poole, UK) at 37°C and in a 5% CO2 atmosphere. Proliferating myoblasts were seeded onto six-well plates (Nunclon Delta; Thermo Scientific), and after 2–4 days (at ~95% confluency), the medium was switched to differentiation medium consisting of DMEM containing 2% (vol/vol) horse serum (Sigma-Aldrich) as well as the above mixture of antimycotics and antibiotics to induce differentiation of the myoblasts into multinucleated myotubes. Differentiation medium was changed every 24 h, and experiments were performed on days 4 and 5 post induction of differentiation. To stimulate DNA and RNA synthesis IGF1 was used at a final concentration of 50 $ng.ml^{-1}$. The DNA synthesis inhibitor cytosine arabinoside (AraC) was used at a final concentration of 10μM and The RNA synthesis inhibitor CX5461 at 1μM.

3.2.3 Animals

All experimental procedures described in this study protocol are approved by the Animal Care and Use Committee of the Southern Finland, license number ESAVI-2010-07989/Ym-23, STH 534A (21.9.2010) and complements ESAVI/1968/04.10.03/2011, PH308A (30.3.2011) & ESAVI/722/04.10.07/2013, PH275A (1.3.2013). The tests done to animals before arriving at Finland were approved by the Animal Care and Use Committee at the University of Michigan. All experiments are conducted in accordance with the Guidelines of the European Community Council directive 86/609/EEC. All additional stress of animals is avoided. In the procedures, handling and daily care of animals suffering is minimized and

welfare is enhanced by all possible means, and all persons responsible for animal experiments are accordingly educated as recommended by FELASA (Nevalainen et al., 2002,Weiss et al., 2010). Rats were kept in air-conditioned rooms at an ambient temperature of 21 ±2 °C and the relative humidity at 50 ± 10 %. Artificial lighting provided light cycles of 12 h light/12 h total darkness. Rats were provided with a commercially available pelleted rodent diet (R36, Labfor/Lantmännen, Malmö, Sweden) available *ad libitum* for rats throughout the study. The energy content of the feed was 1260 kJ/100 g (300.93 kcal/10 0g). The feed contained raw protein 18.5%, raw fat 4.0%, NFE (nitrogen free extracts) 55.7%, fiber 3.5%, ash 6.3%, and water <12%. The tap water (from the municipal water system of Jyväskylä) was available *ad libitum and* the water in the bottles changed to clean every 3rd day and the bottles every second week. Feed was changed at a rate the rat was consuming it. High Intensity Training (HIT) consisted of Warm-up for 5 min, at 50-60% of VO2max (individually defined VO2 value and speed for each rat) + running for 15 min: 3 min at 85 - 90%, 2 min pauses at 50%, repeated for 3 times; inclination 15o uphill. Training was done 3 times per week, with one day rest between (if possible). Animals were killed 48 hrs after the last HIT-training bout. Animals were anaesthetized with carbon dioxide and killed by cardiac puncture and thereafter immediately necropsied. Left quadriceps were removed and frozen in liquid nitrogen. Rats received a gavage of 7.2 mls/kg of 70% D_2O at the start of the study, with drinking water enriched to 1.4% to maintain body water enrichment over the study period.

3.2.4 Plasma body water, alanine and muscle bound enrichment.

D_2O enrichment of blood plasma was measured using acetone exchange method. 10N NaOH and acetone was added to the sample and vortex mixed for 15s. Following 24h incubation at room temperature, to allow the exchange of hydrogen atoms for deuterium, acetone was extracted into n-heptane and transferred to an auto sampler vial and capped. Deuterium exchanged acetone was determined by GC-MS/MS, alongside a standard curve of known D2O enrichments. Plasma alanine was extracted as described in section 3.2.1. With the resulting MCME derivatives analysed by GC-MS/MS. Muscle myofibrillar, collagen and mitochondrial proteins were extracted by homogenizing ~50 mg of muscle in ice-cold homogenization buffer pH7.5 (Tris-HCL 50mM, EDTA 1mM, EGTA 1mM, B-glycerophosphate 10mM, NaF 50mM) containing a protease inhibitor tablet (Roche) and sodium orthavanadate 0.5mM, rotated for 10 min. The supernatant containing sarcoplasmic proteins was collected after centrifugation at 13,000 *g* for 5 min at 4°C and proteins precipitated using 1M of perchloric acid (PCA). After washing, the remaining pellet was dounce homogenized in mitochondrial extraction buffer (MOPS 20mM, KCl 110mM and EGTA 1mM) and spun at 1000 g for 5 min to separate mitochondria from myofibrillar and collagen fractions. The supernatant containing mitochondria was removed and pelleted by centrifugation at 11000g. Myofibrillar proteins were extracted from myofibrillar and collagen fractions by solubilizing in 0.3M NaOH and separated from the insoluble collagen by centrifugation, with myofibrillar proteins

precipitated using 1M of perchloric acid (PCA). Myofibrillar, mitochondrial and sarcoplasmic protein-bound AA were released using acid hydrolysis by incubating in 0.1M HCl in Dowex H^+ resin slurry overnight before being washed and eluted from the resin with 2M NH_4OH and evaporated to dryness. Resulting AA were derivatised to MCME's and analyzed on GC-MS/MS.

3.2.5 Peripheral blood mononuclear cell extraction

Peripheral blood mononuclear cells (PBMC's) were extracted using histopaque 1077 (Sigma). Specifically, 3 ml of histopaque was placed in a 15 ml falcon tube and allowed to rise to room temperature. 3 ml of whole blood collected into lithium heparin tubes was layered on top of the histopaque and centrifuged at 400 g for 30 min. The opaque interface containing mononuclear cells was removed to a fresh 15ml falcon tube. 10mls of ice cold PBS was added to wash the mononuclear cells and centrifuged at 2000g to pellet cells and repeated. Cell pellets were subsequently frozen at -80^{o}C

3.2.6 DNA extraction, digestion and derivatisation

To extract DNA ~20-30mg of muscle was homogenized in extraction buffer (5µl/mg) containing 0.1M Tris-HCL pH 8, 0.01M EDTA pH 8 and 1M NaCL. Proteinase K was added to a final concentration of 50µg/µl and placed at 55^{o}C for ~2hrs with occasional mixing until complete digestion had occurred. For cell culture each well was scraped in 200 µl of extraction buffer and PBMC pellets resuspend in 200 µl extraction buffer, both

without proteinase K. To the extractions an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, inverted several times to mix and the upper aqueous layer removed to a clean eppendorf after centrifugation at 13000 rpm for 10 min. To remove additional protein an equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous layer and repeated as above. To precipitate DNA and RNA an equal volume of isopropanol was added to the aqueous layer, inverted several times and centrifuged at 13000 rpm for 20 min. The pellet was washed three times in 70% ethanol; air-dried, re suspended in 22 µl of molecular biology water and quantified using nanodrop. 20 µl of combined DNA and RNA was digested with 5 µl of 375 mM sodium acetate (pH 4.8), 750 µM $ZnSO_4$ containing 0.5 units of nuclease S1 and 0.25 units potato acid phosphatase and placed at 37^{o}C overnight. Hydrolysates were then reacted with 10 µl of benzyl hyrdoxylamine (2% w/v) and 7.5 µl of acetic acid at 100^{o}C for 30 min. Samples were then cooled at room temperature before the addition of 10µl of 1-methyimidazole and 100 µl of acetic anhydride. This reaction rapidly produces heat and samples are cooled at room temperature for ~ 20 min. The reactions are transferred to a boiling tubed and quenched by the addition of 2 ml ddH2O. The newly formed derivatives were extracted by the addition of 750 µl dichloromethane (DCM) vortex mixed and phases allowed to separate. By pre wetting the tip with DCM, the lower layer is removed to a clean boiling tube and the procedure repeated. DCM extracts were then dried and re suspended in 40 µl of ethyl acetate for GC-MS/MS analysis.

3.2.7 GC-MS/MS instrument conditions

To measure DNA and RNA enrichment 2 µl of sample was injected into a trace 1310 gas chromatograph connected to TSQ 8000 triple quadrupole GC-MS/MS (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK). Samples were injected on splitless mode with inlet temperature at 280^{o}C. GC ramp conditions were 120^{o}C for 1 min, ramp to 280 ^{o}C at 10 ^{o}C /min and hold for 3 min. The benzyl hydroxylamine triacetate of deoxyribose was measured on an SRM of 203 to 82 with collision energy of 6. The benzyl hydroxylamine tetraacetate of ribose was measured on an SRM of 273-111 with collision energy 6. Samples were run in triplicate alongside standard curves of known deoxyribose and ribose standards. The average of both peaks were used in the results. Additionally, unlabeled samples were injected in different quantities to determine abundance effects.

3.2.8 Statistical Analyses

Descriptive statistics were produced for all data sets to check for normal distribution (accepted if $P>0.05$) using a Kolmogorov-Smirnov test. All data are presented as means ± SEM. Differences between plasma alanine ratios and the effects of HIIT on DNA and RNA synthesis were analyzed by t-test. Elimination rates were determined by fitting a one phase decay and rise to plateau by one phase association. All other data sets were analyzed by repeated measures one-way or two-way ANOVA with a Bonferroni correction using GraphPad Prism (La Jolla, CA) Software Version 5. Correlations were assessed using Pearson's product moment correlation coefficient. The α level of significance was set at $P<0.05$.

3.3 Results: Development of methods using D_2O in the measurement of protein synthesis

3.3.1 Pilot to determine D_2O dosing for MPS detection

A pilot study was performed to assess changes in body water enrichment in response to a moderate bolus of D_2O and further if this is sufficient to detect MPS. One subject consumed a 150 ml bolus of 70-atom% D_2O, enriching body water to 0.15% and gradually declining to 0.043% after 16 days. In contrast, another subject consuming 50 $mls.d^{-1}$ resulted in a progressive and continual increase in body water enrichment from 0.12% to 0.45% (Fig 3.1). After 7 days of increased body water enrichment muscle ^2H APE increased to 0.0021% in bolus and 0.0038% in continuous with both giving similar muscle fractional synthetic rates of ~1 $\%.d^{-1}$ (Table 3.1).

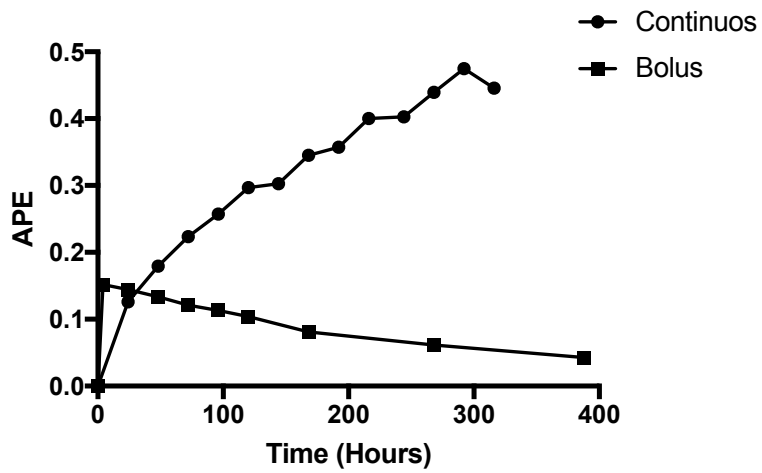


Figure 3.1 APE of deuterium in the body water measured in saliva via TC-EA IRMS after either continuous or bolus administration of 70-atom% D_2O. Continuous 50 $mls.d^{-1}$ of D_2O vs. bolus 150 ml D_2O on day 0

	d^2H	Muscle Alanine APE	Body Water Average APE	FSR (%/d)
Subject 1	131.687	0.0021	*0.147*	1.05
Subject 2	244.232	0.0038	*0.262*	1.09

Table 3.1 APE of deuterium in *vastus lateralis* muscle biopsies taken from subject 1 and 2 after 7 days of either bolus or continuous labeling.

3.4.2 D_2O equilibration in saliva, plasma water and plasma alanine

Once we had shown MPS rates could be determined from a single 150 ml bolus of 70-atom% D_2O (Section 3.4.1), we next investigated the acute equilibration of D_2O with body water and plasma alanine. Regular blood and saliva samples were collected after 5 subjects consumed 150 ml 70-atom% D_2O. 1 hour after D_2O consumption saliva plasma water enrichment increased to 0.216±0.013%, plasma water to 0.197±0.013% and corrected plasma alanine (x 11/3.7) to 0.171±0.016%. The enrichment in plasma alanine was significantly different than that of saliva after 1 hour ($P<0.05$). At 2 and 4 hrs full equilibration had taken place being 0.205±0.01%, 0.206±0.01% and 0.197±0.01% in saliva water, plasma water and plasma alanine with no difference between the measures.

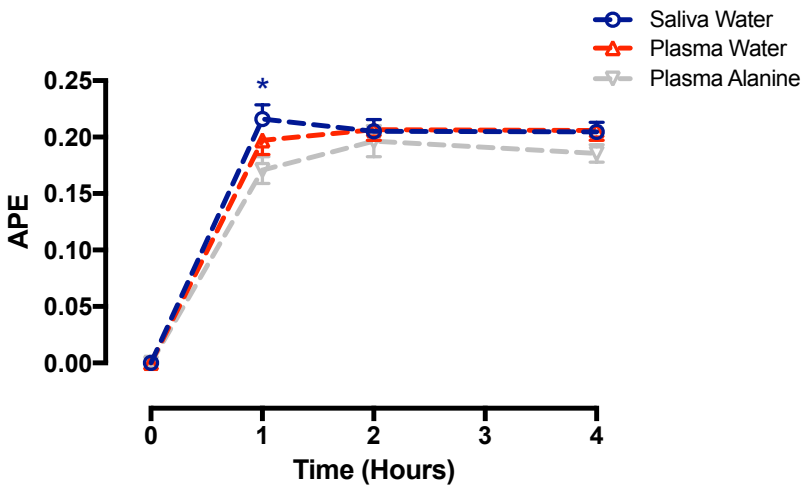


Figure 3.2 Time course of deuterium APE measured in saliva, plasma water and plasma alanine after consumption of a 150 ml bolus of 70-atom% D_2O. * Significantly different than that of plasma alanine $P<0.05$.

3.4.3 Validation of saliva enrichment in the representation of plasma alanine

After determining the acute equilibration of saliva, plasma water and plasma alanine (section 3.4.2) we next looked if this relationship remained constant over the course of a bolus of D_2O. The elimination rate of saliva and plasma water enrichment mirrored that of plasma alanine being $0.0042.h^{-1}$, $0.0038.h^{-1}$ and $0.0041.h^{-1}$ respectively. Elimination rate constants were not significantly different between groups such that one curve is applicable to all data sets being $0.0041.h^{-1}$ (Fig 3.3). Further log transformation of the slopes revealed no significant differences such that one slope could represent all data, with a pooled slope -0.00431 (Fig 3.4).

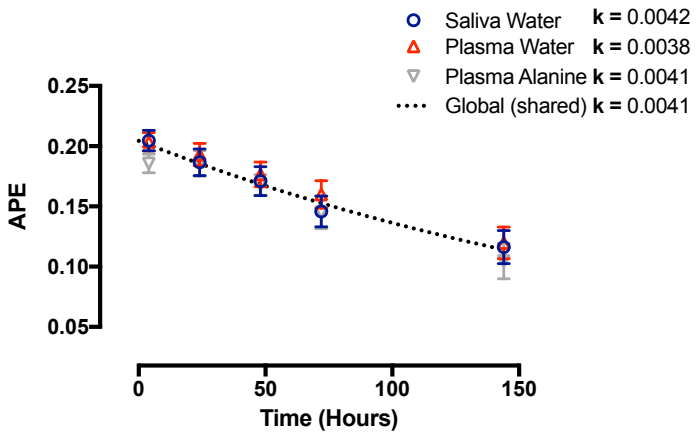


Figure 3.3 Time course of deuterium APE in saliva, plasma water and plasma alanine (x 3.7/11) after 150 ml bolus 70-atom % D_2O.

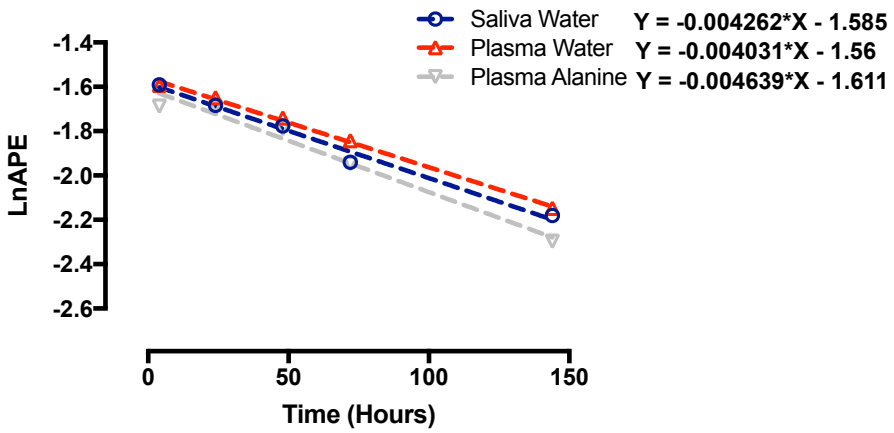


Figure 3.4 Natural log of the time course of deuterium APE in saliva, plasma water and plasma alanine (x 3.7/11) after 150 ml bolus 70-atom % D_2O

3.4.4 Long term relationship between plasma water and plasma alanine

After showing over 1 week saliva enrichment reflects that of plasma water and plasma alanine in humans (Section 3.4.3), we next investigated if this relationship remains long term in rats. Plasma ^{2}H enrichment in rats receiving continuous D_2O in drinking water remained constant from 3 to 6 weeks at 0.67±0.1% and 066±0.1% respectively. Additionally alanine enrichment remained constant over 3 to 6 weeks of labeling being 2.61±0.03% and 2.57±0.04% respectively. The ratio between body water enrichment and plasma alanine enrichment remained constant throughout being 3.84±0.03 and 3.89±0.05 at 3 and 6 weeks

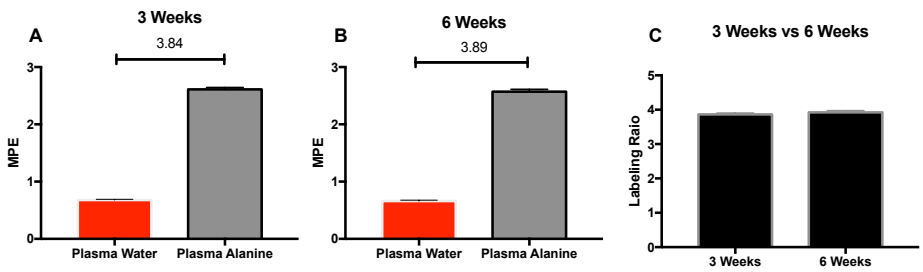


Figure 3.5 Mole percent excess of plasma body water and plasma alanine in rats at **A)** 3 weeks **B)** 6 weeks and the labeling ratio **C)** between plasma water and plasma alanine at 3 and 6 weeks.

3.4.3 Non linear calculations in the determining long term MPS

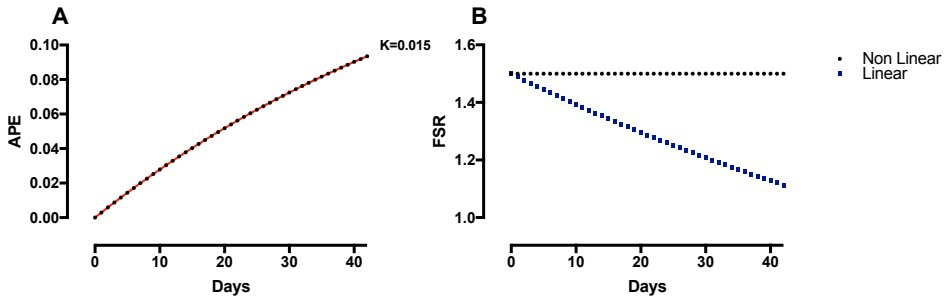


Figure 3.6 A) Time course of theoretical protein enrichment based on the exponential rise curve equation $Eb = 0.2(1 - e^{-0.015t})$ with body water enrichment at a constant 0.2 and FSR 1.5 %d^{-1}. **B)** FSR of **A** calculated using equation 4 (linear) and equation 5 (non Linear)

Protein synthesis can be described of as a zero order reaction, with synthesis thought as a constant amount process in the sense that its rate is independent of the product pool. This does not mean that the rate of synthesis is not altered by other influences and it is well demonstrated that it is by food and activity etc (Atherton & Smith 2012). If protein synthesis is generally seen as a constant amount process, then the incorporation of stable isotope kinetics will follow exponential kinetics. This is most easily described in a steady state (Fig 3.6), in which protein synthesis is occurring at 1.5 %.d^{-1} with a constant precursor enrichment of 0.2%. Here the change in isotopic enrichment can be described as:

Equation 1

$$\frac{dEb}{dt} = K_s Ep - K_b Eb$$

where the change in bound enrichment with time (dEb/dt) is equal to rate of entry (K_s) and enrichment of the precursor (Ep) minus the rate of loss (Kb) of bound enrichment. In the steady state synthesis and breakdown are presumed to be equal and so equation 1 can be expressed as:

Equation 2

$$\frac{dEb}{dt} = K_s(Ep - Eb)$$

Skeletal muscle proteins are relatively long lived, with synthesis and breakdown occurring ~1-2 %.d^{-1} (Phillips et al. 1997; MacDonald et al. 2013). Acute stable isotope experiments are performed over a short period of time where the precursor enrichment (Ep) is much greater than the bound enrichment (Eb). It has traditionally therefore been assumed that the loss of labeled amino acids from the pool over this time period is insignificant and is excluded from equation 2 forming:

Equation 3

$$\frac{dEb}{dt} = K_s Ep$$

Integration and rearrangement to enable calculation of the FSR using precursor product calculations yields:

Equation 4

$$Ks = \frac{Eb}{\int_{t0}^{t1} Ep\ (t)dt}$$

Using equation 4 to calculate *Ks* from figure 3.6A underestimates the true rate, which is held at constant 1.5%.d^{-1}. After 1 day equation 4 calculates *ks* at 1.48%.d^{-1} and progressively declines to 1.1%.d^{-1} after 42 days. Increasing the length of isotope tracer's experiments leads to a significant build up in *Eb* that is progressively released by breakdown and must therefore be taken into account with integration of equation 2 yielding:

Equation 5

$$Eb = Ep(1 - e^{-Kst})$$

Equation 5 can be used to estimate *Ks* when regular samples are collected and in the case of figure 3.6A when ideal data is used the calculated *Ks* is 1.5. Equation 5 can be further rearranged to solve for Ks using precursor product calculations enabling precursor product calculations when only two time points are available:

Equation 6

$$ks = -Ln\left(\frac{1 - \frac{Eb}{Ep}}{t}\right)$$

Using equation 6 to calculate the rate of the rise in protein bound enrichment gives an FSR of 1.5 %.d^{-1}, which remains constant over the 42 days

The equations described above were applied to experimental points obtained from quadriceps of rats maintained at constant body water enrichment. Applying equation 5, the turnover rate was 2.7 %.d^{-1}, with a predicted plateau of 1.01 (Fig 3.7A). Using non-linear precursor product calculations, the turnover rate was 2.75±0.07 %.d^{-1} at 3 and 2.82±0.1 %.d^{-1} at 6 weeks not significantly different. (Fig 3.7B) Applying linear calculations to the same data set turnover rates are 2.11±0.05 %.d^{-1} at 3 weeks and 1.65±0.03 %.d^{-1} at 6 weeks, being significantly different than 3 weeks (P<0.001). The values calculated using linear equations were significantly lower than non-linear (P<0.001).

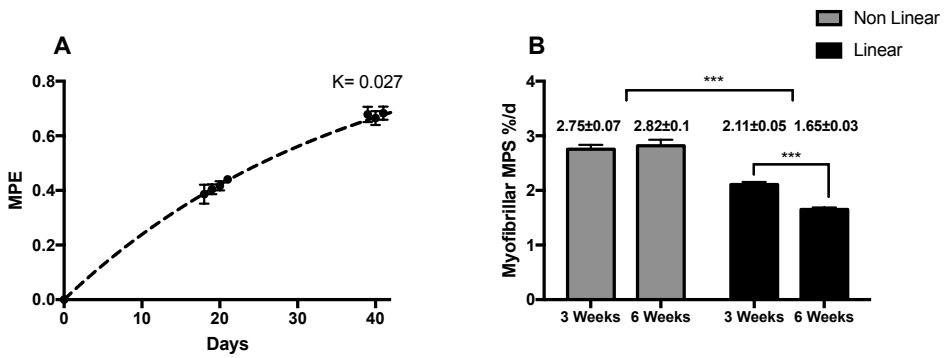


Figure 3.7 A) Fraction new of protein bound alanine from rat quadriceps maintained at constant body water **B)** Myofibrillar muscle protein synthetic rate calculated using linear and non linear precursor product calculations. * P<0.001.

3.5 Results: Development of a method using D_2O in the measurement of DNA and RNA synthesis

3.5.1 Extraction, digestion and derivatisation

Total nucleotides were extracted via phenol-chloroform-isoamyl alcohol, resuspended in molecular biology water and checked for quantity and quality using a Nano drop spectrophotometer. Typical quantities were ~1 ug.mg^{-1} from muscle tissue with a ratio between 1.8-2.0 confirming pure DNA and RNA. Agarose gel electrophoresis was used to confirm effective digestion of DNA/RNA, with the absence of SYBR safe signal confirming complete digestion. Dexoynucleoside standards subjected to identical digestion and derivatisation procedures confirming that deoxyribose is primarily released from purine nucleotides, contributing to 97% of total ribose measured. Similarly nucleoside standards showed that ribose from the purine nucleoside contributes 98 % adenosine and guanosine

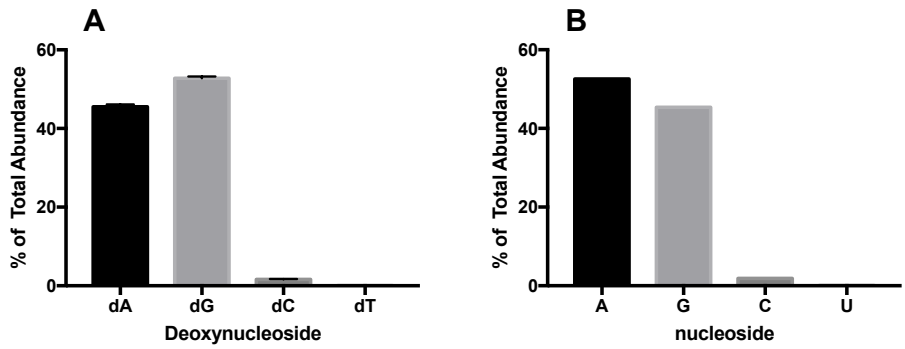


Figure 3.8 A) The percentage of A) deoxyribose and B) ribose from each nucleoside that contributes to total signal after digestion and derivatisation.

Figure 3.9 shows typical ion chromatograms for SRM transitions for the M and M+1 ions of deoxyribose and ribose of a human muscle sample. Due to the anomeric carbon and nucleophilic attack by benzyl hydroxylamine, cis-trans isomers are formed for both that of ribose and deoxyribose (Becker et al. 2013). The extra hydroxyl group of ribose forms a tetraacetate and is larger than the triacetate of deoxyribose that are easily separated upon gas chromatography. Isotopically labeled deoxyribose and ribose were used to confirm the ion peaks and progression of the label through individual SRM transitions. Additionally, C2C12's cultured in media containing U-13C6 glucose showed a progressive increase in the M+5 isotopomer in both ribose and deoxyribose confirming the presence of all back bone carbons (Fig 3.10)

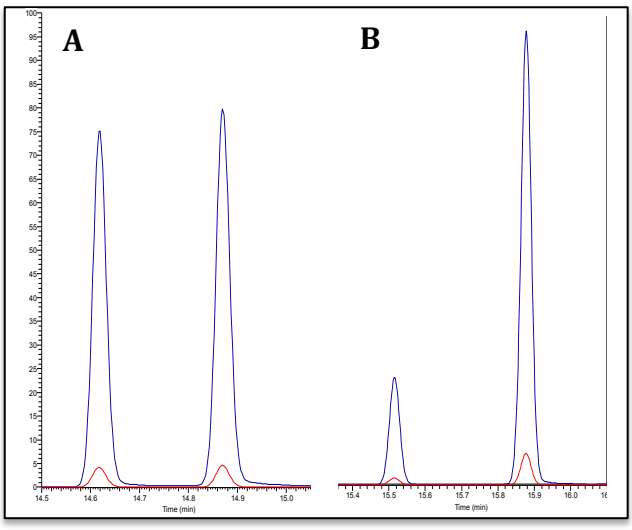


Figure 3.9 Typical GC-MS/MS chromatogram of BHTA derivative of dR and R on a DB-17 column for the SRM transitions of **A)** deoxyribose (203-85) and B) ribose (273-111). Blue line represents the M and red the M+1

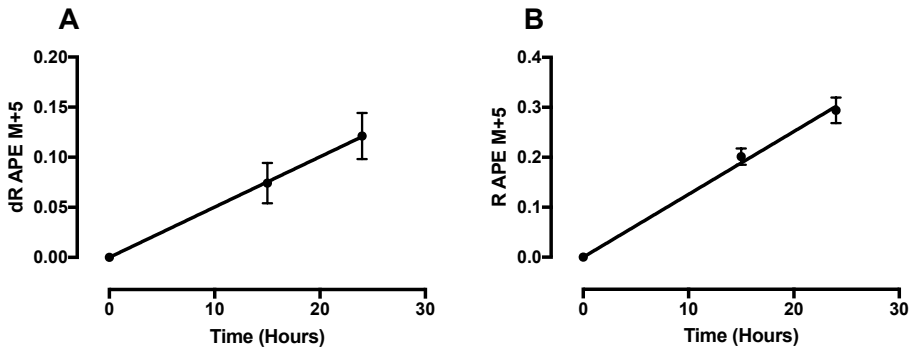


Figure 3.10 The APE of the M+5 isotopomer of **A)** deoxyribose and **B)** ribose of DNA and RNA extracted from C2C12's in U-13C6 glucose enriched media

3.5.2 Calculation of deuterium amplification into DNA and RNA

In section 3.5.1 we have shown our method primarily measures ribose and deoxyribose from that of purine nucleotides. To determine how much deuterium is incorporated into these ribose moieties, nucleotides were extracted from C2C12s grown at a range of D_2O concentrations. The mole percent excess of deoxyribose (Fig 3.11 A) extracted from purine DNA increased linearly with increasing concentrations of media D_2O enrichment being 0%, 2.7±0.03%, 5.9±0.04%, 11.6±0.07% and 22.5±0.09% at media concentrations of 0% 0.67%, 1.9%, 4.6% and 8.9% respectively. Linear regression revealed that on average 3.1 ^2H were incorporated into purine deoxribose during synthesis of new DNA. The mole percent excess of ribose (Fig 3.11 B) extracted from purine RNA increased linearly with increasing concentrations of media D_2O enrichment being 0%, 1.6±0.08%, 4.1±0.1%, 9.5±0.15%, 18.8±0.18% at media concentrations of 0% 0.67%, 1.9%, 4.6% and 8.9% respectively. Linear regression revealed that on average 2.1 ^2H are incorporated into purine ribose during synthesis of new RNA. The mole percent excess of deoxyribose and ribose SRM transitions were compared against that of whole deoxyadeoninse (Fig 3.11 C) and adenosine (Fig 3.11 D) and showed excellent agreement with an r^2 of 1 for deoxyadenosine and 0.99 for adenosine

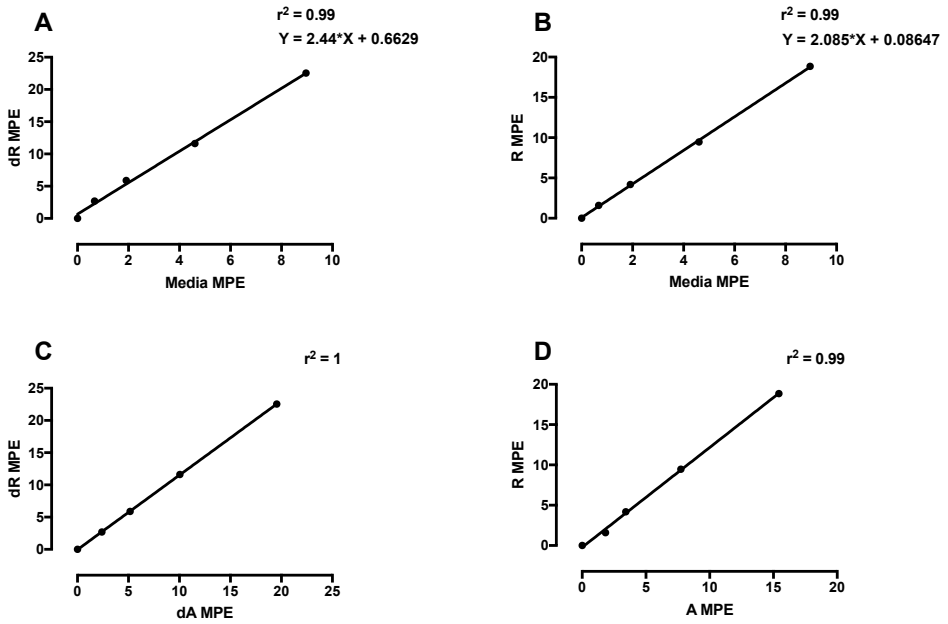


Figure 3.11 MPE of deuterium in A) deoxyribose and **B)** ribose at increasing concentrations of media D_2O enrichment. MPE of deuterium in C) deoxyribose vs. deoxyadenosine and **D)** ribose vs. adenosine at increasing concentrations of media enrichment

3.5.3 Validation of D_2O incorporation in determining cell division

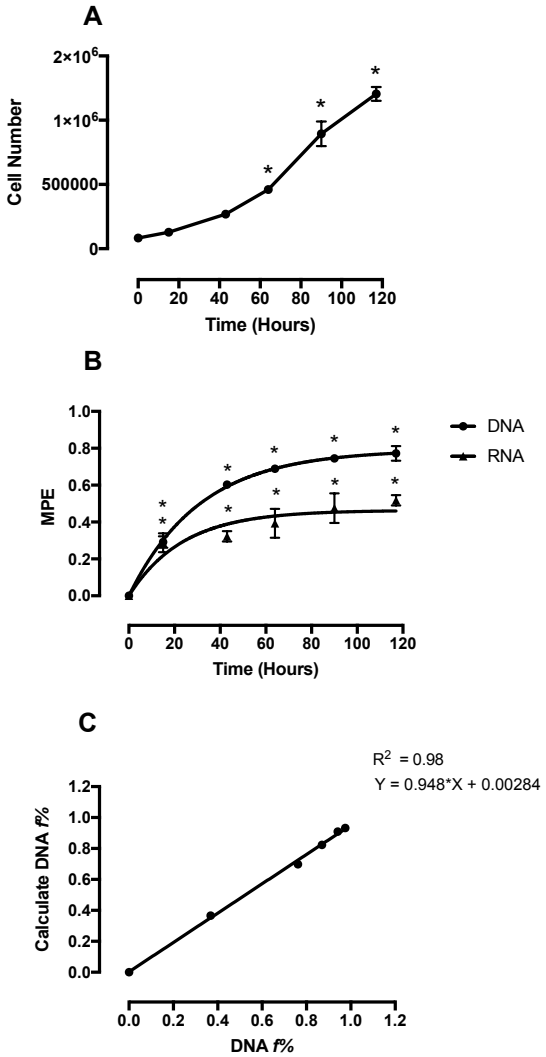


Figure 3.12 Time course of **A)** cell number **B)** MPE of DNA and RNA in proliferating C2C12's and **C)** the fraction of new DNA vs. the fraction of new DNA calculated by replication number. * Significantly different from baseline $P<0.05$

We have confirmed that the amplification of deuterium into backbone hydrogen's is constant (Section 3.5.2) and therefore bound enrichment will be determined by body water enrichment and rate of

synthesis. To validate that our method is reflective of cellular proliferation and therefore DNA and RNA synthesis, D_2O incorporation was compared to cell proliferation. The average C2C12 number increased from 0.083±0.001 million cells per well to 1.2±0.03 million cells after 117 hrs of cell culture representing ~3.8 cell divisions in 5 days. The DNA MPE increase followed a rise to plateau relationship from 0.29±0.02% after 15 hrs to 0.77±0.02% by 117 hrs. Similarly the RNA MPE increased followed a rise to plateau relationship from 0.28±0.03% after 15 hrs to 0.52±0.02% by 117 hrs. The expected fraction new of DNA was predicted from the number of cell divisions and compared with the fraction of new DNA using precursor product relationship. The two methods showed excellent agreement with an R^2 of 0.98.

3.5.4 Stimulation of DNA and RNA synthesis

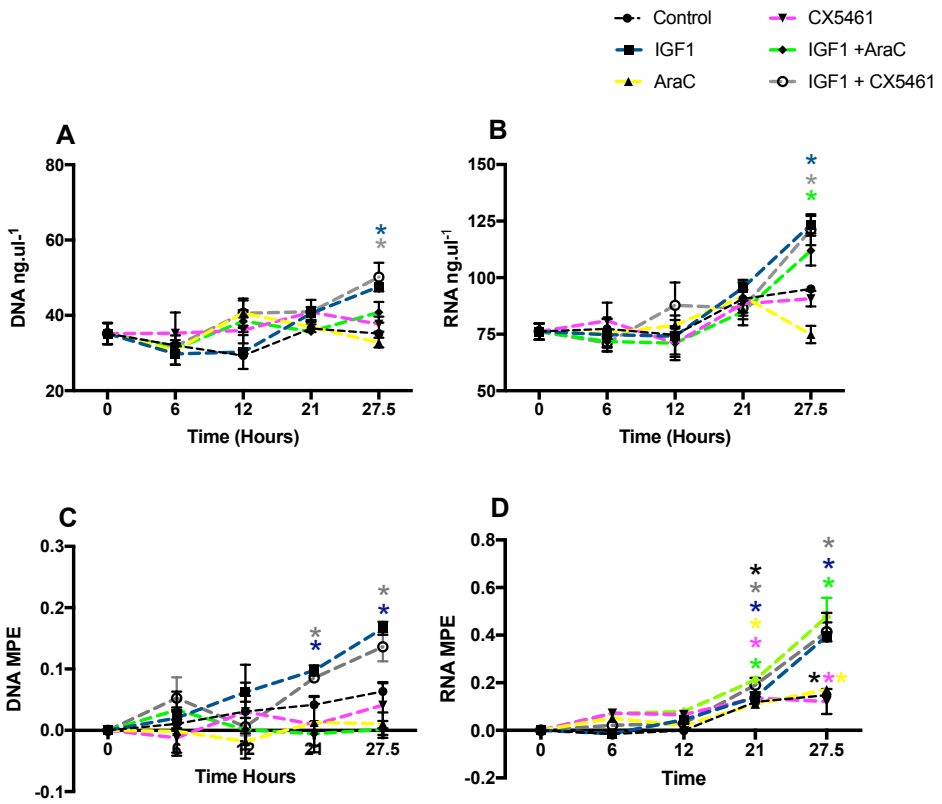


Figure 3.13 The time course of concentration of **A)** DNA **B)** RNA and the MPE in **C)** DNA **D)** RNA in non-treated condition (Control) and in responses to IGF1, AraC, CX5461, IGF1 + AraC and IGF1 + CX5461. * Significantly different from baseline $P<0.05$

We have confirmed that our method is reflective of the synthesis of new DNA and RNA (Section 3.5.3). To validate that changes in the rates of DNA and RNA synthesis can be detected differentiated C2C12's were treated with known stimulators and inhibitors of DNA and RNA synthesis (Fig 3.13). After 27.5hrs the DNA content in differentiated C2C12's increased in responses to IGF1 from 35.2±0.1

ng.ul^{-1} to 47.6±0.6 ng.ul^{-1} and in response to IGF1 + CX5461 to 50.8±0.3 ng.ul^{-1}. However treatment with the DNA synthesis inhibitor AraC prevented the IGF1 stimulated increase in DNA with no other significant changes throughout. After 27.5 hrs the RNA content increased in responses to IGF1 from 76.1±3 ng.ul^{-1} to 123.4±3 ng.ul^{-1} and in response to IGF1 + AraC to 111.9±6 ng.ul^{-1}. Treatment with the RNA synthesis inhibitor CX5461 did not prevent the IGF1 stimulated increase in RNA and increased to 121.1±6 ng.ul^{-1}. Matching the increases in concentration of DNA, the MPE of DNA increased to 0.16±0.01% in responses to IGF1 and to 0.14±0.02% in response to IGF1 and CX5461 with no significant increase in any other treatment. After 27.5 hrs the RNA MPE significantly increased in all groups to ~0.12%. At 27.5 hrs the RNA MPE increased in control, Arac, CX5461 to ~0.15% with the increase in IGF1, IGF1 + Arac and IGF1 + CX5461 significantly greater than the other treatments being 0.39±0.1%, 0.47±0.08% and 0.41±0.04% respectively

3.5.5 Measurement of DNA and RNA synthesis in muscle *in vivo*

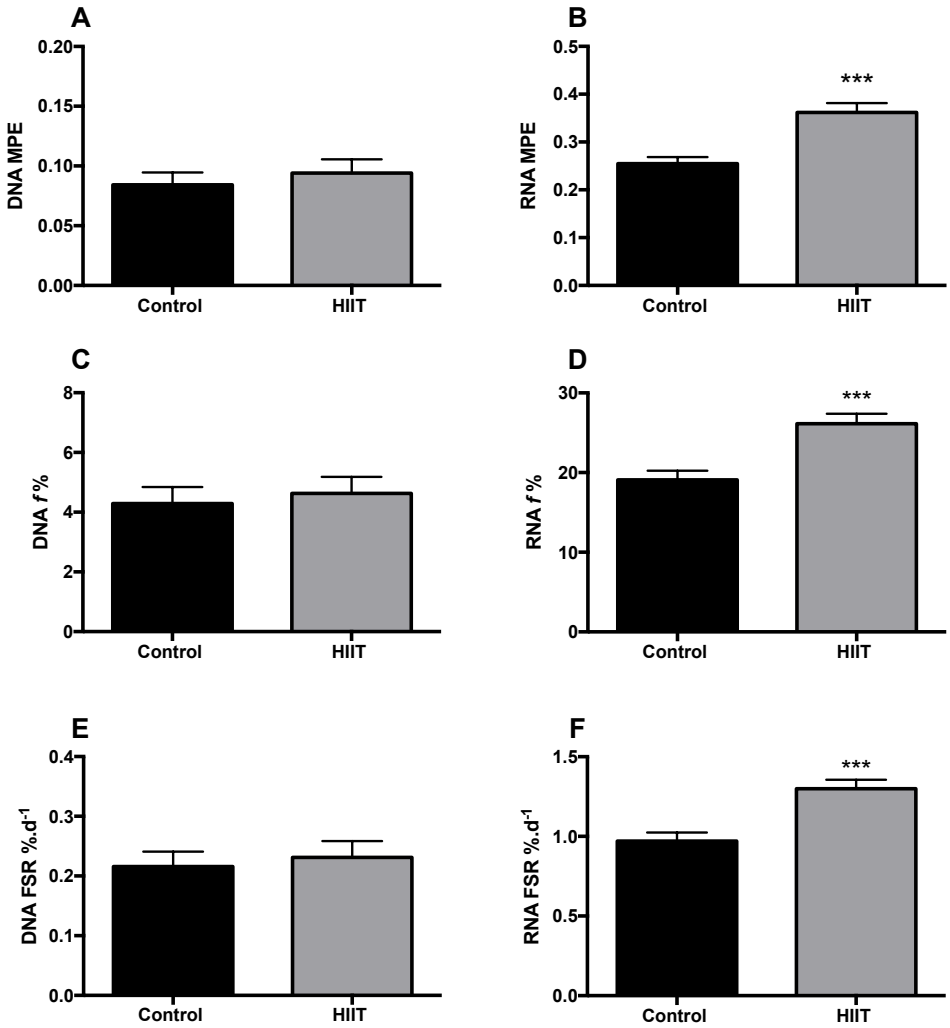


Figure 3.14 MPE of **A)** DNA **B)** RNA, fraction new of **C)** DNA **D)** RNA and FSR %.d^{-1} of **E)** DNA **F)** RNA in quadriceps of control and HIIT of rats. *P<0.001

We have shown the ability to accurately measure the synthesis of new DNA and RNA (Section 3.5.4). Next, we applied these methods *in vivo*, to determine if measurable DNA and RNA synthesis rates are achievable in mature muscle by long term body water enrichment in rats. After 3 weeks of continuous D_2O administration DNA MPE significantly increased to

0.084±0.01% with no additional effect of HIIT on the rate of incorporation being 0.094±0.01%. RNA MPE significantly increased to 0.25±0.01% and was significantly greater with HIIT exercise to 0.362±0.01%. The fraction of new DNA and RNA was calculated using the body water enrichment over the labeling period multiplied by the amplification values determined *in vitro* of 3.1 and 2.1 for DNA and RNA respectively. The fraction of new DNA over 3 weeks was 4.2±0.5% with no effect of HIIT running being 4.62±0.5%. The fraction of new RNA was 19.1±1% and was significantly increased with HIIT to 26.1±1%. The FSR %.d^{-1} of muscle quadriceps was 0.21±0.02 %.d^{-1} with no significant effect of HIIT at 0.23±0.03 %.d-1. RNA FSR %.d^{-1} was 0.97±0.05 %.d^{-1} with a significant increase in response to HIIT running exercise to 1.3±0.05 %.d^{-1}. The FSR %.d^{-1} of muscle subfractions from the same rats showed a main effect of HIIT exercise over the 3 weeks (Fig 3.15).

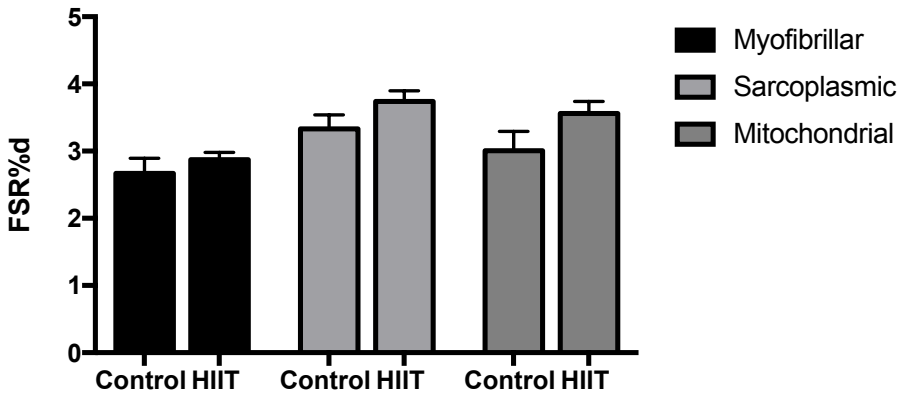


Figure 3.15 FSR %.d^{-1} of myofibrillar, sarcoplasmic and mitochondrial protein fractions from rat quadriceps in control and HIIT trained rats. Main effect of exercise P<0.05

3.5.6 Determination of DNA and RNA synthesis rates in humans

Having confirmed that we can measure the incorporation of deuterium into muscle *in vivo* (Section 3.5.4), we further tested out methods in humans. After 6 weeks continuous D_2O administration human blood peripheral blood mononuclear cells MPE increased to 0.37±0.04% at 3 weeks with no further increase at 6 weeks being 0.38±0.07% (Fig 3.16). The amplification of deuterium into dR at 3 and 6 weeks was 2.36±0.01% and 2.56±0.35% respectively, with no significant difference. Unfortunately measures of RNA enrichment were not made.

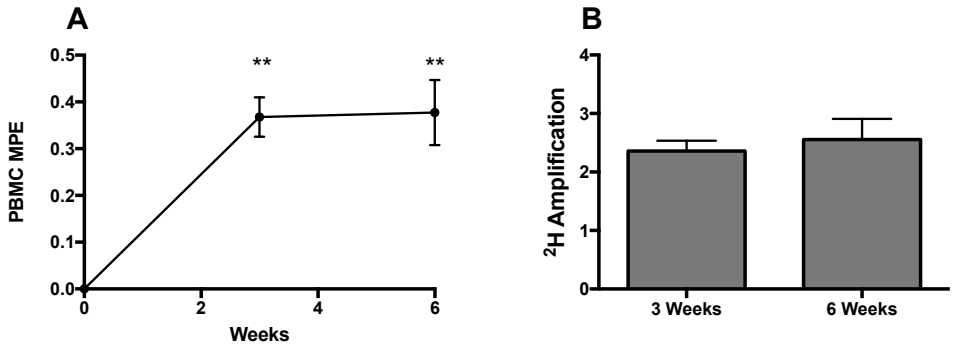


Figure 3.16 Time course of MPE in peripheral blood mononuclear cells after 3 and 6 weeks of continuous body water labeling. Significantly different from baseline ** P<0.01

The deuterium incorporation into DNA and RNA was measured from human *vastus lateralis* after continuous administration of D_2O (Fig 3.17). There was a progressive increases in the MPE in DNA from 0.022±0.22% to 0.035±0.22% at 3 and 6 weeks respectively. There was a significant

increase in RNA MPE at 3 weeks 0.071±0.01 to 0.14±0.02 at 3 and 6 weeks respectively

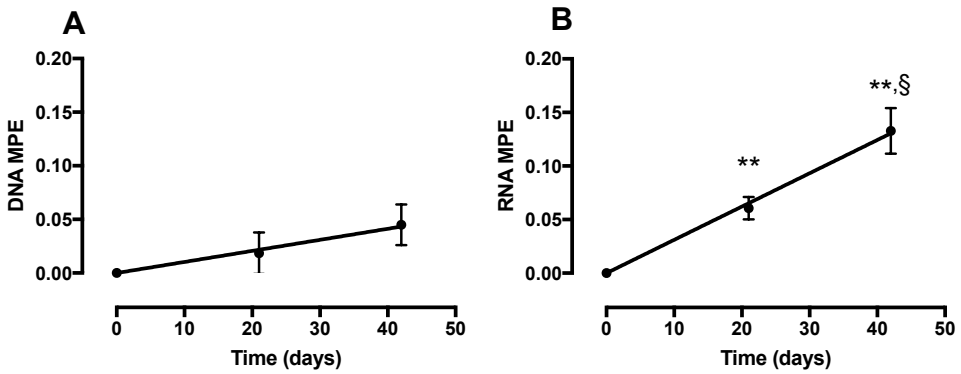


Figure 3.17 Time course of MPE in **A)** DNA and **B)** RNA from human *vastus lateralis* muscle

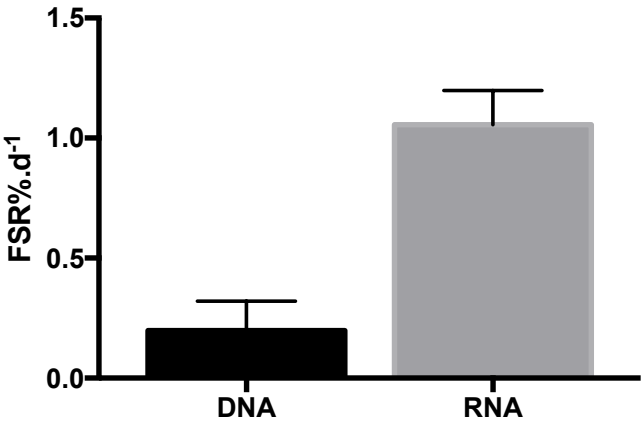


Figure 3.18 Fractional synthesis rates of DNA and RNA 0-6 weeks from human *vastus lateralis muscle*

An FSR %.d^{-1} was calculated using the 6 week MPE of DNA and RNA the average body water enrichment over the 6 weeks multiplied by 3.1 for DNA and 2.1 for RNA . The average rates of synthesis were 0.19±0.1 %.d^{-1} and 1±0.1 %.d^{-1} for DNA and RNA respectively.

3.6 Discussion

3.6.1 Application of D_2O in the measurement of MPS

The ability to make longer-term measures of MPS is of great importance in understanding the processes involved in growth and maintenance of muscle mass throughout life. D_2O has recently been re introduced in human muscle research (Gasier et al. 2012; Robinson et al. 2011), yet Relatively few measures of muscle metabolism have been made and as such defining optimal experimental approaches is in early stages. Here we have further validated the use of D_2O in the measurement of protein synthesis and developed a method for the simultaneous measurement of human muscle DNA and RNA synthesis.

A fundamental aspect of an isotope tracer study is the ability to detect the incorporation of the tracer into its biological end point and ultimately relies on the sensitivity of the analytical machine (Rennie 2007). It must be considered therefore what is the appropriate D_2O loading regime to make accurate measures of MPS. Using GC-MS, Robinson et al made measures of MPS over 6 weeks using 150 $ml.d^{-1}$ for the first week and 100 $ml.d^{-1}$ thereafter (Robinson et al. 2011). Similar, Gasier et al was able to make measure of MPS over 24 hrs using 300 ml of D_2O, although accurate data quantification was difficult due to analytical sensitivity (Gasier et al. 2012). Here, in a pilot study, using GC-pyr-IRMS we have shown that an accurate profile of body water enrichment can be made from either continuous or bolus ingestion of D_2O. Further we have shown that after single 150 ml bolus of D_2O, accurate measures of MPS can be made,

negating the need for daily consumption of D_2O that can be costly and cause nausea in subjects (Jones & Leatherdale 1991).

In order to make precursor product relationship calculations it is essential that a measure of the precursor is made (Wolfe & Chinkes 2005). Studies using D_2O in the measure of MPS typically rely on body water through that of saliva (Robinson et al. 2011). For the first time here we have shown the acute equilibration of body water with that of plasma alanine. After a bolus of D_2O there was no significant difference in normalized plasma alanine enrichment to that of body water 2-4 hrs after consumption. As such, sampling of saliva should wait at least 2 hrs after a bolus of D_2O. Further we have shown that the elimination rates of body water measured by saliva, plasma water or plasma alanine are equal such that saliva sampling alone represents that of body water in humans. Further we also showed that the labeling relationship between body water and plasma alanine was not altered in rats when body water is maintained for up to 6 weeks. As such these results demonstrate that plasma alanine levels are maintained during every day life and further supports the use of D_2O for long-term studies of protein turnover rates.

Body water is believed to be representative of intracellular amino acid enrichment as labeling occurs intracellulary (Gasier et al. 2010), with transamination rapid enough that any considerable flux in alanine pool size would immediately take on the body water enrichment (Belloto et al. 2007; Gasier et al. 2010). We did not measure intracellular amino acid

enrichment, however in mice the intracellular enrichment has shown to follow the kinetics of plasma alanine (Belloto et al. 2007). In humans one study has shown a relationship of r^2=0.73 between plasma alanine and intracellular alanine being 305-455 ppm and intracellular 205-450 ppm respectively (MacDonald et al. 2015). This suggests that the relationship may not be perfect, although it was acknowledged that there was greater analytical variance with intracellular measures due to the small sample size; as such further validation is required in determine both intracellular and t-RNA labeling. However here we have shown long term labeling in rats follows a rise to plateau in the fraction of newly synthesized protein over the 6 weeks. The experimental curve applied to these points predicted the plateau to be 1.01. This demonstrates that muscle is a homogenous tissue that can achieve complete renewal. Further this shows that when reaching complete renewal the enrichment in bound alanine is equal to that of plasma alanine and therefor suggests that there is no intracellular dilution.

3.6.2 Measurement of Muscle DNA RNA synthesis

We have developed and validated methods using D_2O in the measurement of muscle DNA and RNA synthesis that can be used safely in human subjects. With controversy over the role DNA and RNA synthesis has during muscle hypertrophy this method will be highly applicable in human exercise studies. Further, we have supported these findings both *in vitro* and *in vivo* with known stimulators and inhibitors of synthesis and for the

first time we have shown a time course in the synthesis of skeletal muscle RNA and DNA synthesis.

Historically, DNA synthesis has been used as a measure of cell division and accordingly the incorporation of labeled nucleoside analogs is used to measure this rate (Waldman et al. 1991). The major limitation in the use of these analogs is there toxicity making them not suitable for human use (Asher et al. 1995; Rocha et al. 1990). The basis of our knowledge on DNA synthesis in human muscle is therefore often inferred from histochemical staining and correlative changes of cell size with myonuclei content (Kadi, Schjerling, et al. 2004; Petrella et al. 2006; Snijders et al. 2016). Similarly, changes in total RNA concentrations are used to make estimates of increases in ribosomal content (Nakada et al. 2016; Figueiredo et al. 2015). Methods using D_2O to determine cellular proliferation rates have been used previously in humans, although primarily in fast proliferating cells such as lymphocytes (Hellerstein et al. 2003; Mccune et al. 1999; Hayes et al. 2010). Here we have advanced and developed these methods to the application of DNA and RNA synthesis.

Due to limitations of using nucleoside analogues discussed above, rates of DNA synthesis using tracers are typically made through the ribose moiety of purine nucleosides, released during digestion and derivatisation in acidic conditions (Macallan et al. 1998; Neese et al. 2001; Neese et al. 2002). Here we validated this premise by treating nucleosides to the sample experimental conditions as extracted samples and showed that in

deoxynucleosides the majority of deoxribose is released from purines, with similar results were obtained for nucleosides.

During nucleoside synthesis deuterium can be incorporated into all carbon bound hydrogen's. As such, the whole ribose moiety has typically been measured using NCI GC-MS to capture all the potentially incorporated deuterium (Neese et al. 2002; Robinson et al. 2011). However GC-MS sensitivity is limited and NCI reproducibility rapidly deteriorates due to source contamination. Here we have developed GC-MS/MS methods that increase the limit of detection and precision 10 fold by selected reaction monitoring of dual fragmentation. The requirement of two fragmentations introduces the possibility of losing labeled hydrogen's, especially as the most abundant ions formed may not be those of interest and therefore care must be taken in choosing an appropriate SRM. To ensure our fragmentation pattern encompassed all carbon bound hydrogen's the enrichment of whole nucleosides was compared to that of ribose moieties and showed comparative results. Further, myocytes were cultured in media containing U-13C6 glucose that labels all 5 carbons in ribose via entry into the pentose phosphate pathway. Ribose was measured by GC-MS/MS monitoring the +5 isotopomer and increased over time validating the measured fragments contain all of the carbons and presumably all hydrogen's due to the unlikely fragmentation of C-H bonds.

To determine actual rates of synthesis the precursor enrichment of the pathway is required. The nucleoside precursor pool is complex and

difficult to measure, with continuous input of unlabeled substrates such that the maximal theoretical plateau is never achieved (Martini et al. 2004). This problem can be over come by measuring the plateau of a population of cells that will be 100% new over the duration of labeling, with the plateau representing that of the precursor (Neese et al. 2002). To determine standard amplification values in which precursor enrichments can be calculated from body water, C2C12's were repeatedly passaged into a range of D2O media concentrations. In doing so we have shown that the amplification of deuterium into deoxyribose is 3.1, agreeing with others showing a range of values from ~3-6 and can vary depending on derivative used (Neese et al. 2002; Hellerstein et al. 2003). Additionally we performed these measures in human PBMC's and showed an amplification of 2.6±0.3. This is slightly lower than previous values determined in human monocytes and granulocytes with reported amplifications of ~3.1 (Messmer et al. 2005; Neese et al. 2002; Robinson et al. 2011). These differences may be due to inter individual variability, as the range was from 1.2-4. Additionally, PBMC turnover has reported to be at rates upward of 20 $\%.d^{-1}$ (Busch et al. 2007). We collected PBMCS at 3 and 6 weeks prior to a weekly top up of D_2O where body water enrichments are low. With such rapid turnover rates PBMC enrichment could begin to reflect lower enrichments at the end of the week that would underestimate amplification. However for the first time in myocytes we have shown that plateau incorporation in RNA is 2.1 x the body water enrichment, although data could not be gathered in humans. This reduced amplification is expected as the ribose has one less carbon bound

hydrogen lost during conversion to deoxyribose by robonucleotide reductase. Further, during reduction of carbon 2 the hydrogen provided is transferred from a diothiol group that will readily interchange with body water, likely responsible for the considerable increase in amplification (Holmgren & Sengupta 2010).

We further validated our methods against cellular proliferation and known stimulators of DNA and RNA synthesis. As expected, cell proliferation occurred in a sigmoidal manner; doubling almost everyday in the main growth phase. In contrast to the curve of cell counting, D_2O incorporation followed a rise to plateau curve becoming rapidly saturated. This is expected as after 1 round of cell division there will be one parent strand and one newly synthesized strand- therefore being 50% labeled. After a 2^{nd} cell division 75% of the DNA will be labeled, 3^{rd} 87.5% and so forth with a plateau rapidly attained after relatively few cellular divisions. To compare the two methods, the number of cellular divisions that had occurred was calculated from a standard curve to interpolate the expected fraction of new DNA. This was compared with the fraction of new DNA determined via D_2O and showed almost identical results. Additional validation was performed *in vitro*, testing the sensitivity of D_2O in determining changes in DNA and RNA synthesis rates in response to known stimulators and inhibitors. IGF1 is a known stimulator of growth in cell culture models, and here we have shown that after 27 hrs there is a significant increase in both DNA and RNA concentrations. This was reflected by increases in that of DNA and RNA enrichment.

We further applied our methods of muscle DNA and RNA synthesis *in vivo*. In mature rat quadriceps we determined a fractional DNA synthesis rate of 0.21±0.07%.d^{-1} and 0.97±0.1%.d^{-1} for DNA and RNA respectively. Previous rates of muscle DNA synthesis rate *in vivo* using D_2O in mice have shown to be 0.1% - 0.25% (Miller et al. 2012; Drake et al. 2015; Drake et al. 2014) although no detectable enrichment was seen in canines over 1 week (Miller et al. 2015). Our results are therefore in good agreement with those previously obtained. This slow rate of DNA synthesis is expected as muscle is a post mitotic tissue and without growth or damage it is presumed to have relatively little cellular proliferation (Zammit 2008), yet it is worth note that there was detectable DNA synthesis at rest in fully grown rats. Muscle contains multiple cell types such as satellite cells, pericytes (Dellavalle et al. 2007), myoendothelial and interstitial cells (Zheng et al. 2007; Mitchell et al. 2010). Satellite cells constitute ~ 3% of muscle nuclei yet are believed to remain quiescent other than in growth and repair to substantial damage in which they are undoubtedly active (Hawke *et al.,* 2001). The newly synthesized DNA over the 3 weeks could be an accumulation of DNA from multiple cell types, with sporadic proliferation and fusion of satellite cells during the wear and tear of daily living that was not increased with treadmill running.

RNA synthesis rates measured here were ~4 times quicker than that of DNA and to our knowledge there are few measures of muscle RNA synthesis *in vivo*. Over a short period of time using an infusion of methyl-

^{3}H methionine a rate of 6.2 %.d^{-1} for RNA in muscle was shown (Grimble & Millward 1976). Although considerably higher than the values reported here, the authors mention this rate was similar to MPS rates for rats at that age. Although the age was not stated this suggests animals used were growing rather than fully mature animals used here. Measures of total RNA synthesis encompass that of mRNA, tRNA and rRNA. mRNA and tRNA have rapid synthesis and degradation rates of min to hrs (Ross 1995), whilst ribosomal rRNA has shown a half-life of 7 days in rat fibroblasts. Ribosomal RNA makes up ~80% of total RNA and so measures of total RNA will primarily reflect that of rRNA. Further the rate of RNA synthesis was significantly increased to 1.3±0.2 %.d^{-1} with HIIT running exercise and may possibly be due to the increased demands of protein synthesis observed.

There are relatively few measures of muscle DNA and RNA synthesis in humans for us to compare our data to. Here we have shown from human VL rates of muscle DNA synthesis of 0.19±0.1 %.d^{-1} and 1±0.1 %.d^{-1} for RNA synthesis. Using D_2O, rates of human DNA synthesis have been previously been reported ~0.03 %.d^{-1} in young sedentary adults and ~0.15%.d^{-1} in older exercising individuals (Robinson et al. 2011). As such the rates we report here of ~0.2 %.d^{-1} are similar to that of older exercising individuals and disparity between the young subjects possibly due to the low subject number and analytical sensitivity in (Robinson et al. 2011). These slow rates of muscle DNA synthesis reflect of what we know about satellite cell activity. Satellite cells undergo proliferation to form two

daughter cells, one maintaining the satellite cell pool and one fusing with the myofibre (Troy et al. 2012). Acute muscle damage can repair without the need of without satellite cell incorporation (Bansal 2003) where as substantial damage requires satellite cell activation (Lepper et al. 2011). Daily wear and tear of muscle is therefore may not require addition of satellite cells. However in adult mice satellite cells have shown to contribute to myofibre without injury (Keefe et al. 2015). Our subjects continued their habitual activity regimes and therefore may induce acute muscle damage at different levels of damage over the six weeks. This may lead to activation and fusion of satellite cells.

To our knowledge there a no previous measures of RNA synthesis reported in human muscle. Further there is very little on the habitual regulation of RNA in mature muscle *in vivo*. RNA content presumably remains relatively constant throughout habitual activity, yet synthesis is sensitive to muscular overload and has shown increases >50% after just two exercise bouts (Bickel et al. 2005). Using observations from animal studies, Grimble suggested that rRNA synthesis in muscle is similar to that of cellular proteins and would therefore agree with results obtained here. Of ~1% (Grimble et al. 2000). Further whole body rRNA turnover by measures of RNA breakdown products in urine is estimated ~2.5%. Therefore relatively low rates of muscle RNA turnover may be expected as if it were substantially greater the large contribution of muscle to whole body mass would be reflected in these rates (Grimble et al. 2000). As such it has been shown that there is a close relationship between whole body

protein and RNA degradation rates, r^2=0.7, suggesting they have similar turnover rates (Topp et al. 2008).

In summary we have further validated the use of D_2O in measure of muscle protein synthesis and developed methods in the measurement of DNA and RNA synthesis. We have determined the acute equilibration of saliva and plasma alanine identifying when post bolus sampling is optimum. Further we have confirmed that saliva is representative of plasma alanine during a long-term study, crucial in the assessment of precursor enrichment over weeks. Additionally long term incorporation of D_2O into rat muscle suggest there is not intracellular dilution of tracer further confirming saliva as a suitable measure of precursor enrichment. In developing methods to measure DNA and RNA synthesis, we have confirmed DNA rates similar to those previously published. Additionally we have provided the first measure of muscle RNA synthesis that's suggests turnover is similar to that of muscle protein. These new methods will likely play significant roles in further elucidating muscle metabolism at rest, in response to exercise and ageing.

Chapter 4 - Application of Novel D_2O Methods to Investigate Skeletal Muscle Hypertrophy at the Onset of Resistance Exercise Training

4.1 Introduction

Skeletal muscles exhibit marked physiologic and biochemical adaptations to RET, for example increased skeletal muscle mass, strength, power and metabolic function (Goldberg et al. 1975; Warburton et al. 2006). Yet, the most defining adaptations to functional overload, via RET, are skeletal muscle hypertrophy and strength. In healthy untrained adults, muscle mass remains constant as a result of a dynamic equilibrium between MPS and MPB. However, in response to RET, hypertrophy occurs as a result of MPS chronically exceeding MPB; a process driven by RET induced increases in MPS after exercise (Laurent & Sparrow 1977) and facilitated via the intake of dietary essential amino acid substrates (Burd et al. 2011; Witard et al. 2014; Phillips et al. 2012). Knowledge of the temporal, metabolic and molecular regulation of skeletal muscle hypertrophy by RET is particularly important in the context of mitigating age and disease-related muscle wasting conditions, with RET being a foremost treatment.

The time course of hypertrophic responses to RET remains a contentious and poorly defined issue, in particular, whether RET-induced hypertrophy is linear over time, or skewed in favor of early or later muscle growth. For example, early studies suggest that neural adaptations dominate in the initial weeks of RET (Moritani & DeVries 1979; Häkkinen, Newton, et al. 1998) and that hypertrophy occurs at a later stage (Staron et al. 1994; Abe et al. 2000). Nonetheless, muscle hypertrophy has also been reported after as little as 3-4 weeks RET, as measured by MRI (Seynnes et al. 2007) CT scan (DeFreitas et al. 2011), fibre CSA (Woolstenhulme et al. 2006) or

muscle thickness via ultrasound (Baroni et al. 2013). These findings suggest that muscle hypertrophy may occur earlier into an RET program than was previously thought. In support of this, hypertrophic responses to RET have been shown to diminish over time (Ogasawara et al. 2013), in addition to hypertrophic potential being minimal in well trained strength athletes (Alway et al. 1992) compared to sedentary, RET-naïve individuals (Ahtiainen et al. 2003). It could be argued that such discrepancies are due to distinct analytical approaches for quantifying hypertrophy. For example, while fibre CSA is often used to detect changes at the cellular level, MRI ("gold standard"), CT and DXA are commonly used for whole muscle(s) group assessments (Heymsfield et al. 1997). Recent work has also revealed that ultrasound represents an accurate non-invasive approach to quantify hypertrophy, via assessing muscle thickness (Bemben 2002). Indeed, evidence is mounting that ultrasound provides a reliable surrogate for changes in muscle CSA (Reeves et al. 2004; Abe et al. 1997) and volume (Esformes et al. 2002). Further, ultrasound permits architectural measures unattainable with other techniques i.e. hypertrophy-related architectural remodeling such as addition of sarcomeres in series vs. parallel (Aagaard et al. 2001; Franchi et al. 2014). Nonetheless, whether discrepant reports in the time course of skeletal muscle hypertrophy (early vs. late) are physiologically or methodologically driven, is poorly defined.

Muscle hypertrophy occurs as a result of cumulative increases in MPS occurring in response to each successive bout of RET. RET induced

increases in MPS are driven via complex and integrated mechanotransduction, hormonal and growth factor responses stimulating activation of "anabolic" intramuscular signaling pathways (i.e. mechanistic target of rapamycin; mTORc1) that coordinate mRNA translation (Drummond, Fry, et al. 2009). Indeed, acute signaling responses to RET are marked (i.e. phosphorylation of mTORc1 substrates) yet varied (Murton et al. 2014) and whilst some correlates with acute MPS have been reported (Fry et al. 2011a; Kumar et al. 2009), adaptive responses of anabolic signaling in relation to hypertrophy remain unclear. Similarly, the quantification of *acute* MPS using amino acid (AA) tracers (carbon, nitrogen or hydrogen) in the hrs following a single bout of RET does not correlate with ensuing hypertrophy (Mitchell et al. 2014; Mayhew et al. 2009); moreover, these methods are limited to short duration studies i.e. <24 h such that they cannot report on long term links between MPS and hypertrophy. Accordingly, other approaches are needed to determine mechanistic links between the time-course of hypertrophy, MPS and anabolic signaling. The recent re-introduction and development of 'heavy water' (D_2O) isotope techniques has enabled longer-term measures of MPS and metabolism to be made (Gasier et al. 2012; Robinson et al. 2011; Busch et al. 2006). We have further developed the use of D_2O in the measurement of human muscle protein, DNA and RNA synthesis (Chapter 3). Using these methods we aimed to determine temporal mechanistic links between hypertrophy, MPS and anabolic signaling.

4.2 Methods

The study protocol and all the methods relating to this section have been previously described in chapter 2

Statistical Analyses

Descriptive statistics were produced for all data sets to check for normal distribution (accepted if $P>0.05$) using a Kolmogorov-Smirnov test. All data are presented as means ± SEM. Myofibrillar protein synthesis 0-6 week and absolute protein synthetic rates were analyzed by paired t-test (two tailed). 1RM was analyzed by using repeated-measures one-way anova with all other data sets analyzed by repeated measures two-way ANOVA with a Bonferroni correction using GraphPad Prism (La Jolla, CA) Software Version 5. Correlations were assessed using Pearson's product moment correlation coefficient. The α level of significance was set at $P<0.05$

4.3 Results

4.3.1 1-RM and MVC in UT and T legs

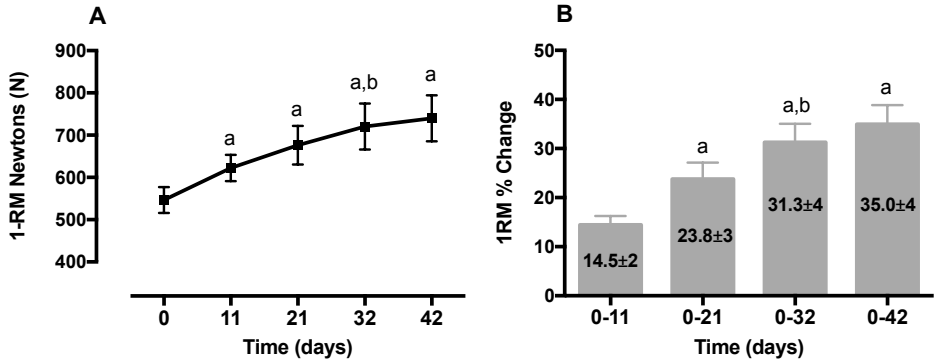


Figure 4.1 Time course of changes in A) T leg 1-RM, B) % change in T leg 1-RM from baseline. a significantly different from baseline $p<0.05$, b significantly different than previous time point $p<0.05$

After 6 weeks RET 1-RM increased from a baseline of 546±31 N to 739±54 N $p<0.01$ in the T leg (Fig 4.1). Further, the change in 1-RM from baseline progressively increased, from 14.5±2% (0-11 d) to 23.8±3% (0-21 d) $P<0.05$ and from 23.8±3% (0-21 d) to 31±3% 0-32 d $P<0.05$. Similarly, MVC increased over a range of angles in the T leg (Fig 4.2), with the greatest increase being at 60^{O} i.e. baseline 245±23 Nm vs. 6 weeks 307±23 Nm $p<0.01$. MVC also increased at 6 weeks in the UT, 235±23 Nm to 269±24Nm, while the UT leg became stronger than the rest leg after 11 days $P<0.01$ and remained stronger over the training period $P<0.01$.

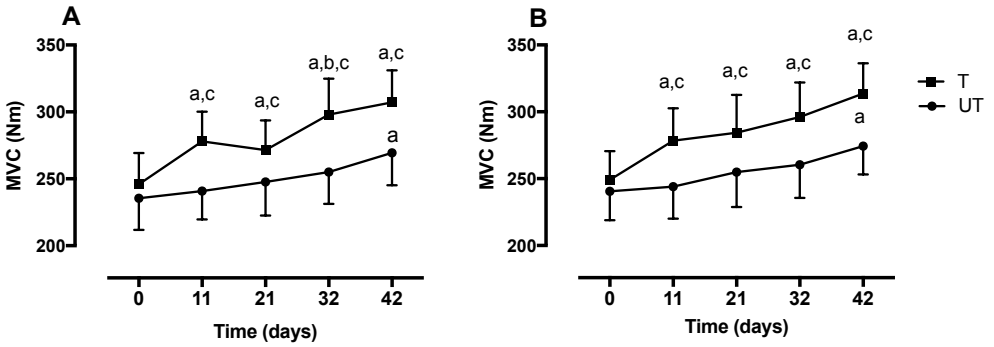


Figure 4.2 Time course of change in MVC in T and UT legs over the 6 weeks of RET at A) 60º and B) 70º. a significantly different from baseline $p<0.05$, b significantly different than previous time point $p<0.05$, c significantly different from rest leg at that time point $p<0.05$

4.3.2 Muscle Mass and Architecture in UT and T legs

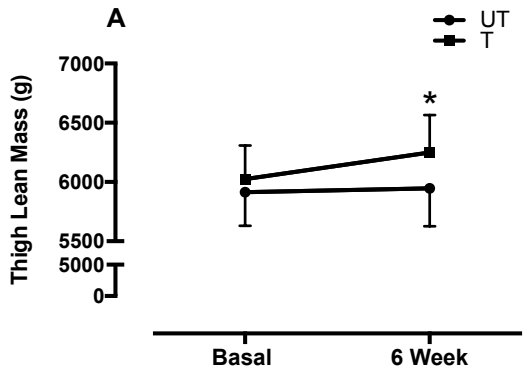


Figure 4.3 Thigh lean mass over the 6-week training period.

Thigh lean mass assessed by DXA remained constant in the UT leg (5914±283 g to 5946±319 g $p<0.05$) whilst there was an increase over the 6 weeks in the T leg (6013±284 g to 6249±315 g $p<0.05$). Measures of VL

architecture revealed increases in MT (2.46±0.1 cm vs. 2.66±0.09 cm $P<0.01$) θ (18.25±1^{o} to 19.49±0.9^{o} $p<0.01$) and L*f* (7.8±0.3cm to 8.2±0.4 cm $p<0.05$) at baseline vs. 3 weeks respectively with no further increase in any of these parameters at 6 weeks (2.72±0.09 cm, 20.09±0.9 ^{o} and 8.28 ± 0.4cm) in MT, θ and L*f*, respectively.

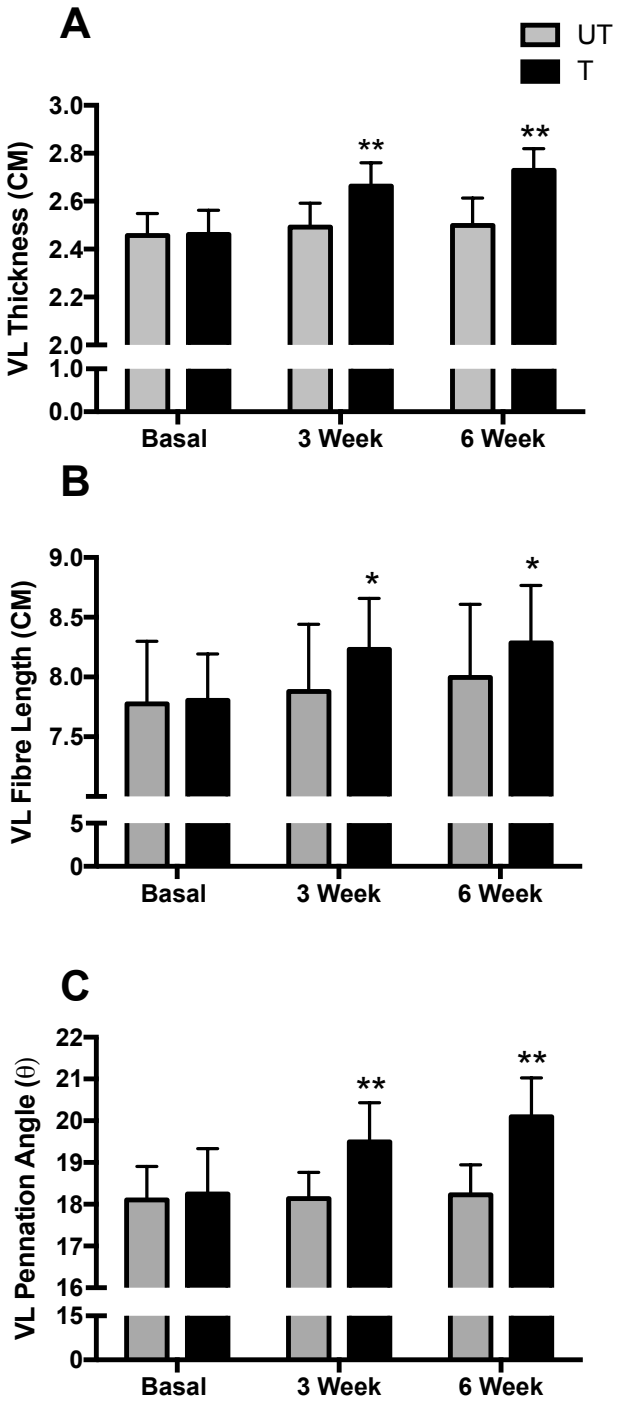


Figure 4.4 Time course of VL A) MT B) L*f* and D) θ in UT and T legs at 0, 3 and 6 weeks. * Significantly different from baseline P<0.05, **P<0.01.

4.3.3 Body water enrichment and D_2O-Derived MPS (MPS) in UT and T legs

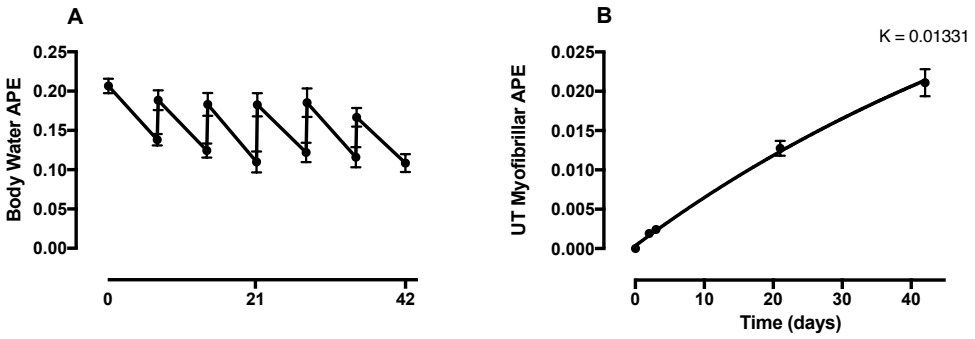


Figure 4.5 A) Time course of body water enrichment over 6 weeks B) One phase association of the change in myofibrillar APE over 6 weeks

After the initial 150 ml bolus of 70% D_2O, body water enrichment was 0.21±0.01% and was thereafter maintained in a pseudo steady state between 0.192 ± 0.01% and 0.122 ±0.01% for the remainder of the 6 week study period. Furthermore, as protein synthesis tends to follow zero order kinetics; FSR was calculated in the UT leg using a one-compartment model in which the rate constant (*k*) was determined. This compartmental fitting describes a constant turnover of protein; fit using additional measures of muscle APE taken from the UT leg at day 2 in 8 subjects and 3 in 2 subjects. Figure 4B shows the fractional turnover k was 1.33 $\%.d^{-1}$ with myofibrillar protein half life 52.07 days and showed excellent correlation between the two calculation methods ($R^2 = 0.94$, $P<0.001$).

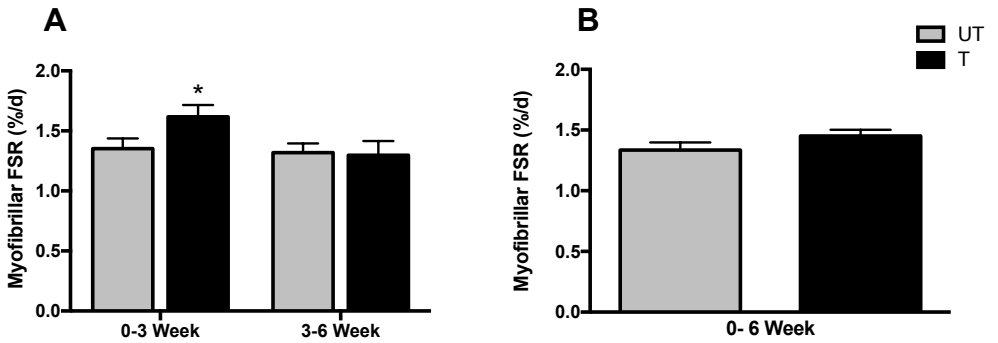


Fig 4.6 Temporal pattern of myofibrillar FSR in UT and T legs over A) 0-3 and 3-6weeks and b) myofibrillar FSR over the 0-6 week training period. * significantly different from UT leg at that time point $p<0.05$.

Over the training period MPS in the UT leg remained constant at 1.35±0.08 $\%.d^{-1}$, 1.32±0.07 $\%.d^{-1}$ and 1.33±0.06 $\%.d^{-1}$ at 0-3, 3-6 and 0-6 weeks respectively. In the T leg, MPS was increased compared to UT 0-3 weeks: 1.6±0.01$\%.d^{-1}$ $P<0.05$ while being 1.29±0.11$\%.d^{-1}$ 3-6 weeks and 1.45±0.05 % at 0-6 weeks, with MPS showing a tendency to be increased 0-6 weeks in the T compared to UT leg P=0.1 (Fig 4.6) The ASR was increased in the T leg over 6 weeks 8.1±0.1$g.d^{-1}$ to 9.3±0.7 $g.d^{-1}$ $P<0.05$ while ABR did not change 8.5±0.6$g.d^{-1}$ to 8.1±0.8 $g.d^{-1}$(Fig 4.7).

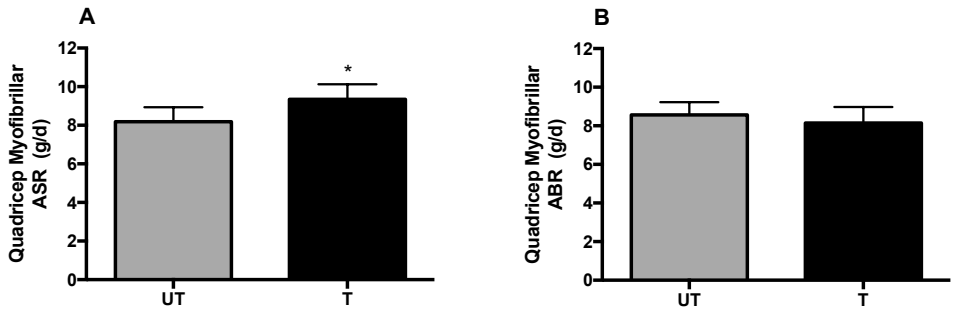


Figure 4.7 A) Absolute quadriceps synthesis 0-6 weeks. B) Absolute quadriceps breakdown 0-6 weeks E * significantly different UT

4.3.5 Muscle Protein, RNA and DNA concentrations

Table 1 shows ASP μg.mg dry weight^{-1} was unchanged in the UT and T legs throughout. When μg ASP is normalized μg DNA, a measure of cell size, shows T legs trend towards significance by 6 weeks with RET (88±8μg/μg to 103±5 μg/μg P=0.06) (Fig 4.8A). RNA μg.mg dry weight^{-1} increased at 3 and 6 weeks in T legs (Table 1) with the cell capacity for protein synthesis μg RNA/μg DNA (Fig 4.8B) increased at 3 weeks (0.47±0.05 μg/μg to 0.62±0.05 μg/μg P<0.01) and 6 weeks (0.64 ±0.03 μg/μg p<0.01). There was no change any of the above parameters in UT legs throughout the study

	Basal		3 Week		6 Week	
	Rest	Exercise	Rest	Exercise	Rest	Exercise
ASP μg.mg dry weight^{-1}	526±41	521 ±34	529±20	613 ± 25	520.8±15	552 ±28
RNA μg.mg dry weight^{-1}	3.2±0.3	2.75 ±0.2	3.1±0.2	3.91 ±0.2**	3.1±0.1	3.38 ±0.1*
DNA μg.mg dry weight^{-1}	6.3±0.6	6.20 ±0.4	5.75±0.2	6.65 ±0.6	5.74±0.2	5.39 ±0.3

Table 4.1 Skeletal muscle protein RNA and DNA concentrations

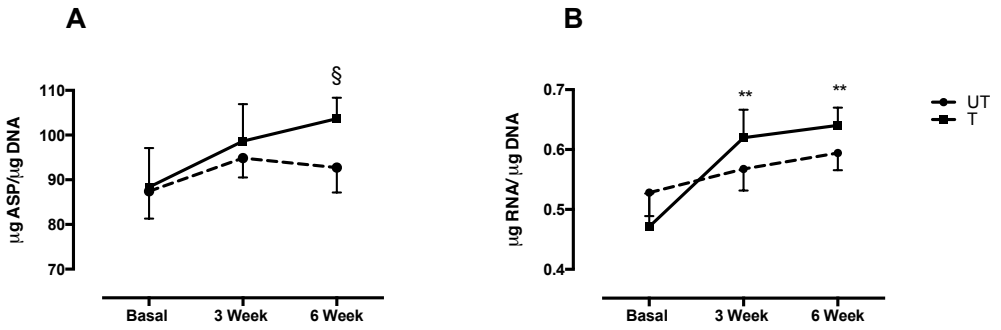


Figure 4.8 Change in A) μg ASP/μg DNA and F) Change in the μg RNA/μg DNA over the 6 weeks RET in T and UT legs * significantly different from baseline P<0.05, **P<0.01

4.3.5 Muscle DNA and RNA synthesis

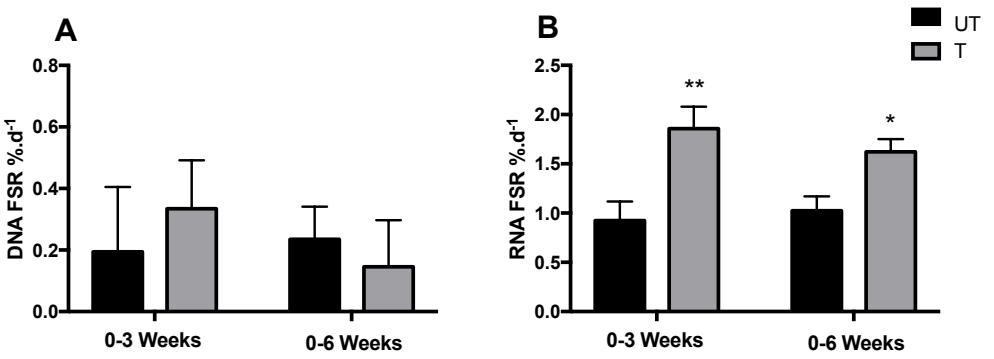
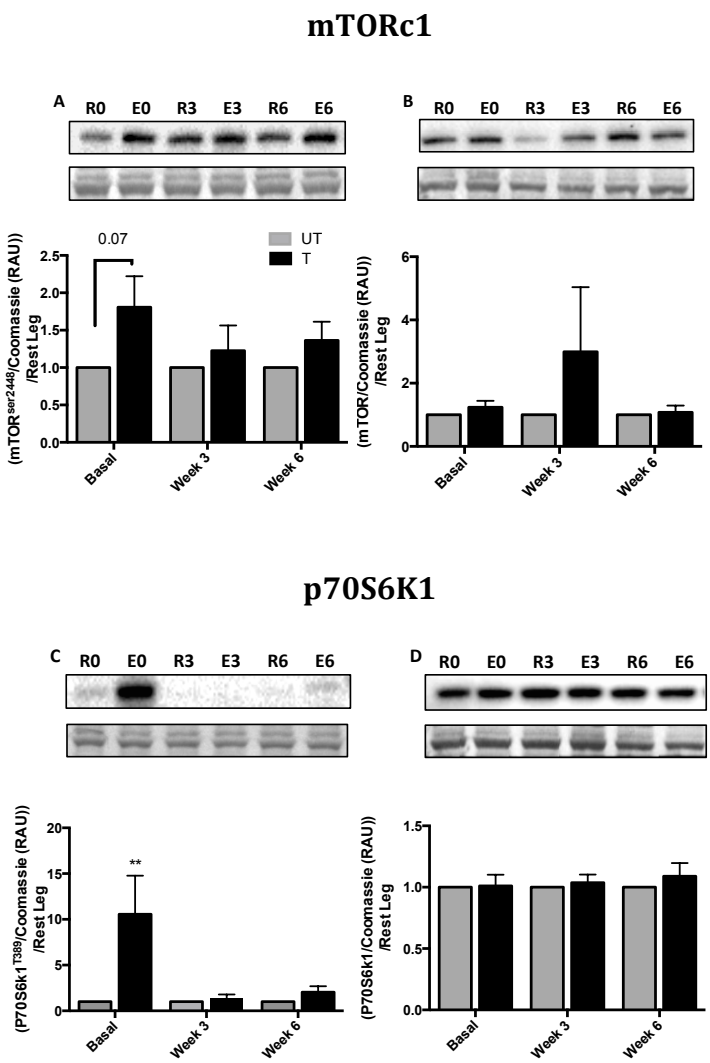


Figure 4.9 FSR %d^{-1} of A) DNA B) RNA in T and UT legs over the 6 weeks. * significantly different from UT $P<0.05$ ** $P<0.01$

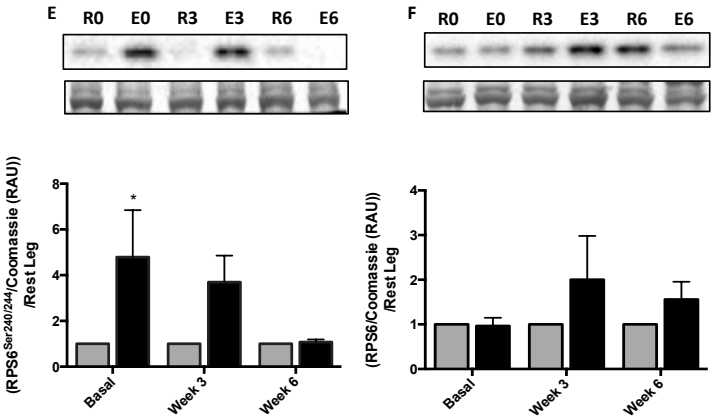
Over the 6 week training period DNA FSR %d^{-1} was 0.19±0.2 %d^{-1} and 0.23±0.1 %d^{-1} at 3 and 6 weeks respectively, with no significant difference (Fig 4.8). There was no effect of RE on DNA FSR %d^{-1} being 0.33±0.15 %d^{-1} and 0.15±0.15 %d^{-1} at 0-3 and 0-6 weeks respectively. Muscle RNA synthesis was constant over the 6 weeks being 0.92±0.19 %d^{-1} and 1 ±0.15 %d^{-1} at 3 and 6 weeks respectively. In contrast to DNA, RNA was significantly increased with exercise at both 3 and 6 weeks being 1.85±0.2 %d^{-1} $P<0.01$ and 1.62±0.1 %d^{-1} ($P<0.05$) respectively (Fig 4.9).

4.5.6 Intramuscular Anabolic Signaling

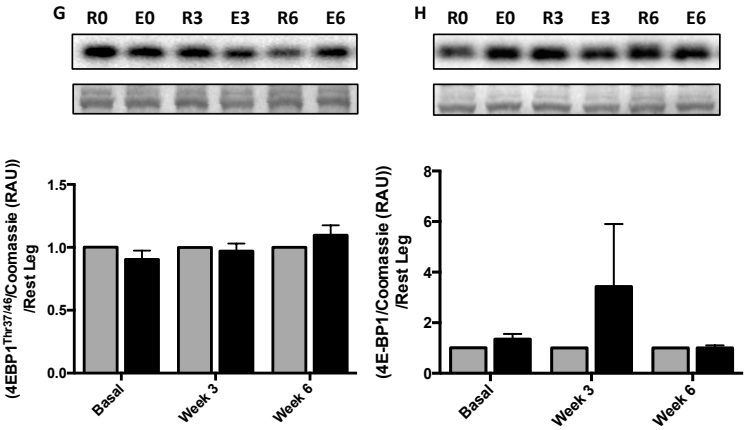
Figure 4.9 shows the acute post exercise response (60-90 min) at 0, 3 and 6 weeks. After the first exercise bout there was a significant increase (compared to UT) in phosphorylation of p70SK1^{T389} P<0.01, RPS6$^{Ser240/244}$ P<0.05, a tendency for mTOR^{ser2448} P=0.07, and a decrease in eEF2^{Thr56} P<0.05. No changes were observed in phosphorylation of any proteins at 3 weeks although eEF2^{Thr56} exhibited acute reductions in phosphorylation at 6 weeks P<0.01. No change was observed in the total concentrations of any proteins over the 6 weeks study.



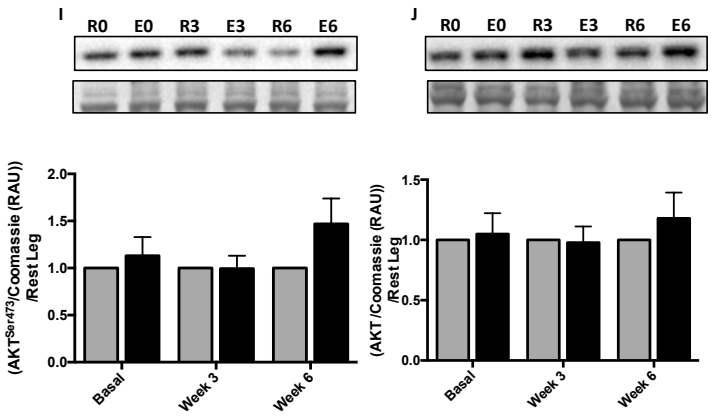
RPS6



4EBP1



AKT



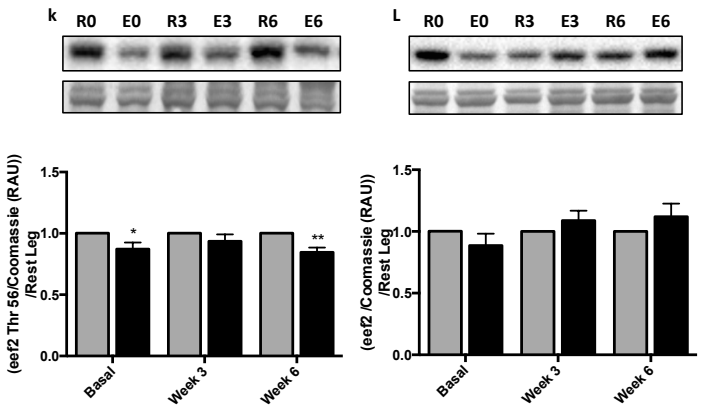


Figure 4.10 Relative change compared to UT in A) mTORc1 ^{Ser2448} B) total mTOR, C) p70S6k1 ^{Thr389} D) total p70S6K1 E) rps6$^{ser240/244}$ F) total rps6 **G)** 4EBP1$^{Thr37/46}$ H) total 4EBP1 I) Akt^{Ser473} J) total AKT K) eEF2^{Thr56} L) total eEF2. * Significantly different from baseline P<0.05, **P<0.01

4.5.7 Correlations

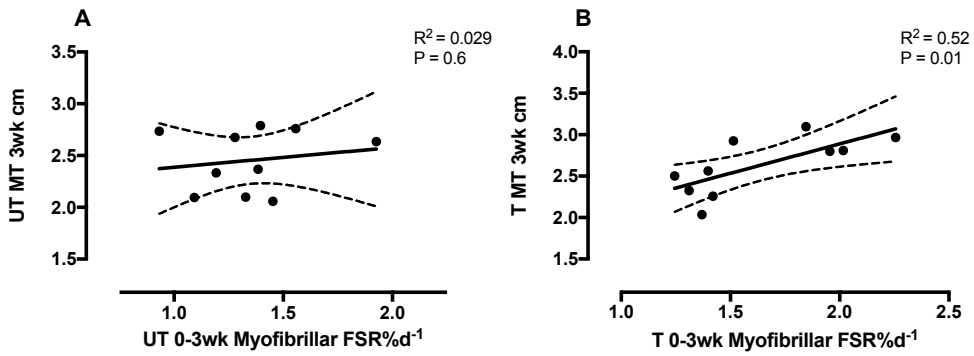


Fig 4.11 A) Correlation of MT at 3 weeks in UT vs. myofibrillar MPS 0-3-week in UT B) Correlation of MT at 3 weeks in T vs. myofibrillar MPS 0-3wk in T

Rates of myofibrillar FSR were significantly correlated with MT at 3 weeks in T legs, P=0.01 R^2=0.52; a relationship not evident in UT legs (Fig 4.10) P=0.6 R^2=0.029.

4.4 Discussion

A considerable amount of research has focused on longer-term physiological adaptations to exercise, particularly RET-induced muscle hypertrophy. Yet, the temporal links between skeletal muscle hypertrophy and regulatory metabolic and molecular processes remain poorly defined. Therefore, we have used novel D_2O tracer techniques (Wilkinson et al. 2014) to investigate long term, temporal hypertrophic and MPS adaptations of skeletal muscle to RET. We have shown that skeletal hypertrophy occurs rapidly with RET, is driven by sustained increases in MPS and RNA synthesis that wanes despite progressive intensity over time and is associated with reduced RET induced mTORC1 signaling.

Imaging techniques including MRI, CT and DXA are the most commonly used techniques to quantify muscle mass (Heymsfield et al. 2014). Nonetheless, recent developments in ultrasonography offers a more readily available non-invasive method for measurement of muscle mass and has shown utility in detecting early physiological adaptations (Baroni et al. 2013; Seynnes et al. 2007). Indeed, numerous indices of muscle hypertrophy such as increases in muscle thickness and alterations in architecture (θ, L*f*) are observed in hypertrophied muscle using ultrasonography (Franchi et al. 2014; Aagaard et al. 2001). Moreover, ultrasound quantification of MT correlated well with individual muscle measures of mass derived from more established techniques such as MRI (Thomaes et al. 2012; Giles et al. 2015), and also represents a reliable predictor of whole muscle group volume changes with RET (Miyatani et al.

2002; Miyatani et al. 2004; Giles et al. 2015). When applying these methods to our study, we found, in contrast to certain studies reporting delayed muscle hypertrophy (Moritani & DeVries 1979) that increases in MT, pennation angle and fascicle length were evident after just 3 weeks RET. Moreover, by 6 weeks none of these parameters had further increased despite the intensity-progressive nature of our RET protocol. While seemingly controversial, our results show muscle hypertrophy to be most active during the early weeks of RET, matching that of other studies showing a bias towards early hypertrophy i.e. after 3 weeks (Seynnes et al. 2007) and as early as 1-wk with high training intensities (DeFreitas et al. 2011). Moreover, the trajectory of hypertrophy with longer term RET studies is known to decrease, with the rate of muscle growth significantly less after the first 6 weeks (Ogasawara et al. 2013; Wernbom et al. 2007; Reidy et al. 2016). The rate of muscle adaptation is unclear and is most likely to display heterogeneity between individuals, although agreeing with our results the majority of muscle adaptation has shown to occur rapidly. For instance during a 5 and 8 week RET program, ~ 70 % of the total increase in quadriceps CSA occurred during the first 3 weeks (Seynnes et al. 2007; DeFreitas et al. 2011). Collectively, our findings suggest muscle hypertrophy is rapid in response to RET, along with rapid functional improvements suggestive of increased neural recruitment i.e. 1-RM/ MVC (Häkkinen & Komi 1983; Seynnes et al. 2007). Our 6-week RET training protocol yielded increases in DXA-derived muscle mass over the 6-week training period (Tesch et al. 2004; Constantin et al. 2011) in T but not UT legs, agreeing with ultrasound measures. These adaptations

occurred early into the RET program matching gradual declines in MPS, yet prolonged RET has shown to induce greater adaptations than those in the present study. The relationships between MPS/MPB and continued hypertrophy in response RE are still somewhat incompletely defined, although training progression will be influenced by varied nutrition and RE paradigms, with ones genetic potential for hypertrophy sure to be a substantial factor (Phillips et al. 2013).

'Absolute' rates of MPS are open to interpretation, and may vary considerably between individuals and indeed the analytical methodologies employed; resting postabsorptive and postprandial rates of MPS are reflected by the muscle fraction measured i.e. mixed vs. myofibrillar, and both the amino acid tracer and precursor/surrogate used to calculate synthesis (Smith et al. 2011), yet with these caveats the magnitude of change induced by exercise and nutrition are reasonably consistent (Kumar et al. 2009). Using these traditional AA-tracer techniques and measurements over periods of hrs, resting myofibrillar postabosrptive MPS ranges from ~0.03-0.06 $\%.h^{-1}$ equating to ~0.75 -1.45 $\%.d^{-1}$ (Cuthbertson et al. 2005a; Kumar et al. 2009; Smith, Patterson, et al. 2011). The use of D_2O enables a measure of 'free living' MPS, taking into account postabsorptive, postprandial and active/ inactive periods over periods of days to weeks in a cumulative manner. Although few studies have reported longer term rested rates of MPS in young individuals, using D_2O approaches we previously reported ~1.4 $\%.d^{-1}$ over 8 d, matching that by Macdonald et al of 1.45 $\%.d^{-1}$ (Wilkinson et al. 2014; MacDonald et al.

2013), with others reporting slightly slower rates of ~ 5.5 %.wk^{-1} (0.8 %.d^{-1}) over 6 weeks (Robinson et al. 2011). In the present study, our "time control" UT exhibited rates of MPS over 6 weeks of 1.33 %.d^{-1}, which remained constant 0-3 and 3-6 weeks. Importantly, Busch et al. showed continuous labeling with D_2O permits reliable measurement of turnover rates of long lived, slow turnover proteins, with mixed muscle proteins showing a continuous rise for up to 15 weeks following single exponential kinetics (Busch et al. 2006). As such, MPS was further calculated using a one compartmental model of protein labeling (Kasumov et al. 2011; Kasumov et al. 2013), providing an identical turnover of 1.33 %.d^{-1} validating steady state turnover of MPS.

Overload-induced muscle hypertrophy is primarily driven through post-exercise increases in MPS, resulting in positive net balance (Phillips et al. 2012). Reflecting this, MPS is robustly increased after a single bout of RE (Kumar et al. 2009; Kumar et al. 2012) for up to 72h under protein nourished conditions (Burd et al. 2011; Miller et al. 2005). Since we, and others have found that muscle growth is not a process that is linear with time (Ogasawara et al. 2013; DeFreitas et al. 2011), it would be expected this would be reflected in diminishing acute MPS responses to exercise throughout a RET program. However this assumption is clouded by conflicting reports i.e. with trained individuals demonstrating reduced post exercise muscle protein turnover (Phillips et al. 1999) and progressive RET shown to either attenuate (Tang et al. 2008) or have no effect on these measures (Kim et al. 2005; Wilkinson et al. 2006). These

discrepancies are likely due to differences in acute measures (i.e. muscle fractions examined or fasted/ fed and length of measurement). Instead, the advantage of our application of D_2O in this study is that it permits readouts reflecting temporal and cumulative responses to RET. Indeed, using such approaches, we have previously shown that increased MPS remains elevated over 8 d RET (Wilkinson et al. 2014) such that accretion of new proteins would be expected relatively soon and that the magnitude of MPS is decreased after just 3 repeated exercise bouts (Wilkinson et al. 2014); again supporting the notion of a progressive "desensitization" to the anabolic effects of RET. Moreover, in the present study we have tracked temporal responses to unilateral RET (to permit comparison to a time control UT leg). For the first time, we have shown that predominating early increases in hypertrophy are underpinned by sustained increases in long-term MPS and that, despite exposure to a progressive RET program, MPS decreased to that of the UT in the 3-6-wk RET-period. This was mirrored by muscle growth as assessed by ultrasound, supporting the notion that hypertrophy is rapid, occurs early in RET, and is linked to sustained (vs. the rest leg) increases in MPS. Furthermore, the fact that we observed significant correlations between FSR of myofibrillar proteins in the T leg, but not the UT leg, suggests there to be a mechanistic link between long-term MPS and muscle hypertrophy. Finally, although the stimulation of MPS in the T leg was dampened 3-6 weeks, increases in muscle mass means the *absolute* amount of MPS is greater, despite FSR remaining constant; this was reflected by our observations of increased

ASR (in the absence of alterations in ABR) in the T leg after 6 weeks of RET.

It has been suggested that ribosomal biogenesis plays a key role at the onset of skeletal muscle hypertrophy (Figueiredo et al. 2015; Stec et al. 2016). We chose to investigate indices of myocellular translational efficiency and capacity in order to further explain the temporal changes in muscle mass and metabolic responses to RET. In doing so, we observed that in addition to increases in muscle mass and MPS (over 0-3 weeks), we observed increases in RNA/DNA ratios (a measure of the capacity for protein synthesis) with increases in protein/DNA ratios (a measure of cell size) approaching significance at 6 weeks (Cheek 1968; Goldberg 1967; Forsberg et al. 1991). Further, for the first time we supported these findings with dynamic measures of RNA synthesis using D_2O. In response to RE training RNA synthesis was increases at both 3 and 6 weeks further supporting ribosomal biogenesis having a significant roles in muscle hypertrophy. In past studies, an acute bout of RET showed no change in RNA content and so increases in MPS were associated with greater RNA activity i.e. polysome aggregation (Chesley et al. 1992). However, overload-induced muscle hypertrophy in animal models has been shown to increase RNA content, presumably in order to increase synthetic capacity to support larger muscle masses (Laurent & Sparrow 1977). In humans total RNA content has also been shown to increase after a few bouts of RE (Bickel et al. 2005) or following several weeks of RET (Haddad et al. 2005). Moreover, we observed increases in the capacity for protein

synthesis at 3 weeks with no further increase at 6 weeks, supporting our observations of predominating early hypertrophy. In contrast to the increased synthesis of RNA, we saw no increases in DNA synthesis or content. The amount of satellite cells and myonuclei per fibre has shown not to increase until 8-10 weeks of training (Snijders et al. 2016). A lack of increased DNA synthesis here suggests there was minimal proliferation and fusion of satellite cells with myofibres. It has been suggested that myonuclear addition only occurs beyond certain increase in fibre CSA (Petrella et al. 2006). However we do not know increases in fibre CSA here and so further research is needed.

In an attempt to further elucidate the underlying regulation of these temporal muscle mass and metabolic responses we chose to investigate the activation status of signaling pathways involved in the control of MPS (Drummond et al. 2009). Using a carefully designed study we ensured muscle biopsies were taken 60-90 min after RE at baseline, 3 and 6 weeks such that we could chart relationships between anabolic signals and longer term MPS and muscle mass changes. After the first bout of RE, we observed increased phosphorylation of the key mTORC1 cognate substrates, p70S6K1 and RPS6, in addition to decreased eEF2 phosphorylation (reflecting increased activity), all signals mechanistically linked with RE-induced increases in MPS (Dreyer et al. 2006; Kumar et al. 2009) and muscle hypertrophy. Intriguingly, matching the plateau of MPS by 3 weeks, activation of these proteins diminished over time (all but eEF2), reflecting attenuated MPS and hypertrophic responses seen with

training progression (Nader et al. 2014; Gonzalez et al. 2015). Indeed, these time adaptive responses are likely to become more specific, such that the synthesis of new proteins will be needed in order to support the phenotypic adaptation of the training stimulus (Wilkinson et al. 2008). The mechanisms regulating these refractory responses to hypertrophy are unclear. Skeletal muscle is efficient in rapidly remodeling to external demands placed up on it, likely becoming less perturbed by mechanical and metabolic stress with proceeding habituation. This could explain the diminished anabolic signaling responses observed, and explain the observed waning in muscle mass gains beyond 3 weeks.

We also acknowledge a few study limitations. Firstly, our resolution is limited to 3 time points (for metabolic/ molecular end-points), and secondly, this is a means based analysis and it is likely that individuals' trajectory of hypertrophy could diverge depending on "responder status' (Phillips et al. 2013). Nonetheless, that changes in muscle size are dominated by early hypertrophy is in little doubt. In summary, our data shows that muscle hypertrophy occurs rapidly in response to RET and that this is driven by sustained increases in MPS, which subsequently decline with rates of muscle hypertrophy and with anabolic signaling. Since the early rise in MPS was correlated with MT, measures of MPS with D_2O may hold some predictive capacity for hypertrophic outcomes. Using these techniques, subjects were allowed to undergo their usual activities of daily living; thus our measures of MPS reflect that of true free-living situation. There is no doubt these measures will prove crucial to determine the "real

life" biology of the control of muscle mass and efficacy of interventions in health, ageing and catabolic diseases.

Chapter 5 - The Effects of Age on Chronic MPS and Hypertrophic Mechanisms in Response to Resistance Exercise Training

5.1 Introduction

Advancing age is associated with incipient declines in skeletal muscle mass, strength and quality, processes termed sarcopenia and dynapenia (Mitchell et al. 2012). Since skeletal muscles are central for locomotion and the performance of everyday habitual activities, declines in muscle mass and function lead to impaired quality of life and independence. Furthermore, as skeletal muscle plays a substantial role in whole body metabolic health, muscle loss results in decreased metabolic rate (Tzankoff & Norris 1977) and increased risk of morbidity (Luukinen et al. 1997) and mortality (Laukkanen et al. 1995). With increases in life expectancy showing no signs of slowing (Christensen et al. 2009) and those >60 y to represent 25% of the population by 2050, increased strains on the healthcare system are expected to rise (Janssen et al. 2004).

Muscle mass is controlled by the diurnal balance between MPS and muscle MPB (Atherton & Smith 2012). Therefore, declines in mass occur when MPS is chronically lower than MPB. The most significant regulators of MPS and MPB are food intake (in particular protein (Bukhari et al. 2015; Wilkinson et al. 2015; Atherton et al. 2010b)) and physical activity (Breen et al. 2013; Wilkinson et al. 2014; Kumar et al. 2009; Kumar et al. 2012). Both feeding and exercise transiently stimulate MPS, and when combined result in sustained positive net balance and eventually protein accretion i.e. following a RET program (Phillips et al. 1997; Bukhari et al. 2015; Moore et al. 2009; Moore et al. 2015; Fry et al. 2011b). Whilst basal rates of MPS and MPB are not detectably lower in old age (Volpi et al. 2001;

Kumar et al. 2009), older individuals do exhibit 'anabolic resistance', manifesting as a reduced anabolic response to these key growth signals regulating homeostasis (Kumar et al. 2009; Fry et al. 2011b; Cuthbertson et al. 2005a; Wall et al. 2015). Over time, inadequate replenishment of muscle protein lost during fasting or inactivity leads to a progressive loss of muscle with age.

RET arguably offers the most effective countermeasure and lowest risk profile strategy to mitigate age-related declines in skeletal muscle mass and strength (Fiatarone et al. 1994). However in response to the same RET-program, older individuals generally show attenuated muscle hypertrophy (Greig et al. 2011; Kosek et al. 2006; Mero et al. 2013; Welle et al. 1996; Peterson et al. 2011), although equal adaptations have been reported (Häkkinen et al. 1998; Mayhew et al. 2009). The exact mechanisms leading to RET-induced anabolic resistance are unclear. Aging can be associated with neuromuscular deficits (e.g. loss of motor units (Campbell et al. 1973)), sustained malnutrition (Morley 2012), physical inactivity (Troiando et al. 2008), suboptimal anabolic hormonal profiles (Feldman et al. 2002) and various co-morbidities and chronic diseases (Marengoni et al. 2011); all of which theoretically represent unfavorable attributes for hypertrophy. Additionally at the cellular level, muscle-signaling responses linked with increased translational efficiency (e.g. mTORC1 signaling) and capacity (ribosomal biogenesis), have shown to be blunted "acutely" with aging (Kumar et al. 2009; Fry et al. 2011a; Stec et al.

2015). Nonetheless, the coordinate mechanisms remain elusive and are likely to be multifactorial in nature.

Current assumptions on the mechanisms of muscle hypertrophy in youth and aging have been largely derived from "acute" metabolic and molecular studies i.e. those undertaken after a single bout of RE (Kumar et al. 2009; Bukhari et al. 2015; Fry et al. 2011b; Miller et al. 2005). Nonetheless, due to the disparity in acquisition of these acute response i.e. nutritional provision, length of measurement and exercise paradigms, the exact relationships between acute responses and blunted adaptations are unclear (Mitchell et al. 2014; Atherton et al. 2015). Similar issues are associated with RET-studies where hypertrophic adaptations are assessed with a single beginning and end-point (Mayhew et al. 2009; Häkkinen et al. 1998) in which physiological insight is great but metabolic and molecular insight is limited.

To circumvent these limitations we have recently developed (Chapter 2) and used novel D_2O methods to investigate temporal mechanistic links between hypertrophy, MPS and anabolic signaling (Chapter 3). Using these methods we further aim quantify MPS responses to 6 weeks RET in young and older individuals and simultaneously assess indices of cellular translational efficiency and capacity.

5.2 Methods

The study protocol and all the methods relating to this section have been previously described in chapter 2

5.2.8 Statistical Analyses

Descriptive statistics were produced for all data sets to check for normal distribution (accepted if $P>0.05$) using a Kolmogorov-Smirnov test. All data are presented as means ± SEM. Subject physical characteristics were measured via t-test with all other data sets were analyzed by repeated measures two-way ANOVA with a Bonferroni correction using GraphPad Prism (La Jolla, CA) Software Version 5. Correlations were assessed using Pearson's product moment correlation coefficient. The α level of significance was set at $P<0.05$.

5.3 Results

5.3.1 Subject characteristics

	Young	Old
Age *(years)*	23±1	69 ±1*
Height *(m)*	1.80±0.02	1.75 ±0.02
Weight *(kg)*	76±3	80 ±3
BMI *($kg.m^{-2}$)*	23.6±1	25.8 ±1
FFM *(kg)*	62±2	59±2
ALM *(kg)*	27.7±1	25.2 ±1
ALM/h^2 *($ALMkg.m^{-2}$)*	8.5±0.2	8.1 ±0.1
SMI	33±1	29±1*

Table 5.1 Physical characteristics of young and older subjects. * Significantly different that young * P<0.05. Data are mean ± SEM

Subject baseline characteristics are shown in Table 1. Young and old subject were well matched on most physical characteristics, having no difference in height, weight or BMI. The only significant baseline difference was that of skeletal muscle mass index (SMI) that represents the total muscle mass in relation to total body mass. As older subjects had slightly less FFM and were slightly heavier, this is reflected as a lower SMI.

5.3.2 Activity

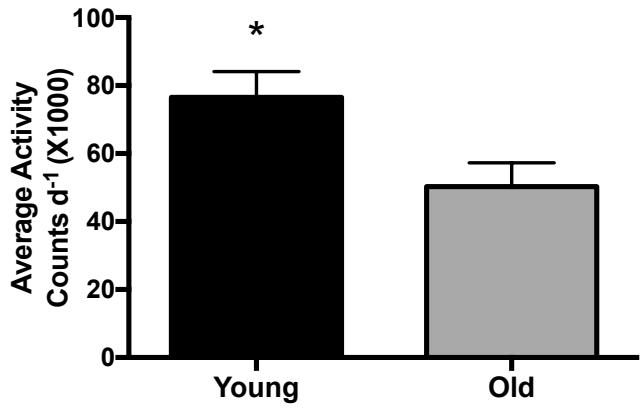


Figure 5.1 Average activity counts in young and old individuals measured over one week of the RET program. * P<0.05. Data are mean ± SEM

	Young	Old
MET min.day^{-1}		
METs <1.5	932±65	932±18
1.5≤ METs< 3	231±32	314±94
3≤ METs< 6	150±30	149±32
6≤ METs< 10.2	3±1	4±1
10.2≤ METs	1.3±0.4	0.9±0.4

Table 5. 2 The amount of time spent at levels of physical activity in young and old individuals over one week of the training study define by the metabolic equivalent of task

Monitoring of habitual activity behaviors over the study period revealed that the younger subjects registered greater activity counts; 72548±5746 counts vs. 52445±7462 counts (P<0.05) in young and old respectively (Fig 5.1). However looking at the amount of minutes spent at levels of physical activity in young and old subjects defined by metabolic equivalent of task there was no difference Table 5.1.

5.3.3 Diet

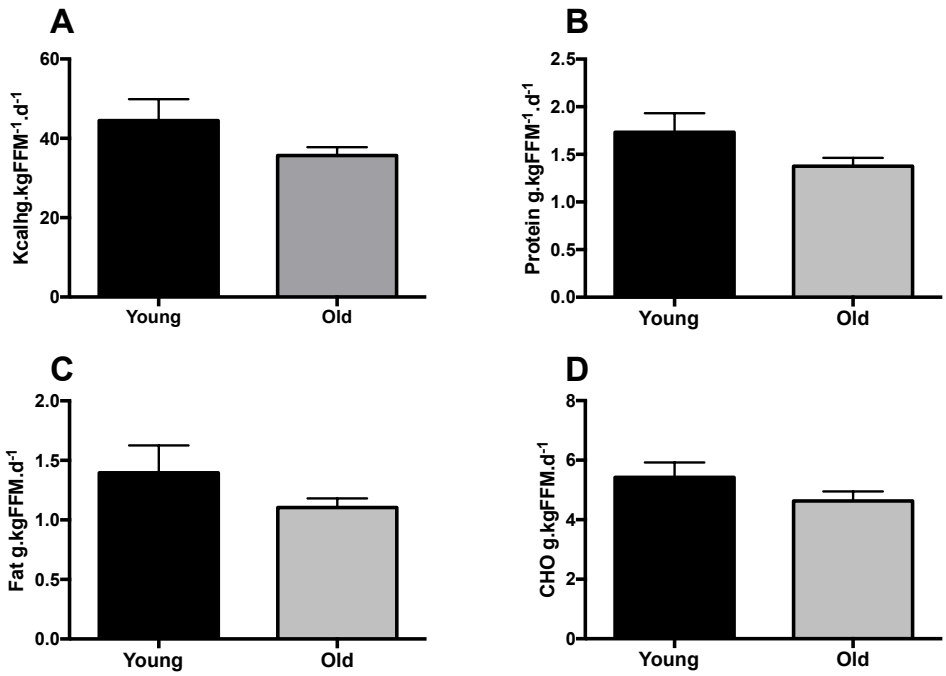


Figure 5.2 The average amount of A) Kcal B) protein C) fat and D) carbohydrate consumed per Kg of FFM per day in young and old subjects.

Habitual Dietary intake was assessed via 4-day diet diaries from one week over the training period. This revealed there was no significant difference in the intake of Kcal 44±5 $g.kgFFM^{-1}.d^{-1}$ vs. 36±7 $g.kgFFM^{-1}.d^{-1}$, fat 1.39±0.2 $g.kgFFM^{-1}.d^{-1}$ vs. 1.1±0.07 $g.kgFFM^{-1}.d^{-1}$ and carbohydrate 5.4±0.49 $g.kgFFM^{-1}.d^{-1}$ vs. 4.63±0.31 $g.kgFFM^{-1}.d^{-1}$ in young vs. old respectively. There was a trend (P=0.1) for older subjects to consume less protein 1.73±0.19 $g.kgFFM^{-1}.d^{-1}$ vs. 1.37±0.09 $g.kgFFM^{-1}.d^{-1}$

5.3.4 Muscle strength

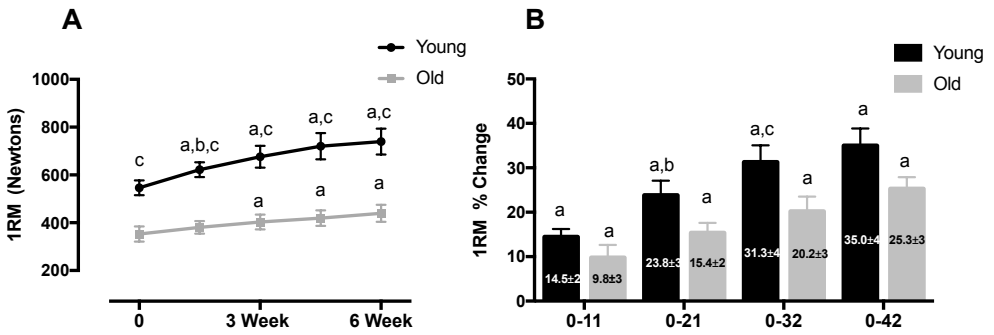


Figure 5.3 Time course of changes in A) 1RM and B) % change in 1RM in trained legs over 6 weeks in young and old subjects. a significantly different from baseline P<0.05. b significantly different from previous time point P<0.05. c significantly different than old P<0.05

At the onset of training young subjects had a greater 1RM 546±31 N vs. 352±31 N (P<0.05) and remained significant stronger over the training period. During the 6 weeks young and old subjects significantly increased 1RM young reaching 739±54 N and old 439±36 N both P<0.01 (Fig 5.3A). This equated to an increased of 35.0±4%, P<0.01 in young and O 25.3±3% (P<0.01) in old after 6 weeks with no difference in the increase between groups (Fig 5.3B).

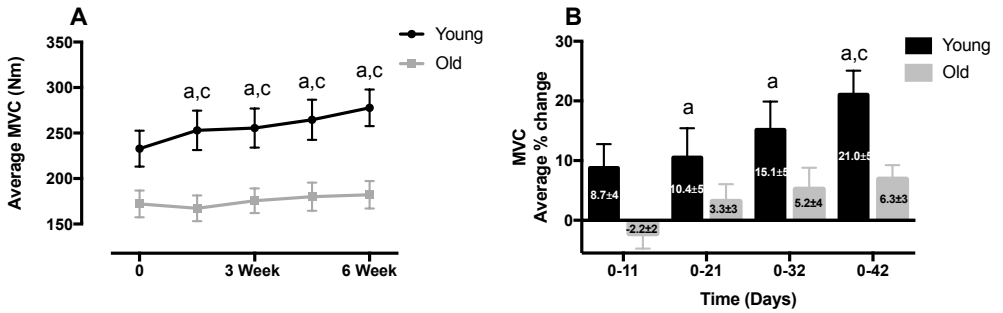


Figure 5.4 Time course of changes in A) average MVC and B) % change in average MV 1RM in trained legs over 6 weeks in young and old subjects. a significantly different from baseline P<0.05. b significantly different from previous time point P<0.05. c significantly different than old P<0.05

At the onset of training the average MVC across joint angles was 232±19 Nm in young and 172±14 Nm old (P=0.09). Over the 6 week training young showed a gradual and significant increase in MVC to 277±20 Nm P<0.01 with no change in old 182±15 Nm (Fig 5.4A). In young subjects the average change across all joint angles at 6 weeks was 21±5%, P<0.01 in Y and 6.3±3% in O (Fig 5.4B). However, taking the peak performance at any given angles for each individual (since optimal performance may vary by angle within individual subjects) both Y and O showed increased MVC (Y 36.2±5%, P<0.001) and O (16.4±4%, P<0.05)

5.3.5 Muscle mass and architecture

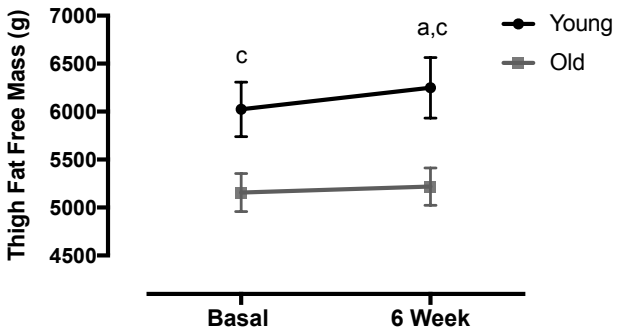


Figure 5.5 TFFM of young and old subjects at baseline and after 6 weeks RET. a significantly different from baseline P<0.05. c significantly different than old P<0.05

TFFM measured by DXA showed that in addition to having greater mass before and after RET, only young increased TFFM after the 6-week training program, 6023±284 g to 6249±315 g (P<0.05) vs. old 5156±198 g to 5218±194 g (Fig 5.5). Temporal changes in VL architecture measured via ultrasound revealed that by 3 weeks RET induced increases in young muscle thickness MT, 2.46±0.1 cm to 2.66±0.1 cm (P<0.01)(Fig 5.6A), pennation angle θ, 18.6±1 º to 19.7±1 º, (P<0.01), (Fig 5.6C) and fibre length Lf, 7.8±0.4 cm to 8.2±9 0.4 cm, (P<0.01), (Fig 5.6B). By 6 weeks of training there was no further increase in any of these variables (2.72±0.1 cm, 20.2±1 º and 8.3±0.4 cm, MT, θ and Lf respectively). In contrast, old did not significantly increase any of these measures over the 6 weeks: MT 2.0±0.1 cm to 2.1±0.1 cm and 2.1±0.1 cm, θ 15.6±0.6 º to 16.3±0.8 º and 16.4±0.8 º and Lf 7.3±0.1 cm to 7.33±0.1 cm and 7.36±0.1 cm at 0, 3 and 6

weeks respectively. The untrained (UT) legs showed no changes in muscle architecture from baseline over the 6 weeks.

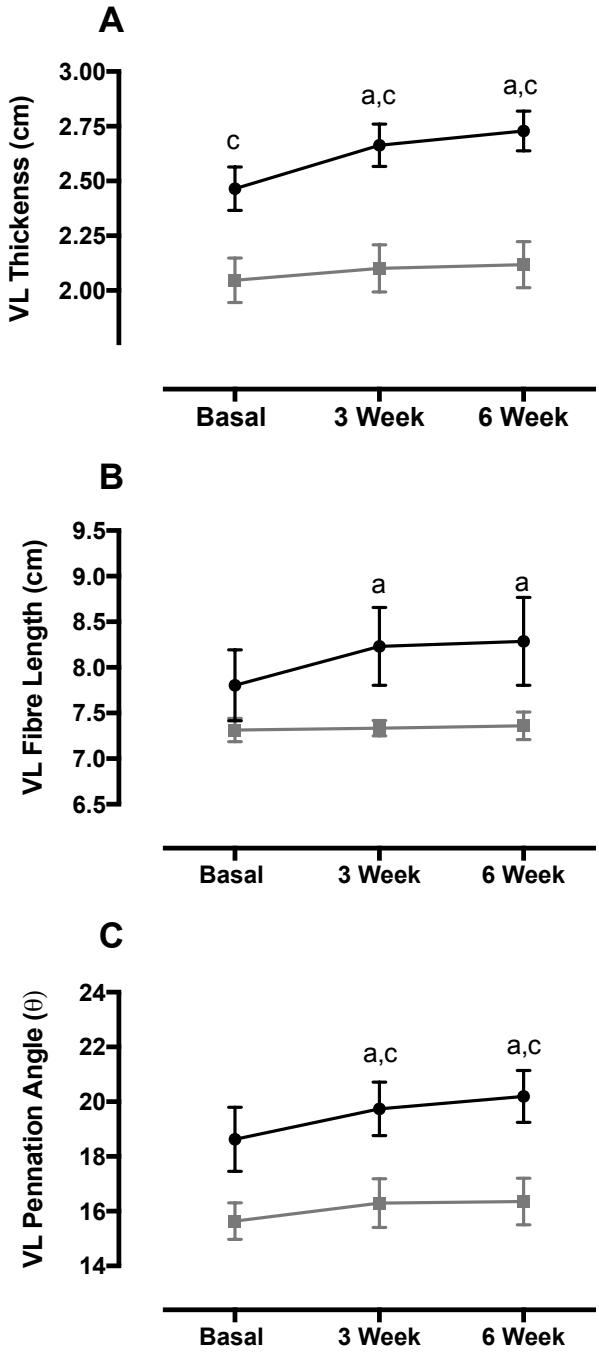


Figure 5.6 Time course of changes in A) MT B) L*f* and C) θ over 6 weeks in young and old subjects. a significantly different from baseline P<0.05. c significantly different than old P<0.05

5.3.6 Muscle RNA, DNA and protein concentrations

There were no changes in the alkali soluble protein per wet weight muscle (ASP,μg.mg^{-1}) in trained legs of either group throughout the 6-week RET period, although young had greater ASP at 3 weeks than old (613.5±25 vs. 480.5±33, P<0.05, Fig 5.7A). The ratio of ASP:DNA (μg.μg^{-1}), a measure of cell size, displayed a trend for increase by 6 weeks (88±8 to 103±5, P=0.1), whilst old showed no change at any time point (Fig 5.7B). RNA content (μg.mg^{-1}) increased in young only at 3 weeks (2.75±0.2 to 3.91±0.2, P<0.01) with a trend for increase at 6 weeks (3.38±0.1, P=0.1), whilst there was no change in RNA content in old (Fig 5.7C). The DNA content (μg.mg^{-1} wet weight of muscle, Fig 5.7E) did not change in either group over the training period, however the ratio of μgRNA:μgDNA a reflection of ribosomal capacity per unit DNA, increased in young at 3 weeks (0.47±0.05 to 0.62±0.05, P<0.05) and remained increased at 6 weeks (0.64±0.03, P<0.01, Fig 5.7B). In contrast to young, there was no change in RNA:DNA in old (0.53±0.05 to 0.57±0.05 and 0.58±0.05, NS, at 0, 3 and 6 weeks respectively). Finally the ratio of RNA:ASP (μg.mg^{-1}), primarily a measure of ribosomal capacity (Waterlow et al. 1978; Laurent et al. 1978), was elevated at 3 weeks (5.0±0.3 to 6.0±0.3, P=0.06) and increased at 6 weeks (6.1±0.3, P<0.05) with no change in old (4.9±0.5 to 5.7±0.4 and 5.6±0.5, NS) at 0, 3 and 6 weeks, respectively (Fig 5.7F).

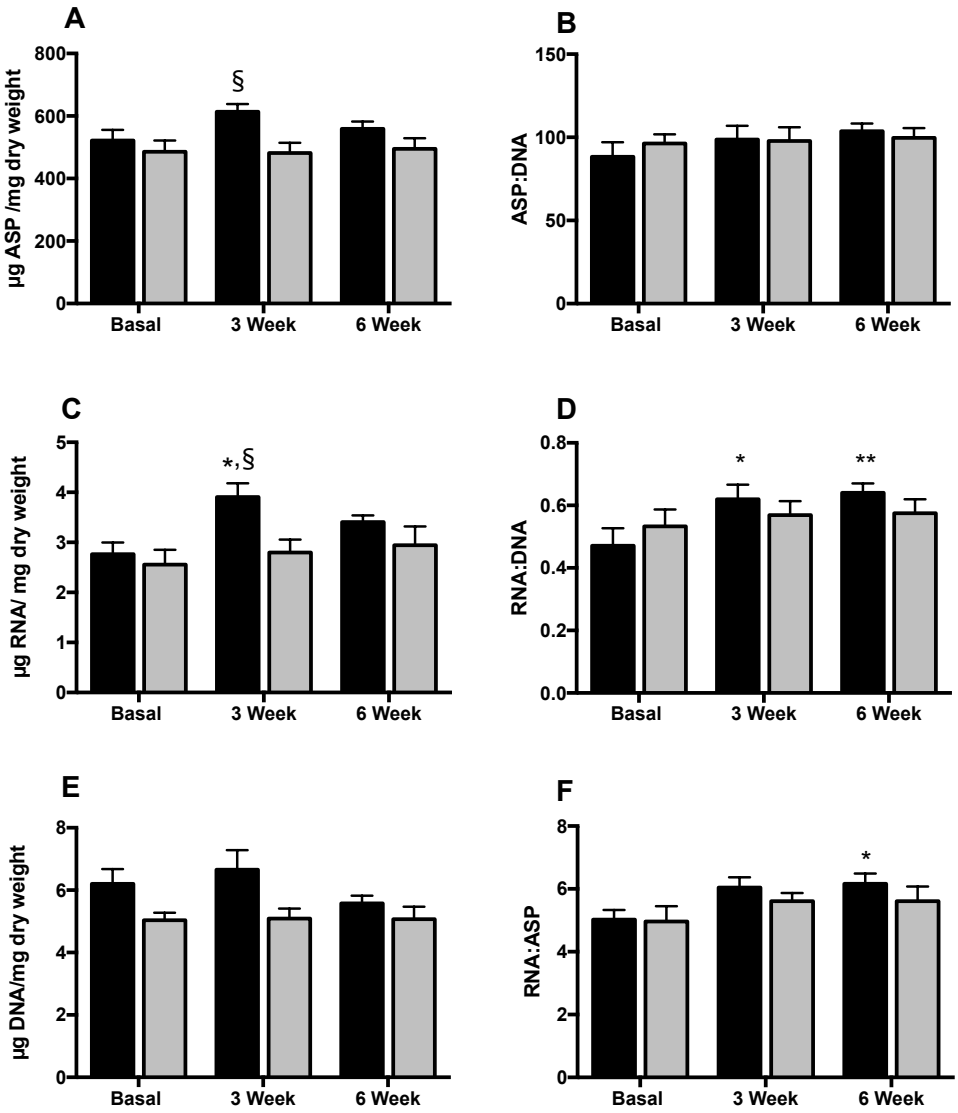


Figure 5.7 Time course of the changes in µg.mg^{-1} dry weight muscle A) ASP, C) RNA, E) DNA and ratios of B) ASP:DNA, D) RNA:DNA and F) RNA:ASP. Values are mean ± SEM. Significantly different from baseline * P<0.05, ** P<0.01, significantly different from old § P<0.05.

5.3.7 Muscle Protein Synthesis (MPS), absolute (ASR) and fractional growth rates (FGR)

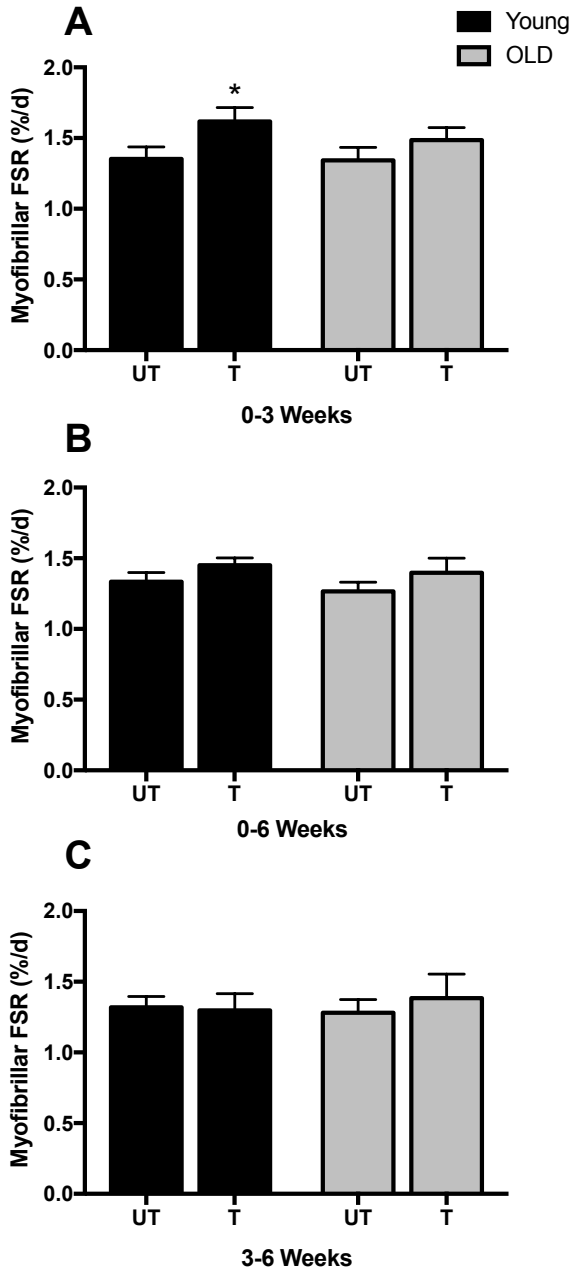


Figure 5.8 Myofibrillar FSR %.d^{-1} at A) 0-3 weeks B) 0-6 weeks and C) 3-6 weeks in young and old. Values are mean ± SEM. * Significantly different from rest $P<0.05$

There was no difference in MPS in the rest leg over the 6 week training program and no difference between Y and O being ~1.3 %.d^{-1} throughout (Fig 5.8A). With the onset of RET, only Y increased MPS from 0-3 weeks 1.6±0.01 %.d^{-1} (P<0.05) vs. O 1.49±0.08 %.d^{-1}. By 6 weeks MPS was no longer increased in either Y 1.45±0.05 %.d^{-1} and remained unchanged in O 1.39±0.1 %.d^{-1} (Fig 5.8A) reflected by no significant increases in the 3-6 week MPS of 1.29±11 %.d^{-1} and 1.37±0.2 %.d^{-1}

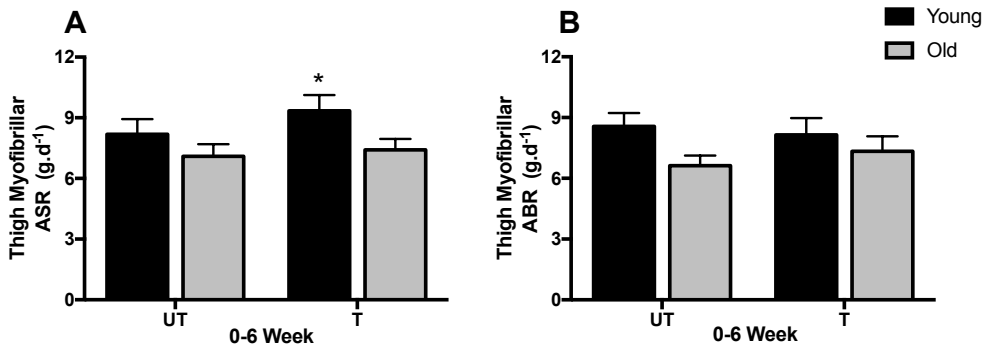


Figure 5.9 A) Thigh myofibrillar ASR over 0-6 weeks and B) Thigh myofibrillar ABR over 0-6 weeks in young and old. * Significantly different from rest P<0.05

Absolute synthetic rate, (ASR, g.d^{-1}) was increased in the T leg only in the young group over the 6 weeks 8.1±0.1 to 9.3±0.7 (P<0.05) (Fig 5.9A) In contrast there was no change in absolute breakdown rate (ABR) g.d^{-1} observed with either group over the 6 week RET period 8.5±0.6 to 8.1±0.8 in young and 6.6±0.5 to 7.3±0.7g in old (Fig 5.9B). Similarly FGR %.d^{-1} increased in Y only(-0.002±0.016 to 0.086±0.02, P<0.001 (Fig 5.10).

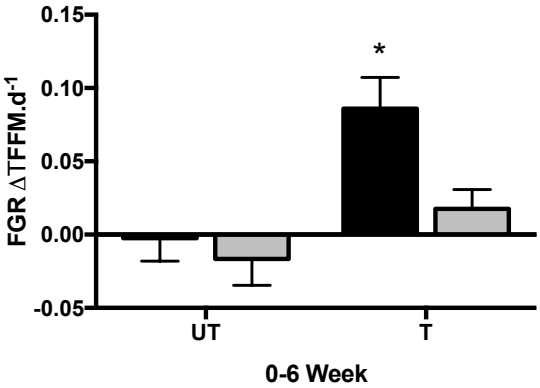


Figure 5.10 FGR in TFFM.d^{-1} over 0-6 weeks in young and old. * Significantly different from UT P<0.001

5.3.8 Hormones

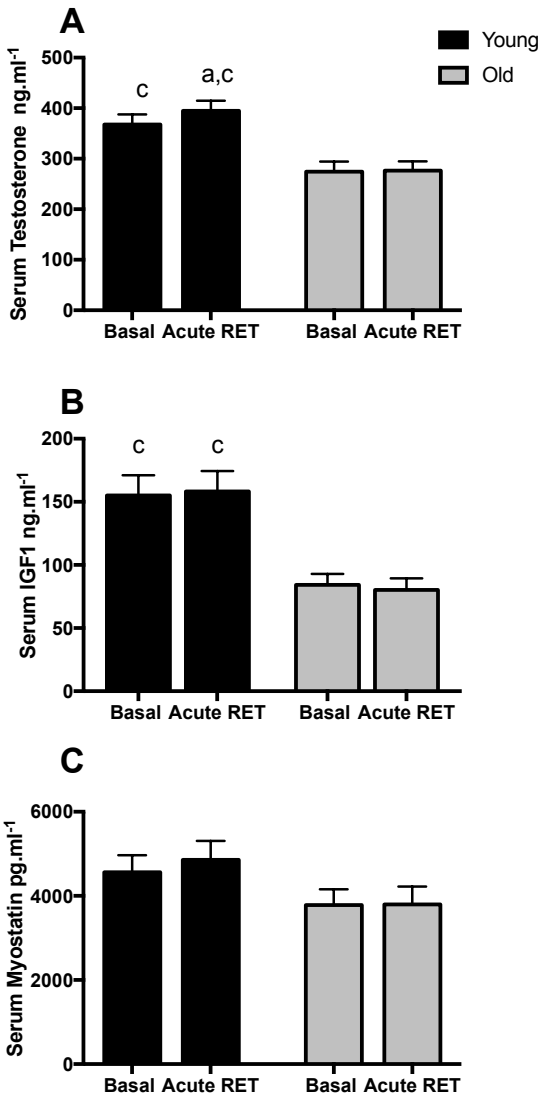


Figure 5.11 Serum concentrations of A) Testosterone B) IGF-1 and C) myostatin at baseline and 60-90min after the first bout of RET in young and old subjects. a significantly different from baseline $P<0.05$. c significantly different than old $P<0.05$

At baseline and after acute RE, serum concentrations of testosterone and IGF1 were significantly higher in Y than O. Basal testosterone was 367±19ng.ml^{-1} in young and 274±19ng.ml^{-1} in old $P<0.01$ (Fig 5.11A). Whilst levels of IGF-1 were 155±16ng.ml^{-1} in young and 84±9 ng.ml^{-1} in old $P<0.01$ (Fig 5.11B). However there was no significant difference in levels of myostatin either before or after exercise being 3781±373pg.ml^{-1} in young and 4563±403 pg.ml^{-} in old at baseline (Fig 5.11C). After the first RE bout there was only a significant increase in testosterone and this occurred inly in young individuals to 394±20ng.ml^{-1} $P<0.05$.

5.3.8 Intramuscular signaling

Muscle protein synthesis

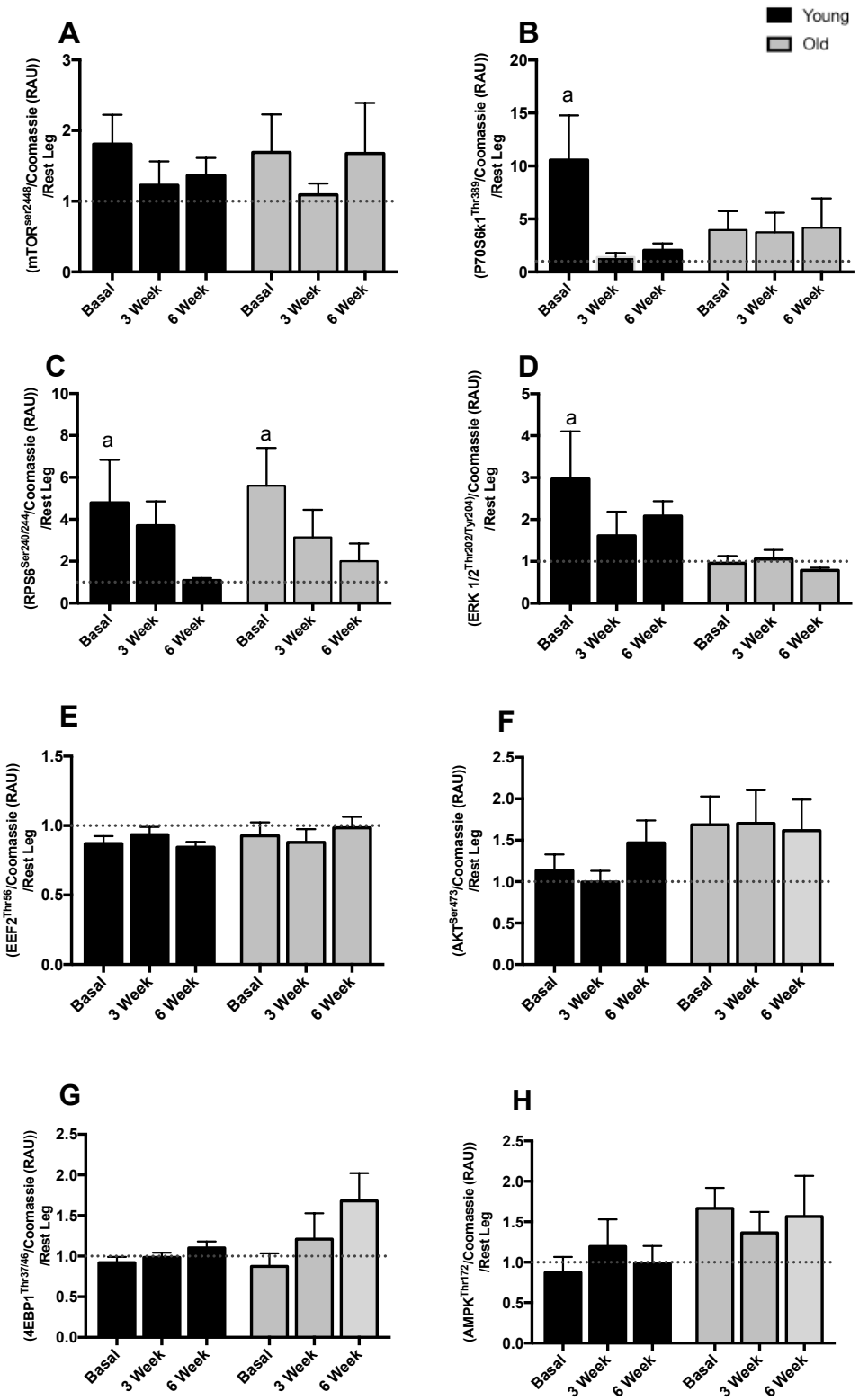


Figure 5.12. Relative change compared with UT in A) mTOR^{Ser2448} B) p70S6K1^{Thr389} C) rps6$^{ser240/244}$ D) ERK1/2^{Thr202Tyr204} E) eEF2^{Thr56} F) Akt^{Ser473} G) 4EBP1$^{Thr37/46}$ H) AMPK^{Thr172}.Values are mean ± SEM. [a] significantly different from UT P<0.05. Untrained (UT)

Muscle Protein Breakdown

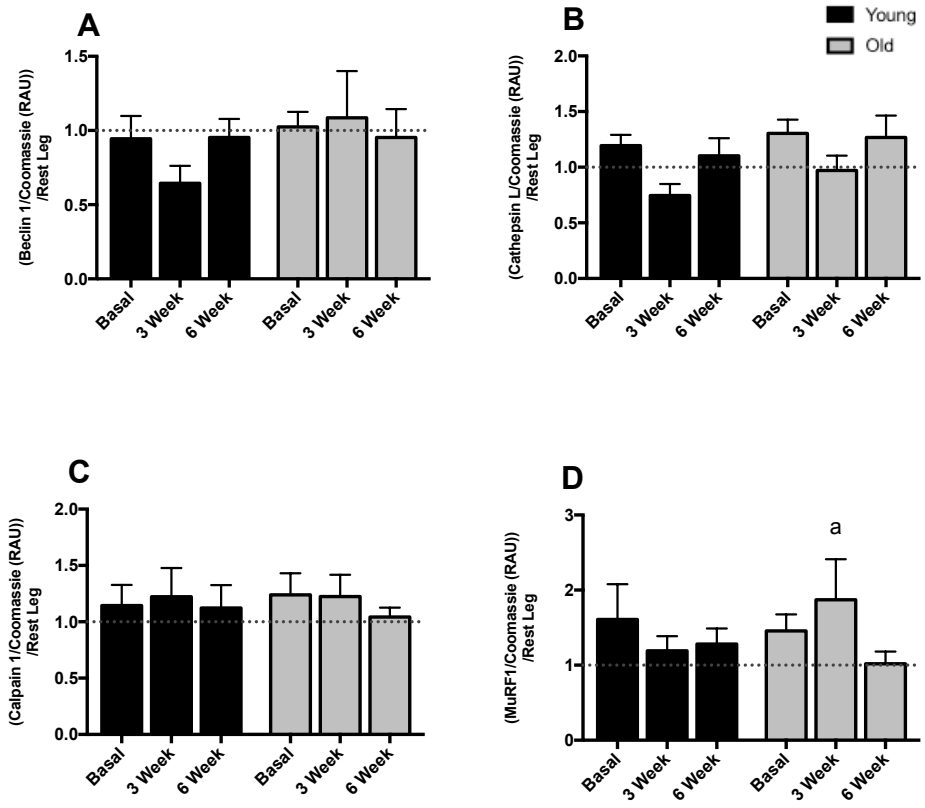


Figure 5.13 Relative change compared with UT in A) Beclin 1 B) Cathepsin L C) Calpain 1 D) Values are mean ± SEM. [a] significantly different from UT P<0.05. Untrained (UT)

Ribosomal Biogenesis

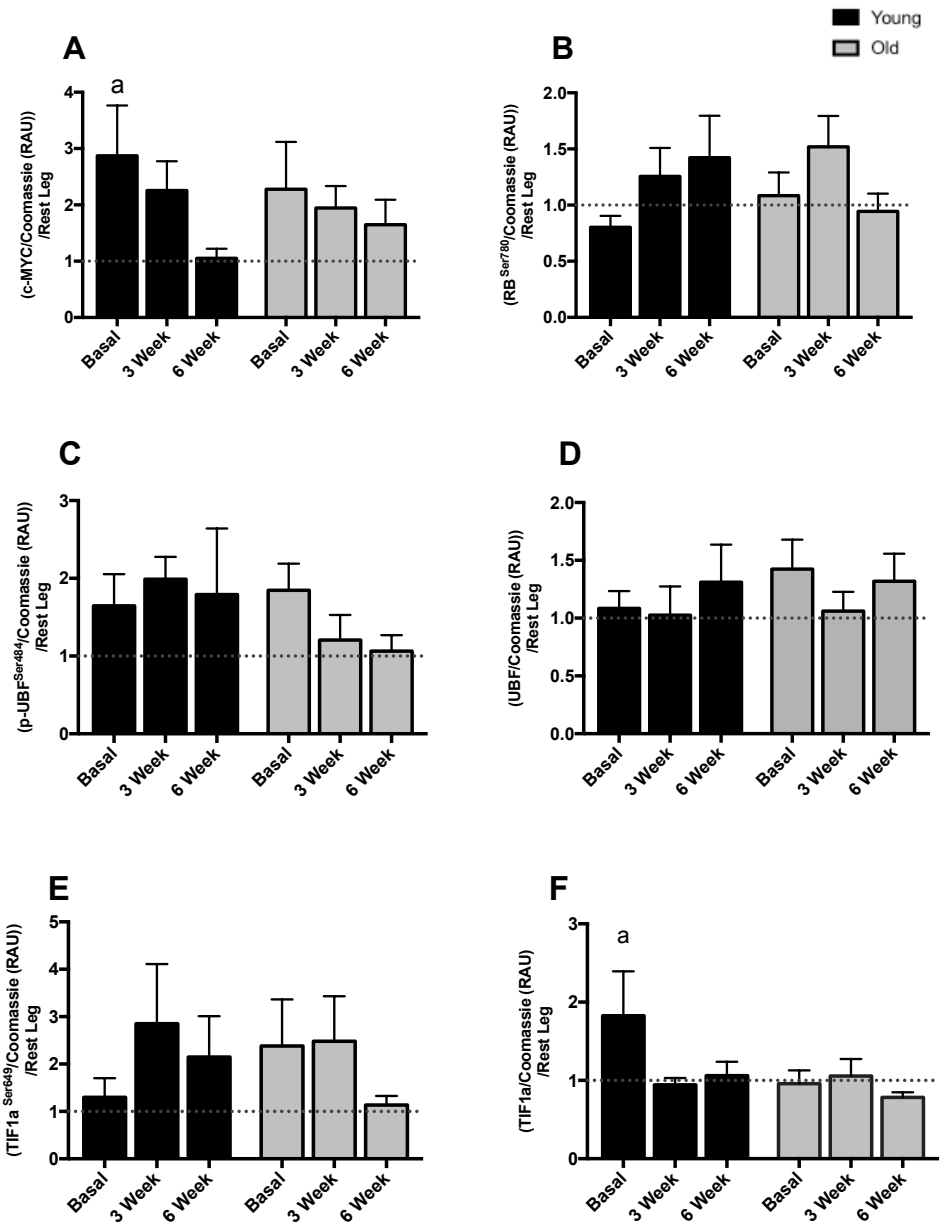


Figure 5.14 Relative change compared with UT in A) c-MYC B) pRB^{Ser780} C) UBF1^{Ser484} D) UBF1 E) TIF1a^{Ser649} F) TIF1a. Values are mean ± SEM. [a] significantly different from UT P<0.05. Untrained (UT)

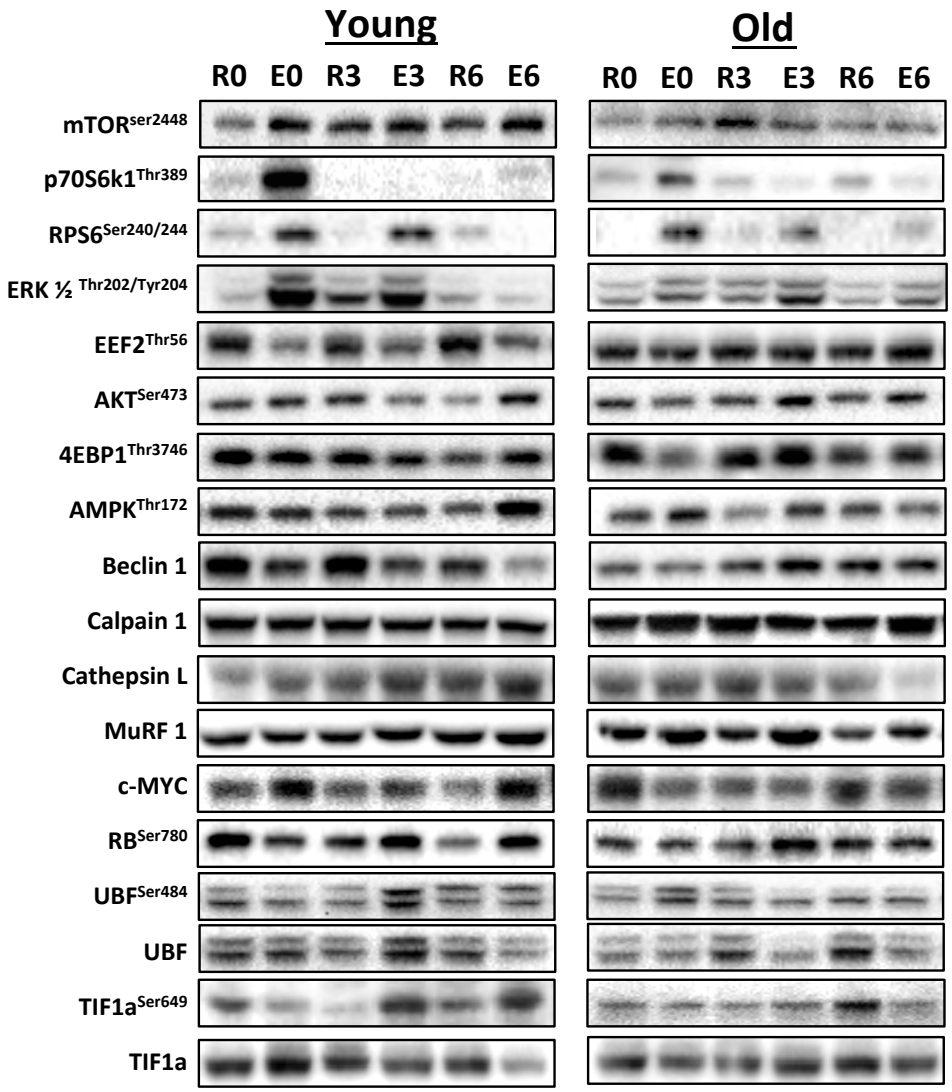


Figure 5.15 Representative blots of signaling targets used in Fig 5.12, Fig 5.13 and Fig 5.14 for young and old subjects

Muscle anabolic signaling 60-90 min after the first exercise bout showed there were significant increases compared to UT in phosphorylation of P70SK1^{Thr389}, RPS6$^{ser240/244}$, ERK$^{Thr202/Tyr204}$). Further only Y increased total levels of c-MYC and TIF1a after the first exercise bout, whilst O increased phosphorylation of RPS6 $^{ser240/244}$ after the first exercise bout and MuRF-1 60-90 min after RE at 3 weeks (Fig 5.12, 13, 14) .

Intramuscular mRNA expression

Ribosomal Biogenesis

		Basal		3 Weeks		6 Weeks	
		Rest	**Acute RET**	**Rest**	**Acute RET**	**Rest**	**Acute RET**
C-MYC	Y	1.00±0.4	**4.01±1.6***	1.52±0.6	2.45±0.6	1.16±0.3	2.49±0.5
	O	0.75±0.3	1.89±0.8	0.66±0.2	2.95±0.9	0.73±0.2	2.73±0.7
POL1RA	Y	1.00±0.1	1.41±0.2	1.25±0.2	1.49±0.2	1.41±0.2	1.03±0.2
	O	1.21±0.2	1.36±0.2	0.95±0.1	0.97±0.2	0.81±0.1	1.41±0.3
POL1RB	Y	1.00±0.2	1.62±0.4	1.93±0.4	1.39±0.3	1.71±0.3	1.39±0.2
	O	1.24±0.4	1.22±0.3	1.47±0.5	1.69±0.5	1.33±0.3	2.60±0.6
TAF1A	Y	1.00±0.3	1.62±0.4	1.47±0.3	0.84±0.2	1.21±0.1	0.69±0.1
	O	1.14±0.3	1.28±0.4	1.27±0.3	1.44±0.3	1.55±0.6	2.01±0.6
TIF1A	Y	1.00±0.3	1.43±0.5	1.61±0.5	0.87±0.3	1.61±0.3	0.98±0.3
	O	1.29±0.4	1.25±0.5	1.04±0.2	1.18±0.3	1.49±0.4	1.57±0.4
UBF	Y	1.00±0.2	1.59±0.4	1.34±0.2	1.77±0.4	1.51±0.2	1.79±0.6
	O	1.42±0.4	2.00±0.6	1.98±0.6	0.69±0.2	1.58±0.3	3.03±0.6
RPS5	Y	1.00±0.3	1.52±0.7	1.24±0.3	1.13±0.3	1.22±0.2	1.18±0.2
	O	1.42±0.4	1.24±0.4	1.80±1.0	1.05±0.3	1.32±0.4	1.68±0.3
RPSA	Y	1.00±0.2	1.07±0.2	0.96±0.2	0.78±0.2	0.78±0.1	0.81±0.2
	O	0.77±10.1	1.19±0.4	0.75±0.3	0.45±0.1	0.65±0.1	0.76±0.1
RPS26	Y	1.00±0.1	0.97±0.1	1.11±0.1	0.97±0.1	1.01±0.1	0.87±0.1
	O	1.00±0.1	**1.48±0.2***	1.16±0.1	1.32±0.2	1.04±0.1	1.17±0.2
RPS3	Y	1.00±0.1	1.23±0.2	1.19±0.2	1.27±0.2	1.17±0.1	0.92±0.1
	O	1.22±0.3	1.49±0.3	1.26±0.2	1.35±0.2	1.21±0.2	1.82±0.3
RPL13A	Y	1.00±0.2	1.31±0.2	1.52±0.2	1.19±0.2	1.37±0.1	1.11±0.2
	O	1.44±0.4	1.31±0.3	1.19±0.3	1.31±0.4	1.11±0.3	1.53±0.3
RPL11	Y	1.00±0.2	1.91±0.5	2.19±0.4	1.12±0.3	1.73±0.2	1.09±0.4
	O	1.33±0.4	1.55±0.5	1.05±0.3	1.31±0.4	1.15±0.3	1.95±0.4

Table 5.3 Muscle mRNA expression of genes involved in ribosomal biogenesis. Data expressed as fold change from young (mean ±SEM) Significantly different from UT leg at that time point.

Muscle Protein Breakdown

		Basal		3 Weeks		6 Weeks	
		Rest	Acute RET	Rest	Acute RET	Rest	Acute RET
MuRF1	Y	1.00±0.2	2.86±0.8	0.67±0.1	1.61±0.4	1.22±0.5	1.01±0.4
	O	1.41±0.3	3.86±1.5	1.01±0.3	1.81±0.5	1.74±0.9	2.43±0.8
MAFbx	Y	1.00±0.2	2.05±0.4	1.19±0.3	2.07±0.3	1.47±0.2	1.26±0.2
	O	1.26±0.2	**3.12±0.9***	1.20±0.2	2.23±0.7	1.38±0.4	2.12±0.6
20S proteasome	Y	1.00±0.31	1.31±0.4	1.36±0.3	1.10±0.3	1.41±0.3	1.13±0.2
	O	1.21±0.22	1.47±0.5	1.18±0.3	1.27±0.3	1.69±0.5	1.94±0.6
Caspase 3	Y	1.00±0.3	1.45±0.4	1.38±0.3	1.00±0.2	1.73±0.4	1.52±0.4
	O	0.79±0.2	0.91±0.3	1.09±0.3	1.27±0.3	1.27±0.4	1.85±0.6
Calpain L	Y	1.00±0.3	1.81±0.8	1.53±0.4	1.01±0.2	2.35±0.9	0.80±0.2
	O	1.39±0.3	1.95±0.7	1.82±0.5	2.01±0.6	2.11±0.7	2.55±0.7

Table 5.4 Muscle mRNA expression of targets involved in MPB. Data expressed as fold change from young (mean ±SEM). * Significantly different from UT leg at that time point $P<0.05$

		Basal		3 Weeks		6 Weeks	
		Rest	Acute RET	Rest	Acute RET	Rest	Acute RET
Androgen Receptor	Y	1.00±0.2	0.99±0.2	0.99±0.3	1.04±0.2	1.82±0.4	0.83±0.1
	O	0.88±0.2	1.15±0.3	1.06±0.3	1.45±0.3	0.90±0.2	1.21±0.3
Myostatin	Y	1.00±0.2	1.42±0.4	2.08±0.5	1.17±0.4	2.74±1.1	1.39±0.3
	O	2.32±0.5	1.96±0.6	2.78±0.6	2.01±0.3	2.58±0.9	1.72±0.3

Table 5.5 Muscle mRNA expression of androgen receptor and myostatin Data expressed as fold change from young (mean ±SEM).

There were no differences between young and old in the baseline expression of any of the genes measured (Table 5.3, 5.4, 5.5). However 60-90 min after the first exercise bout young showed increases in c-MYC expression ($P<0.05$) (Table 5.3), indicating activation of ribosomal transcription ($P<0.05$). Additionally, only old increased MAFBx (Table 5.4) and RPS26 (Table 5.3) 60-90 min after the first exercise bout in T legs compared to UT ($P<0.05$) and showed a trend for increase with MuRF-1 ($P=0.06$)

Correlations

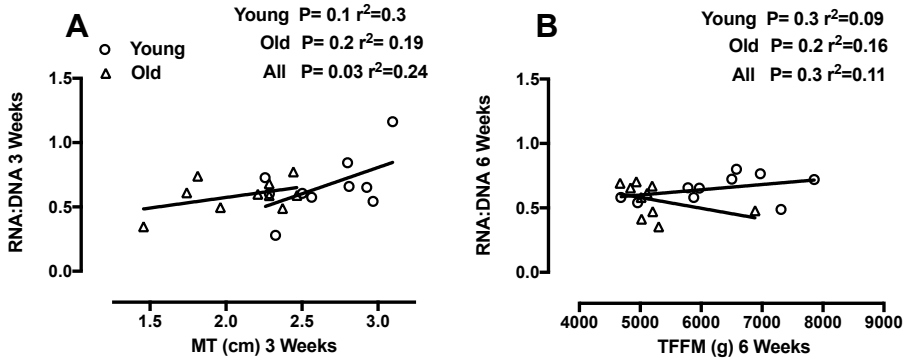


Figure 5.16 Correlations between A) RNA:DNA at 3 weeks and MT at 3 weeks B) RNA:DNA at 6 weeks and TFFM at 6 weeks

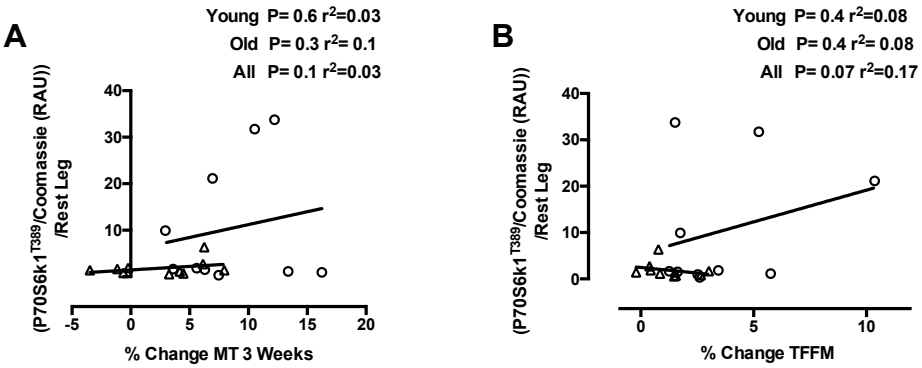


Figure 5.17 Correlations between A) The increase in P70S6K1^{T389} after first RET bout and the % change in MT at 3 weeks B) The increase in P70S6K1^{T389} after first RET bout and the % change in TFFM at 6 weeks

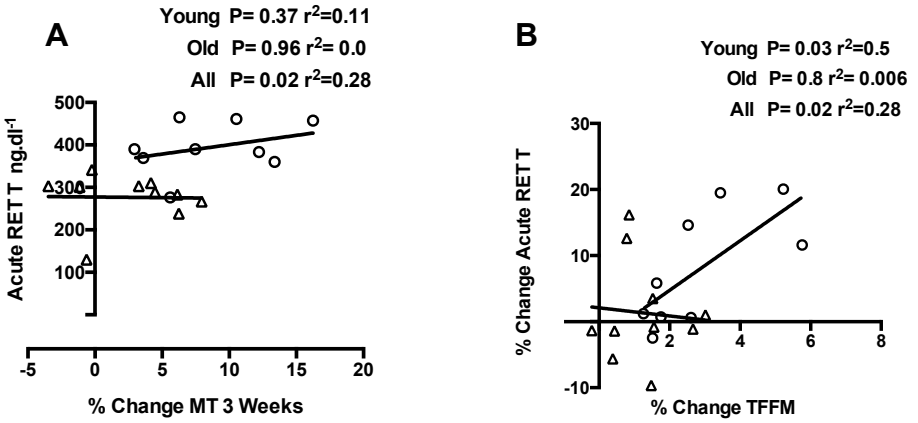


Figure 5.18 Correlations between A) Testosterone after first RET bout and the % change in MT at 3 weeks B) % change in testosterone after first RET bout and the % change in TFFM at 6 weeks

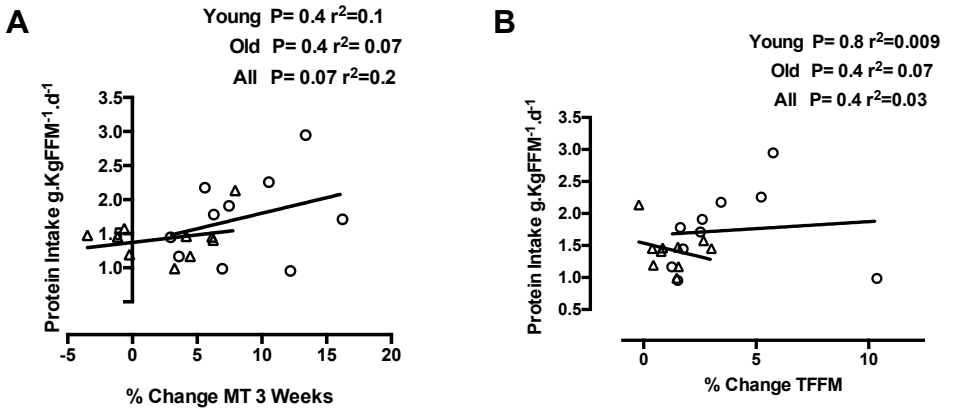


Figure 5.19 Correlations between A) Protein intake g.kgFFM^{-1}.d^{-1} and % change in MT at 3 weeks H) Protein intake g.kgFFM^{-1}.d^{-1} and % change in TFFM at 6 weeks

RNA:DNA was not correlated with MT in the UT state, however at 3 weeks MT was correlated with the RNA:DNA content being driven primarily by changes in the young subjects (Fig 5.16 A), although RNA:DNA was not correlated with TFFM at 6 weeks (Fig 5.16 B). The change in P70S6K1 phosphorylation showed a trend for association with the change in TFFM across all subjects (P=0.07) (Fig 5.17 A), although was not correlated with the change in MT at 3 weeks (Fig 5.17 B). The acute RE induced levels of testosterone were correlated with the change in MT at 3 weeks (Fig 5.18 A) and this change in acute testosterone levels was correlated with the change in TFFM at 6 weeks (Fig 5.18 B). Protein intake across all subjects showed a trend for correlation with the early changes in MT at 3 weeks (Fig 6G, P=0.07) (Fig 5.19 A), although was not correlated with the change in TFFM at 6 weeks (Fig 5.19 B)).

Discussion

There has been a great deal of interest in recent years in uncovering the mechanisms underlying hypertrophic adaptations to exercise, and how these adaptations are influenced by age. Yet much of this information has been largely derived from "acute" metabolic and molecular studies, which may not accurately reflect the true chronic adaptation. Therefore we have used novel D_2O tracer techniques to investigate the temporal effects of RET on longer-term MPS, hypertrophy and strength adaptations in groups of young and older individuals. This provides the first evidence that blunted hypertrophic responses in older individuals are underpinned by chronic deficits in MPS reflecting blunted ribosomal biogenesis, translational efficiency and lower anabolic hormone profiles, thus highlighting the multifactorial nature of the blunted hypertrophic responses.

Whether aging impairs hypertrophic responses to RET remains contentious. For instance, RET-induced hypertrophy of thigh muscles has been shown to be equal (Häkkinen et al. 1998; Mayhew et al. 2009) or impaired (Kosek et al. 2006; Mero et al. 2013; Greig et al. 2011) with age. In the present study, we utilized a 6-week long progressive RET-training program during which we detected no significant increases in either TFFM (DXA) or hypertrophy-related alterations in architecture (ultrasound) in old individuals indicative of blunted RET induced hypertrophy in thigh muscles which are among the muscle groups more profoundly affected by

aging (Janssen et al. 2000) and most important for stability and preventing falls. Similarly, studies of an equal time period in older males have reported no increases in total LBM (Fragala et al. 2014) and similarly no change in VL muscle architecture or TFFM after 6 weeks RET (Scanlon et al. 2014). Indeed, even with long term (~20 weeks) progressive RET, muscle gains are subtle e.g. ~1 kg in whole body (Peterson et al. 2011) and diminished compared to younger counterparts (Phillips et al. 2012). Intriguingly, these data reflect the notion that RET induced hypertrophy occurs rapidly (first 3-4 weeks), diminishing thereafter (Brook et al. 2015). As such, long-term stimuli are neither a pre-requisite for muscle growth nor likely to significantly alter age-related blunted responses. Thus, our present data support the prevailing notion of impaired hypertrophic responses to RET with aging. Although, with time, old individuals will see benefits in whole body strength (Peterson et al. 2010) and noticeable increases in 1RM and MVC were seen here after 6 weeks. Improvements in strength, whether dynamic or static, with little or no increases in muscle mass essentially derive from improvements in 'muscle quality'. Whilst improvements in muscle quality are generally attributed to increased neural activity (Häkkinen et al. 1998; Tracy et al. 1999), improved strength may occur by many other modifiable aspects of muscle contraction (Fragala et al. 2015) improving physical function (Fiatarone et al. 1994; Fragala et al. 2014) and ultimately quality of life (Masel et al. 2009).

Skeletal muscle mass is controlled by the balance between MPS and MPB. Acute amino acid stable isotope tracer studies have shown no difference in fasting or resting MPS or MPB as a result of aging (Volpi et al. 2001). The present study provides the first report of longer-term MPS (over weeks) in young and older individuals in the same study under "basal", conditions. The longer-term D_2O derived measures represent free-living rates of MPS incorporating integrated responses to habitual nutritional intake and physical activity behaviors in young and older recruits. Since loss of muscle mass beyond ~70 y of age occurs at a rate of 0.5-1 $\%.y^{-1}$ (and assuming no changes in MPB), daily MPS would only need to be decreased 0.0015-0.003$\%.d^{-1}$ to account for such a loss. Thus, it is unsurprising that cumulative MPS (even over weeks) was not detectably modified by age. Instead, older muscle demonstrates blunted responses to anabolic stimuli key to regulating muscle homeostasis i.e. nutrition and physical activity (Kumar, et al. 2009; Cuthbertson et al. 2005a). For example, the RET-induced stimulation of MPS is attenuated (<6h) after a bout of RE in older vs. young subjects (Kumar, et al. 2009). Similar has been reported for responses to RET at 3, 6 and 24 h (Fry et al. 2011a) with others also showing reduced rates in older individuals being sustained 24 h after a bout of RET (Mayhew et al. 2009). This study shows that findings from the acute setting translate into longer-term deficits in MPS in the elderly; a notion that would not necessarily be obvious given that acute bouts of exercise (on which the foundation of anabolic resistance has been built) do not correlate to longer term hypertrophy (Mitchell et al. 2014). Finally, it is noteworthy that measures of MPS using D_2O incorporate behavioral

activities outside of study control i.e. habitual physical activity and dietary behaviors. Although the older subjects registered fewer activity counts in this study, they were more active than an average population of their age group (Schrack et al. 2014) and additionally spent equal time in physical activities requiring the same level of metabolic equivalent (MET) as younger recruits. Studies where drastic decrements in activity are enforced, have negative effects on muscle (Breen et al. 2013; Olsen et al. 2008; Krogh-Madsen et al. 2010); in contrast our old subjects are likely to maintain lower levels of activity (Dipietro 2001) in which we are unaware of any evidence that habitual "inactivity" has deleterious consequences upon RET-induced muscle hypertrophy. Habitual behaviors may differ both among individuals and across age- so it cannot be entirely ruled out that slight age-related behavioral differences could contribute to anabolic resistance – in both acute experimental settings and chronic accumulation of these over time. However, intervention studies would be needed to resolve these interesting concepts and may warrant further study.

One of the key facets regulating MPS is ribosomal activity (so called "translational efficiency"). The acute simulation of MPS by RE relies upon increased translational efficiency (Chesley et al. 1992; Drummond et al. 2009). Acute RE (and nutrient interactions) converge on the pleiotropic protein kinase mTORc1, with subsequent downstream signaling enhancing translation initiation and elongation; a pathway essential to acute RE induced stimulation of MPS (Drummond et al. 2009). We and others have previously shown that P70S6K1 signaling after RE is blunted

in older individuals (Kumar et al. 2009; Fry et al. 2011b). In the present study we were able to look at the temporal activation of mTORc1 signaling in young and older individuals. In doing so, we found that P70S6K1 increased only after the first bout of exercise and that this was only observed in the younger group, while RPS6 phosphorylation was increased in both young and older individuals, again only after the first bout. The increase in P70S6K1 activation displayed a trend to be correlated with the increase in TFFM, adding to previous associations between P70S6K1 activation and hypertrophy (Baar & Esser 1999) and dysregulation in MPS responses with age (Kumar et al. 2009; Fry et al. 2011b). Additionally, these findings are in agreement with the notion that hypertrophy occurs early and also that RE trained individuals display attenuations in P70S6K1 (Gonzalez et al. 2015; Coffey et al. 2006). The absolute onset of attenuated signaling responses is unclear, acute stimulation of anabolic signaling responses may remain up until before the 3-week time point, with the duration and pattern of these responses unknown. However, early transcriptional responses are quickly modifiable (Murton et al. 2014) and activation of key signaling proteins are attenuated with repeated exercise stimulus (Ogasawara et al. 2013). This suggests that with prolonged training these signaling pathways become refractory to loading accounting for reduced rates of hypertrophy with training progression. Nonetheless, while age-related blunting was seen in P70S6K1, this was not a uniform response across signals following the first exercise bout, suggesting that blunted translational efficiency is one

aspect, but perhaps not the entire explanation for blunted muscle hypertrophy that is seen in older individuals.

In addition to increasing translational efficiency, prolonged muscle overload produces increases in RNA content and therefore capacity for protein synthesis (Laurent & Sparrow 1977; Kirby et al. 2015), a subject matter which has been the interest of renewed research (Kirby et al. 2015; Stec et al. 2015; Figueiredo et al. 2015; Nader et al. 2014; Figueiredo et al. 2016; West et al. 2015). Indices of synthetic capacity (RNA:protein and RNA:DNA ratios (Smith 2011; Laurent et al. 1978)) increased early into RET in young individuals, plateauing by 3 weeks RET and furthermore these responses were blunted with age. Additionally, with 3 weeks RET, MT was correlated with indices of synthetic capacity (RNA:DNA), which was not evident in the UT state, suggesting early hypertrophy is supported by early increases in synthetic capacity. To gain insight into the molecular mechanism regulating ribosomal biogenesis, upstream signaling pathways controlling rDNA transcription such as c-MYC, which controls many aspects of cell growth and ribosomal biogenesis, were investigated. c-MYC gene expression and protein abundance were increased in young but were blunted with age, which is in line with previous reports showing that markers of ribosomal biogenesis are reduced in older adults 24h after a bout of RET and suggesting rDNA transcription is compromised with age (Stec et al. 2015). Successful rDNA transcription requires multiple transcription factors including UBF and TIF1a with their activities controlled by many phosphorylation sites and upstream signaling

pathways (reviewed in (Kusnadi et al. 2015)). However, similar to previous studies TIF1a increased only in young individuals ostensibly driving rDNA transcription (Stec et al. 2015). Together, our data point to impairments in the regulation of translational capacity in addition to the aforementioned translational efficiency.

Beyond mechano-sensitive pathways, muscle mass and RET-induced muscle hypertrophy are also regulated by hormones, e.g. testosterone, IGF-1, myostatin etc (Bhasin et al. 1996; Kvorning et al. 2006; Whittemore et al. 2003; Barton-Davis et al. 1998). Aging negatively regulates the "anabolic' environment with declining endocrine function resulting in chronic low levels of many hormones such testosterone and IGF-1(Leifke et al. 2000). Whilst myostatin negatively regulates muscle mass, enhancing levels of GH/IGF-1 and testosterone, or restoring them in older age, act as powerful anabolic agents and can substantially increase muscle mass (Sattler et al. 2009; Neto et al. 2015; Bhasin et al. 2001). Compromised hormonal balances with age are therefore likely to impair exercise adaptations, yet the precise influence in early exercise adaptations is unclear. Upon examination of these, we found no difference in myostatin between young and old individuals and this was not acutely affected by RE. Similarly sensitive LC-MS/MS methods have demonstrated that in males, serum myostatin acts as a homeostatic regulator of muscle mass rather than a cause of sarcopenia (Bergen et al. 2015), further adding to oppose evidence that myostatin is implicated in the age relate muscle mass loss (Yarasheski et al. 2002). Circulating levels of many anabolic

hormones decrease with age and we found significantly lower circulating levels of testosterone and IGF-1; moreover only younger individuals increased testosterone after the first bout of RE. Prevention of testosterone production in young males through provision of a GnRH inhibitor has been shown to prevent RET induced hypertrophy (Kvorning et al. 2006), whilst blunted hypertrophy has previously been reported in older men displaying reduced RE induced increases in testosterone (Kraemer et al. 1999). This highlights impaired endocrine response in older age, yet whether a lack of acute hormonal responses contributes to diminished hypertrophy is unclear. Acute RE induced increase in testosterone was correlated with the increase in TFFM and this was strongest in Y where testosterone had increased. Further, whole body RET in older subjects with low testosterone show minimal (~+0.6%) changes in FFM after 12 months RET (Hildreth et al. 2013) while no change in FFM occurred in elderly males with low normal testosterone after 24 weeks RET (Kvorning et al. 2013). Both studies showing greater improvements in FFM when testosterone supplementation and RET are combined. Together with our data this demonstrates that chronic low levels of testosterone and possibly a lack of acute increases may play a significant role in attenuated hypertrophy with age.

In addition to transient increases in MPS with RE, MPB is also increased (Phillips et al. 1997), likely to remove old or damaged proteins. The protein ligases MuRF-1 and MAFBx are markers of ubiquitin proteasome pathway activity which is thought to be predominant in acute RE

responses (Fry et al. 2013). However, after a single bout of exercise expression of MuRF-1 and or MAFBx have shown either no change (Greig et al. 2011; Fry et al. 2013), equal increases (Stefanetti et al. 2014; Fry et al. 2013) or a greater increase with age (Raue et al. 2007) and therefore much uncertainty exists within the literature. We found an initial increases in MAFBx expression and a tendency towards greater MuRf1 expression that only occurred in our older volunteers. Our measures were made 60-90 min after RET, in comparison to ≥2 hrs in these other studies and so our results may highlight key early differences in MPB. Measures of the fractional breakdown rate (FBR) 24h after RET have shown no difference between young and old, however if there are any earlier differences is unknown (Fry et al. 2013). Nonetheless, despite greater initial increases in MPB markers with age, there was no increase in ABR highlighting lack of muscle hypertrophy in older age is likely predominantly driven by blunted MPS responses rather than increases in MPB.

We also need to acknowledge limitations. The data is limited to changes between 3 time points and each individual's response may temporally differ across the study period. Further, biopsy timings within this study reflect acute responses to RE and how these adapt over time and do not demonstrate how resting levels of gene and protein expression may change with training. Additionally, although D_2O permits long-term measures of MPS, these are highly integrated, incorporating everyday habitual regimes with RE responses. In this study, subjects followed their

usual habitual physical activity (potential impacts as previously discussed) and diet, which we recorded. In this study, older subjects tended i.e. non-significantly to consume lower protein levels than younger subjects, which could theoretically impact our findings since acute protein feeding coupled to RET enhances MPS (Bukhari et al. 2015). That said, results of protein supplementation with RET in ageing have been mixed i.e. shown to enhance (Esmarck et al. 2001) or not affect (Verdijk et al. 2009) muscle hypertrophy, while the effects of habitual protein feeding behaviors in this context of RET, ageing and hypertrophy remain poorly defined. That said, since protein intake was not correlated with muscle mass changes in young, old or all subjects combined in our study, any slight differences in protein intake are highly unlikely to be responsible for the observed anabolic resistance. In conclusion we have shown that age-related deficits in anabolic responses to RET are multifactorial, a product of attenuations in translational efficacy, capacity and unfavorable hormone profiles, culminating in reduced MPS and compromised overall hypertrophy.

Chapter 6 – Overview

Ageing populations will soon represent one of the most significant socioeconomic problems in developed societies with substantial pressure placed upon healthcare systems (Rice & Fineman 2004; Robinson & Reinhard 2009; Harper 2014). This is due to a continuous increases in life expectancy, the result of progresses in technology, health care and social practices (Christensen et al. 2009). By 2050 the world health organization predicts that there will be 2 billion people over the age of 65 compared to 600 million today (Harper 2014). Although increased life span maybe desirable, ageing is associated with lack of independence, frailty and disability (Fielding et al. 2011).

There are many changes in body composition throughout life, with one of the most striking being loss of muscle mass with ageing (Delmonico et al. 2009). Beyond the age of ~50y there is a progressive and gradual decline in skeletal muscle mass and strength, termed sarcopenia (Mitchell et al. 2012). Despite lacking clinical definition, sarcopenia is a widespread condition and is associated with frailty, lack of mobility and mortality (Cruz-Jentoft et al. 2010). As such the estimated health care costs of sarcopenia in the year 2000 were ~18 billion, with significant rises in the ageing population expected this will substantially rise (Janssen et al. 2004)

The mechanisms underlying muscle mass loss with ageing are incompletely defined. However older individuals display reduced MPS in response to anabolic stimuli such as nutrition and exercise (Cuthbertson et al. 2005b; Kumar et al. 2009). Over time 'anabolic resistance' results in

the chronic inability to replenish lost muscle protein and represents one of the primary concepts in age related muscle loss (Atherton & Smith 2012).The most effective strategy at increasing muscle mass, strength and function is through RE and supportive nutrition (Fiatarone & O'Neill 1994), offering a potential countermeasure against sarcopenia. However overall it seems that hypertrophic responses to RE are reduced compared to younger counterparts. There have been many potential aspects of muscle metabolism suggested to contribute to reduce increases in MPS and impaired hypertrophy including translational efficiency (Fry et al. 2011a; Kumar et al. 2009), ribosomal biogenesis (Stec et al. 2015), satellite activity (Snijders et al. 2014) and unfavorable anabolic hormones (Kraemer & Häkkinen 1998). Altered adaptation is therefore likely to involve multiple processes, yet there contributions are unclear.

Many of our current assumptions on muscle adaptation are taken from acute stable isotope tracer studies (Kumar et al. 2009; Smith et al. 2011). Although these methods are informative they are restricted by time, limited to controlled environments and individual substrates. As such there ability to provide insight on long term adaptation has been questioned (Mitchell et al. 2014). Therefore many gaps remain in our understanding of muscle hypertrophy not only just in young individuals but also in the impaired responses seen with age. Without methodological progression our ability to investigate the metabolic and mechanistic basis of skeletal muscle hypertrophy is limited.

Here we have developed and validated methods for the long-term measurement of multiple aspects of muscle metabolism including, protein DNA and RNA. We applied these methods to investigate the temporal mechanistic links between hypertrophy, muscle substrate turnover and anabolic signaling. For the first time we have shown that hypertrophy occurs rapidly with RET, is driven by sustained increases in MPS and RNA synthesis that decline despite progressive intensity and is associated with reduced RET induced mTORC1 signaling

Taking these methods further, we investigated the effects ageing has on chronic muscle protein synthesis and hypertrophic adaptations. Over 6 weeks old subjects displayed a lack of RE induced hypertrophy. This was underpinned by a chronic deficit in MPS. However further to this we also identified significant attenuations in many anabolic factors including decreases in translational efficacy, capacity and unfavorable hormone profiles. These are likely to culminate in compromised overall hypertrophy and thus represent anabolic resistance is multifactorial

Our research will have significant implications on current research within the field as we provide novel methods to simultaneously assess multiple aspects of human metabolism *in vivo*. Further to this we have reported the first data looking simultaneously at the major facets of muscle growth (ribosomal biogenesis, muscle protein synthesis and related signaling pathways) temporally in humans. As such these results provide great insight into hypertrophic adaptations with age and further provide a

platform in which to devise appropriate nutritional and exercise interventions on a longer-term basis.

Of course these results are without inherent limitations. Although we report temporal adaptations during RE induced hypertrophy our resolution is still limited to 3 time points. Individuals show varied RE response and therefore may show altered hypertrophic trajectories depending on "responder status'. Further, biopsy timings within this study reflect acute responses to RE and how these adapt over time and do not demonstrate how resting levels of gene and protein expression may change with training.

A key benefit of using D_2O is the ability to make long-term measures of MPS and will demonstrate the intervention efficacy upon everyday habitual regimes. However this also means that these cumulative measures of MPS are highly integrated and so acute differences maybe become washed out over time. Careful planning of sample collection points is therefore necessary to optimize analytical end points. Further, in this study subjects followed their usual habitual physical activity and diet and introduces a lack of which could theoretically impact our findings. However these behaviors can easily be measured and we have taken such factors into consideration.

A true power in these measures is that it gives you a measure of MPS that reflects that of a true free-living situation. Further, measures of MPS using

D_2O can be made with as little as two biopsies (one if plasma protein is taken as baseline) and sufficient saliva samples throughout the study to measure body water enrichment. These techniques are therefore minimally invasive compared to acute tracer studies. There is no doubt that these methods will lend themselves to investigate muscle metabolism in range of subject populations, including those of more fragile clinical populations and has already showed utility in cancer patients (MacDonald et al. 2015).

Further with the continued development of these methods both within our lab and that of others will be pivotal in the unraveling mechanisms in the regulation of muscle mass. Here we have developed methods for the measurement of muscle DNA and RNA synthesis. We will further our results by applying these methods to our older subjects informing on the role satellite cells and ribosomal biogenesis play in ageing adaptations. With the progress of proteomics and metabolomics we will additionally be able to measure the dynamic response of single proteins or metabolites. This combined with measure of multiple substrate pools and hypertrophic end points will provide holistic view of muscle metabolism.

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Publications, Presentations and Awards:

Publications:

Wilkinson DJ, Franchi MV, **Brook MS**, Narici MV, Williams JP, Mitchell WK, Atherton PJ and Smith K (2014).A validation of the application of $D_{(2)}O$ stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans. Am J Physiol Endocrinol Metab, 306(5), E571–9

Brook MS, Wilkinson DJ, Mitchell WK, Lund JN, Szewczyk NS, Greenhaff PL, Smith K and Atherton PJ (2015) Early skeletal muscle hypertrophy predominates adaptive responses to resistance exercise training, matching long term deuterium oxide (D_2O)-derived measures of muscle protein synthesis and mTORc1-signaling. FASEB J 15-273755. Epub 2015 Jul 13.

Brook MS, Wilkinson DJ, Smith K and Atherton PJ (2015) The metabolic and temporal basis of muscle hypertrophy in response to resistance exercise. Eur J Sport Sci, Epub (1-12)

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Wilkinson DJ, **Brook MS,** Smith K and Atherton PJ (2016 Stable isotope tracers and exercise physiology: Past present and future. J Physiol

Brook MS, Wilkinson DJ, Mitchell WK, Phillips B. E,. Lund JN, Szewczyk NS, Greenhaff PL, Smith K and Atherton PJ (2016) Synchronous deficits in cumulative muscle protein synthesis and ribosomal biogenesis underlie age-related anabolic resistance to exercise in humans. J Physiol

Presentations:

European Conference of Sport Science - July 2014, Amsterdam, Netherlands

British Society for Research on Ageing – July 2014, Liverpool, UK

Biomedical Basis of Elite performance- March 2016, Nottingham, United Kingdom

Posters:

ACSM Integrated Physiology of Exercise - September 2014, Miami, USA

International Conference on Frailty & Sarcopenia Research - April 2015, Boston, USA

Biology of Ageing – October 2015, Singapore

Mini-Symposium on Nutrition: Implications to Ageing Processes and People – October 2016, UK

Awards:

European Conference of Sport Science - Young Investigator Award Winner - July 2014, Amsterdam, Netherlands