Nutritional Manipulation of Exercise Induced Skeletal Muscle Cell Signalling: Implications for Acute Training Adaptations

Samuel G Impey

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"Education is the great engine of personal development. It is through education that the daughter of a peasant can become a doctor, that the son of a mineworker can become the head of the mine, that the child of a farmworker can become the president of a great nation. It is what we make out of what we have, not what we are given, that separates one person from another."

Nelson Mandela

Exeuctive Summary

Traditional nutritional approaches for endurance training typically advise high carbohydrate (CHO) availability before, during and after each training session to suppor high training volume, intensity and recovery. However, during the last decade, accumulating data demonstrate that carefully scheduled periods of reduced CHO availability actually augment training-induced oxidative adaptations of skeletal muscle, the so-called *train-low* paradigm. In accordance with this movement there is also growing rationale ot consume protein before, during and/or after train-low sessions in an attempt to simultaneously promote mitochondrial biogenesis, muscle protein synthesis (MPS) and improve net muscle protein balance. The aim of this thesis was to assess the effects of reduced CHO but high protein availability on the regulation of molecular pathways associated with modulation of the aforementioned components of training adaptation. On the basis of characterising such molecular responses, a secondary aim was to formulate a nevel framework for which to practically apply train-low paradigms.

Given the enhanced oxidative adaptatios observed when training in CHO restricted state is potentially regulated through free fatty acid (FFA)-mediated signalling, the aim of study 1 (chapter 4) was to test the hypothesis that leucine-enriched protein feeding before and during exercise does not impair FFA availability or whole body lipid oxidation during exercise. Here I utulised a novel leucine enriched protein gel and compaired this agains a whey drink or placebo gel in a repeated masures design. This study showed that despite the insulemic effects of protein provision, there was no imparment in FFA availability or whole body lipid oxidation during exercise. Therefore, suggesting that protein feeding does not hinder a key objective of train-low sessions.

Building on the results from study 1, I next saught to characterise the effects of reduced CHO but high leucine availability on exercise capacity and cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis and MPS. While low CHO availability inhibited exercise capacity, comparible mitochondrial signalling responces were seen at the point of fatigue despite participants performing significantly more work in high CHO condition. This demonstrated that training with low CHO is work-efficient in eliciting beneficial signals regulating mitochondrial biogenesis. Despite providing leucine rich protein before, during and after exercise, MPS related signalling could not be rescued during the CHO restriced post-exercise period in the low CHO condition. The data from this study suggest that although there are potential metabolic

benefits associated with reduced pre-exercise CHO availability, the post-exercise meal should contain sufficient CHO to restore muscle glycogen to sufficient levels and/or provide the nexessary energy to support post-exercise remodelling process.

Having identified the potential detrimental effects of low CHO recovery, the aim of study 3 (Chapter 6) was to examine the role of leucine availability in regulating post-exercise skeletal muscle remodelling processes in recovery from a train-low training session. Here I fed one of two protein types, a collagen (naturally low in leucine) or a whey (naturally high in leucine) protein during a low CHO training session, in a repeated measures design. When considered with study 2, the data from this study suggested that leucine is essential for reactivation of signalling mechanisms involved in protein translation, interestingly while low CHO training appeared to activate components of the system that selectively degrades malfunctional parts of the cell, leucine content had no effect on these processes.

When taken together, the novel data presented in this thesis allude to a potential muscle glycogen threshold hypothesis surmising that reduced pre-exercise muscle glycogen may enhance the activation of those pathways regulating mitochondrial biogenesis but also suggest that keeping glycogen (and energy) at critically low levels may impair the regulation if post-exercise remodelling processes. From a practical perspective, data lend support for a potential "fuel for the work required" train-low paradigm in that athletes could strategically reduce CHO availability prior to completing pre-determined training workloads that can be redily performed with reduced CHO availability, thereby inducing a work efficient approach to training. Alternativly, when the goals of the training session are to complete the highest workload possible over more prolonged duration, then adequate CHO should be provided prior to and during the specific training session.

Publications and presented abstracts arising from this thesis:

Publications

Impey SG, Smith D, Robinson AL, Owens DJ, Bartlett JD, Smith K, Limb M, Tang J, Fraser WD, Close GL, Morton JP. Leucine enriched protein feeding does not impair exercise-induced free fatty acid availability and lipid oxidation: beneficial implications for training in carbohydrate restricted states. *Amino Acids* 47(2): 407 – 416, 2015.

Impey SG, Hammond KM, Shepherd SO, Sharples AP, Stewart C, Limb M, Smith K, Philp A, Jeromson S, Hamilton DL, Close GL and Morton JP. Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes. *Phys Reports*. 4(10): e12803, 2016.

Abstracts

Impey SG**,** Hammond KM, Hamilton DL, Glose GL, Sharples AP, Morton JP. Post-exercise co-ingestion of leucine and carbohydrate reactivates skeletal muscle p70S6K1 activity during recovery from a train-low endurance training session. Presented at *International Sport and Exercise Nutrition Conference*, Newcastle, UK, December 2016.

Impey SG, Hammond KM, Sheppard S, Jeromson S, Hamilton DL, Close GL, Morton JP. Low muscle glycogen impairs post-exercise p70S6K activity despite high leucine availability: the critical glycogen hypothesis. Presenting at *European College of Sports Scientists*, Malmo, Sweden, July 2015

Impey SG, Owens DJ, Bartlett JD, Smith K, Close GL, Morton JP. Leucine enriched protein feeding does not impair exercise-induced lipid oxidation: implications for training in carbohydrate restricted states. Presented at *European College of Sports Scientists*, Amsterdam, Nederland, July 2014

Contents

List of Abbreviations

4E-BP1, eukaryotic translation initiation factor 4E binding protein 1

eEF2, eukaryotic elongation factor 2

ATG4b, autophagy related 4b cysteine peptidease

ATG12, autophagy related 12

Atrogin1, F-box protein 32

ADP, adenosine diphosphate

ACC, acetyl-CoA carboxylase

AMP, adenosine monophosphate

AMPK, adenosine monophosphate protein kinase

ATP, adenosine triphosphate

β-OHB, beta-hydroxybutyrate

BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3

CHO, carbohydrate

COXIV, cyclooxygenase 4

FFA, free fatty acid

MPS, Muscle protein synthesis

MuRF1, muscle RING-finger protein-1

ROS, reactive oxygen species

p38MAPK, p38 mitogen activated protein kinase

mTOR, mammalian target of rapamycin

p70S6K, ribosomal protein S6 kinase beta-1

p53, protein of 53 kDa

p62, protein of 62 kDa

Parkin, parkin RBR E3 ubiquitin ligase

PGC-1α, peroxiome proliferator-activated receptor gamma coactivator 1 alpha

PHF20, PHD finger protein 20

PKB, protein kinase B

PPARδ, peroxisome proliferator-activated receptor delta

PPO, peak power output

REDD1, regulated in development and DNA damage responses 1

rt-qRT-PCR, reverse transcriptase quantitative real-time polymerase chain reaction

SIRT1, silent mating type information regulation 2 homolog 1

Tfam, mitochondrial transcription factor A

TSC2, tuberous sclerosis complex 2.

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Chapter 1 – General Introduction

1.1 General Introduction

It is well established that regular physical activity is beneficial for skeletal muscle, cardiovascular, pulmonary and cerebral health (Kodama *et al.* 2009). Additionally, repeated bouts of exercise induce an adaptive effect of these systems thereby establishing new set points in homeostasis (Holloszy and Coyle, 1984). In sport, the deliberate pursuit and attainment of such adaptations is known as the "training" effect, the result of which improves exercise performance (Bassett and Howley, 1999). In the context of endurance exercise, the adaptive response to training on a whole body level is recognised functionally by increases in maximal oxygen uptake ($\dot{V}O_{2max}$) and the classic rightward shift of the lactate threshold curve (Morales-Alamo *et al.* 2015). Such adaptations are underpinned by enhancement in oxygen delivery and utilisation by skeletal muscle, largely mediated via increased cardiac output (Mitchell *et al.* 1958), angiogenesis (Andersen and Henriksson, 1977) and an increase in the size and number of mitochondria within skeletal muscle (Holloszy, 1967). The latter process of mitochondrial biogenesis (an increased mitochondrial mass per gram of muscle tissue) is especially relevant for training-induced improvements in endurance performance (Coffey and Hawley, 2007). Indeed, increased mitochondrial mass and function permits less disturbance to homeostasis for a given workload (e.g. less ADP, AMP accumulation, glycogen utilisation, lactate production etc.), the result of which confers fatigue resistance.

The traditional approach to enhancing endurance training adaptations is to manipulate training intensity, duration and recovery periods where the aim is to optimise the adaptive response to each consecutive training session (Bompa and Buzzichelli, 2015). However, with the increasing utilisation of molecular biology techniques in the sport and exercise science domain, accumulating data over the last two decades has also demonstrated a potent effect of nutrient availability on modulating many of the skeletal muscle cell signalling pathways that regulate components of training adaptation (Philp *et al.* 2012; Bartlett *et al.* 2015). Such nutritional modulation of endurance training adaptation is perhaps most widely recognised for carbohydrate (CHO) availability, where it is now generally accepted that carefully chosen periods of training with reduced CHO availability actually augments oxidative adaptations of human skeletal muscle (Bartlett *et al.* 2015). This approach, is of course, in stark contrast to the long held belief that training should always be completed with high CHO availability before, during and after exercise in order to promote training intensity, training volume and recovery (Bergstrom *et al.* 1967; Burke,

2010; Cermak and Van Loon, 2013). Given the well-documented effects of high CHO availability on "competition" performance (Hawley *et al.* 1997), the strategy that has emerged therefore, is the concept of '*train-low, compete high'*. This approach surmises that athletes deliberately complete specific sections of their training with restricted or 'low' CHO availability in order to enhance the adaptive response of skeletal muscle but yet, competition is always competed with 'high' CHO availability so as to maximise performance (Burke, 2010).

When reviewing the relevant train-low literature to date, numerous independent laboratories have consistently observed that reducing endogenous and/or exogenous CHO availability during short-term (e.g. 3-10 week) endurance training increases mitochondrial enzyme activity and protein content (Morton *et al.* 2009; Yeo *et al.* 2008; Van Proeyen *et al.* 2011), increases both whole body (Yeo *et al.* 2008) and intramuscular lipid oxidation (Hulston *et al.* 2010) and in some instances, improves exercise capacity and performance (Hansen *et al.* 2005; Cochran *et al.* 2015; Marquet *et al.* 2016). The augmented training response observed when "training low" is likely mediated via the complex regulation of cell signalling pathways that co-ordinate increased expression of both nuclear and mitochondrial encoded genes. In the context of nutrient-gene interactions, it is therefore apparent that the acute molecular regulation of cell signalling processes provides a theoretical basis for understanding the molecular mechanisms underpinning chronic training adaptations.

The research designs that have been used to study both acute and chronic train-low adaptations thus far, have largely adopted twice per day training protocols (Hansen *et al.* 2005; Yeo *et al.* 2008; Hulston *et al.* 2010), fasted training (Van Proyen *et al.* 2011) and CHO restriction during (Morton *et al.* 2009) and/or post-exercise (Pilegaard *et al.* 2005). More recently, a "sleep-low, train-low" model has also been developed in which athletes perform an evening training session but sleep with reduced post-exercise CHO intake, followed by completion of a fasted training session on the subsequent morning (Bartlett *et al.* 2013; Marquet *et al.* 2016). Despite the emergence of the aforementioned train-low paradigms, the optimal approach for which to practically apply with athletic populations is not currently known. Such limitations are most well recognised for the potential reductions in absolute training intensity associated with reduced CHO availability (Widrick *et al.* 1993; Yeo *et al.* 2008; Hulston *et al.* 2010), perturbations to immune function and associated increases in muscle protein degradation (Lemon and Mullin, 1980; Howarth *et*

al. 2010), all of which could be detrimental to long term training and athletic performance. To compensate for the latter limitation, one potential strategy may be to consume intact protein or essential amino acids, before, during and/or after exercise. For example, the provision of nutrients in the form of protein (as opposed to CHO) may still allow for the enhanced cell signalling pathways associated with training with reduced carbohydrate availability but also provide an important substrate to promote skeletal muscle remodelling and potentially, minimise any progressive loss of lean mass during training. In this regard, accumulating data suggest that protein provision before (Coffey *et al.* 2011), during (Hulston *et al.* 2011) and after (Breen *et al.* 2011; Howarth *et al.* 2010) endurance or repeated sprint type protocols, enhances post-exercise skeletal muscle protein synthesis (MPS) as well as activation of some of the signalling molecules regulating translation initiation and elongation. Furthermore, increased protein availability reduces lean mass loss during heavy training periods when energy and CHO availability are restricted (Morton *et al.* 2010; Mettler *et al.* 2010; Pasiakos *et al.* 2013). Taken together, the above data suggest that protein ingestion before, during and after train-low sessions may therefore prove beneficial as it is likely to still allow for the enhanced cell signalling responses associated with reduced CHO availability whilst also improving net protein balance. Nonetheless, it is also important to characterise the effects of protein feeding (and associated insulin response) during train-low sessions on circulating free fatty acid (FFA) concentrations, given that some of the training adaptations arising from "train-low" may actually be mediated by increased FFA availability, secondary to the effects of CHO restriction *per se* (Philp *et al.* 2013).

In real world training environments of elite endurance athletes, it is also likely that athletes practice an "amalgamation" of the aforementioned train-low paradigms (either through default of their current training structure or via coach and sport scientist-led practices), as opposed to undertaking one potential strategy in isolation. The complexity of practical train-low models is also further exacerbated by the observations that many endurance athletes (especially cyclists) also practice day-to-day or longer term periods of energy periodisation (as opposed to CHO *per se*) in an attempt to reduce both body mass and fat mass in preparation for key competitive events (Stellingwerff, 2012; Vogt *et al.* 2005). When taken together, such data highlight the requirement to study train-low paradigms that may be more reflective of real world athletic practice (i.e. both CHO and energy

restriction) and that are representative of an amalgamation of the train-low protocols previously studied in the research setting.

The cellular recycling process of autophagy has emerged as an integral component of exercise induced remodelling processes. Indeed studies performed both in rodant and human muscle show that autophagy is activated in response to exercise in order to eliminate damaged or aberrantly folded proteins, contribute to remodelling of muscle as part of the adaptive response to exercise and mobilise proteins as alternative energy substrates for neoglucogenesis by the liver in cases of energetic stress (Schwalm *et al.* 2015). Nonetheless, the understanding of how exercise affects autophagic flux is currently limited due to between species (rodent versus humans), nutritional status (fed versus fasted) and differences in exercise protocols (e.g. modality, intensity and duration) between studies (Vainshten and Hood, 2016). Furthermore, to the best of my knowledge, no investigators have yet examined the effects of endurance exercise on autophagy markers whilst also quantifying muscle glycogen availability. Given the well-documented effect of reduced muscle glycogen availability on exercise-induced protein degradation (Lemon and Mullin, 1980; Howarth *et al.* 2010), there is also a need to better understand the effects of exercise on markers of autophagy when completed under the conditions of reduced CHO (and energy) but high protein availability.

1.2 Aims and Objectives

The primary aim of the present thesis is to examine the effects of reduced CHO but high protein availability on the regulation of molecular pathways associated with modulating mitochondrial biogenesis, MPS and autophagy. On the basis of characterising such molecular responses, a secondary aim is to formulate a novel framework for which to practically apply train-low paradigms.

This aim will be achieved by completion of the following objectives:

a) To test the hypothesis that leucine-enriched protein feeding does not impair circulating FFA availability or whole body lipid oxidation during exercise (Study 1, Chapter 4).

b) To characterise the effects of reduced carbohydrate (CHO) but high leucine availability on exercise capacity and skeletal muscle cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis and MPS (Study 2, Chapter 5).

c) To examine the role of protein composition in regulating skeletal muscle cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis, MPS and autophagy when in recovery from a train-low endurance training session (Study 3, Chapter 6).

Chapter 2 – Literature Review

Literature review

2.1 Introduction

Endurance exercise training induces many adaptations in skeletal muscle that function to improve exercise capacity and performance. The most important of these adaptations is widely recognised as the increase in mitochondrial mass (i.e. mitochondrial biogenesis) which ultimately, permits athletes to exercise at higher intensities for a longer duration (Holloszy, 1967; Holloszy and Coyle, 1984). The mechanism by which an increase in muscle mitochondria induces an improvement in exercise capacity and performance is by reducing the disturbance to metabolic homeostasis for a given exercise intensity (Coffey and Hawley, 2007; Coyle, 2000; Holloszy and Coyle, 1984). In the trained state, acute customary exercise is therefore associated with smaller decreases in ATP, phosphocreatine and muscle glycogen utilisation and smaller increases in ADP, AMP, inorganic phosphate and muscle lactate accumulation. Such adaptations are recognised physiologically by the classic rightward shift of the lactate threshold curve and an increased reliance upon lipid sources to fuel muscle contraction (Camera *et al*. 2016). An increased understanding of the mechanisms by which regular exercise promotes mitochondrial biogenesis is therefore of paramount importance for those individuals with the common goal of improving endurance performance.

With the introduction of molecular biology techniques to the sport and exercise sciences, a large body of research over the last decade has focused on elucidating the cellular and molecular mechanisms underpinning exercise-induced mitochondrial biogenesis (Holloszy, 1967). The potential exercise-induced molecular 'signals' and signal transduction pathways are, of course, undoubtedly multi-factorial and highly complex. These signals (acting alone or likely in combination with each other) can activate a multitude of signal transduction pathways which initiate gene transcription and translation to create new proteins (Coffey and Hawley, 2007). The cumulative effect of each acute exercise bout therefore leads to a change in the steady-state level of specific proteins (Holloszy and Booth, 1976). Depending on the nature of the exercise stimulus (i.e. endurance versus resistance), the proteins up-regulated are typically mitochondrial or myofibrillar based, respectively. It is these repeated and transient changes in gene expression and protein translation which are thought to form the molecular basis for training adaptation.

In addition to training stimulus, the capacity for nutritional modulation to augment adaptation is receiving increased recognition. The concept of training with low carbohydrate (CHO) availability has especially been identified as a potential component of nutrition-training interactions designed to improve performance (Bartlett *et al.* 2015). However, further research is required to elucidate the molecular mechanisms underpinning the enhanced adaptation associated with CHO restriction. Furthermore, the optimal strategy for which to practically apply a train low strategy with athletic populations is not currently known.

The present chapter presents an overview of some of the key signalling pathways currently thought to mediate mitochondrial biogenesis, skeletal muscle protein turnover and autophagy. A brief review of how specific nutritional strategies affect these pathways is also included. This chapter is intended to provide the reader with an extended introduction and rationale as to the nature of the studies undertaken in this thesis. Where possible, data are reviewed from human studies though use of murine and cell culture models has been included to substantiate discussion points.

2.2 Carbohydrate and Performance

2.2.1 Overview of CHO storage

Glycogen is a branched polymer of glucose that serves as an energy reserve for ATP production and is found principally within muscle and liver cells. In skeletal muscle, the key importance of glycogen as an energy reserve lies in its rapid mobilization in response to elevated energy requirements, its capacity to serve as an energy source in the absence of oxygen, and the fact that the glycogen granule and glycolytic enzymes are widely distributed within the cell. Glycogen molecules are exquisite examples of highly efficient cellular energy storage systems. The branched structure allows the dense compartmentalization of free glucose, providing muscle, or liver, with a readily accessible form of energy (Melendez-Hevia *et al.* 1993). Incorporation of glucose into a branched polymer allows glycogen granules to store large amounts of glucose without significantly affecting cellular osmolality. A single glycogen molecule can contain ~ 55,000 glucose residues in an area of 8,000 nm^3. The branched structure of the glycogen granule enhances surface area, an essential component for phophorlyase-based degradation. In accordance, the regulation of the branch length, number and density appear to be highly regulated by conserved processes.

Beyond the structure and amount of glycogen particles, its location within the muscle also appears to be important. Evidence for compartmentalized glycogen metabolism arise from studies showing simultaneous synthesis and utilization of glycogen (Shulman and Rothman, 2001), lactate production during fully aerobic contractions, and a preferential requirement for glycolytic ATP by Na^+-K^+ pumps in several cell types including skeletal muscle (James *et al.* 1999). In accordance with these observations, glycogen particles are found in distinct locations within skeletal muscle. Three distinct sub-regions of glycogen storage appear in skeletal muscle, 1. Intermyofibrillar which is located between the myofibrils in close proximity to the sarcoplasmic reticulum and mitochondria; 2. Intramyofibrillar which is located in the myofibrils interspersed within the contractile filaments, and most often in the I-band of the sarcomere; and 3. Subsarcolemmal which is distributed just beneath the surface membrane in close association with the mitochondria, lipids, golgi apparatus and nuclei (Nielsen and Ortenblad. 2011). Several studies have demonstrated that the distribution of glycogen is related to distinct processes in skeletal muscle (Nielsen *et al.* 2009; Ortenblad *et al.* 2011). In relative terms, intermyofibrillar glycogen is the major site of glycogen deposition, constituting approximately 75% of the cell's total store, whereas intramyofibrillar and subsarcolemmal account for 5 to 15% of the total respectively (Figure 2.1).

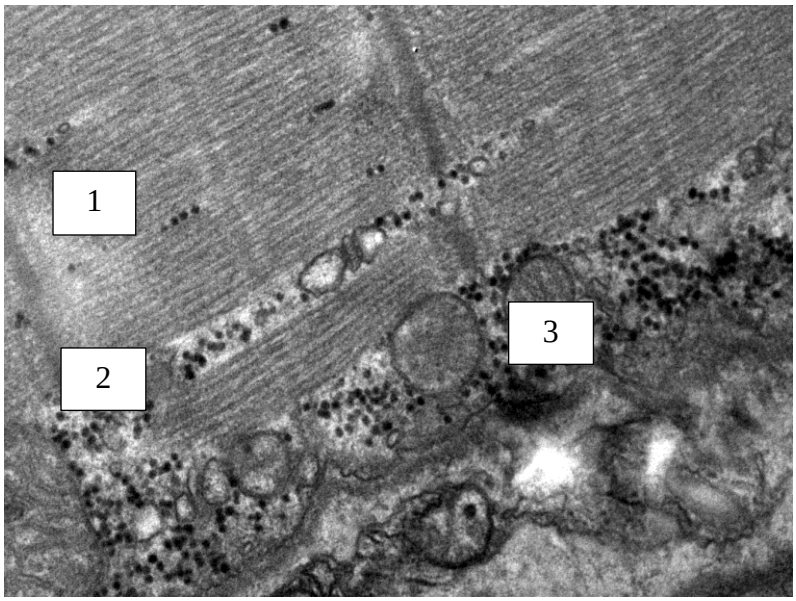

Figure 2.1 – Electron micrograph of human vastus lateralis displaying compartmental glycogen distribution. 1. Intermyofibrillar, 2. Intramyofibrillar and 3. Subsarcolemmal. Image taken at LJMU microscopy laboratory by S. Impey.

Glycogen is stored in liver (~100g) and skeletal muscle (350 – 700g depending on training status and diet). These endogenous carbohydrate stores are relatively small (~5% of total energy storage) although it is well recognised that muscle glycogen represents a significant fuel source for sustained moderate- to high-intensity exercise contributing to more than 50% of total energy requirements (Tsintzas and Williams. 1998). Carbohydrate oxidation yields greater ATP re-synthesis per unit of oxygen delivered to the mitochondria, when compared with fat, thereby making it a logical choice for skeletal muscle to use given the effect of improved gross efficiency (Krough and Lindhard, 1923). Furthermore, given the ability for carbohydrate to support energy production in both anaerobic and aerobic exercise, even when working at very high exercise intensities carbohydrate is still able to derive energy through oxidative phosphorylation (van Loon *et al.* 2001).

2.2.2 CHO availability and exercise performance

Pre-exercise meals

The consensus from the wealth of studies undertaken in the last 40 years is that CHO loading can improve performance and capacity especially when the exercise is greater than 90 minutes in duration (Hawley *et al.* 1997). Initial evidence for the beneficial effect of pre-exercise CHO provision was provided by classic studies from Christensen and Hansen (1939a, 1939b) showing that greater ingestion of CHO extended the duration work at a fixed power output could be sustained. Subsequently two seminal studies of Burgstrom *et al.* (1967) and Karlsson and Saltin (1971) characterised the supercompensation of muscle glycogen following depletion and the beneficial effects this had on exercise capacity. These studies categorically demonstrated that commencing exercise with higher muscle glycogen levels through several days of feeding improved exercise capacity and performance, in an almost linear fashion. A less extreme form of CHO loading was developed in the 1980s where Sherman *et al*. (1991) observed that a simple exercise taper in conjunction with several days of increased dietary CHO intake was also sufficient to increase glycogen storage, It is now generally accepted that trained athletes can increase glycogen storage in both type I and II fibres within 24 – 48 hours of increased CHO intake (Bussau *et al*. 2002).

The enhanced performance effect is likely *initially* mediated by a delay in the time-point at which energy availability becomes limiting to the maintenance of the desired workload,

which in the case of *race pace* is dependent on sustained and high rates of CHO oxidation (O'Brien *et al.* 1993; Leckey *et al.* 2016). Indeed, in reviewing the literature Hawley *et al.* (1997) cited that CHO loading can improve exercise capacity by approximately 20% and time-trial performance can increase by 2-3%. In addition to providing substrate availability for ATP production, it is now recognized that glycogen availability (especially the intramyofibrillar storage pool) can directly modulate contractile function. Indeed, a series of studies from Ørtenblad and colleagues (Geji *et al.* 2014; Ørtenblad *et al.* 2011, 2013) have collectively shown a preferential utilization of this storage pool during exercise in a manner that also correlates with impaired Ca^{2+} release from the sarcoplasmic reticulum. Such impaired excitation-contraction coupling is likely to be of particular importance during those situations where higher power outputs and sprint finishes are required in the very late and finishing stages of races.

Even without several days of high CHO feeding, performance can still be improved by feeding in the 1 (Sherman *et al.* 1991) to 4 hours (Neufer *et al.* 1987; Sherman *et al.* 1989; Wright *et al.* 1991) prior to exercise. While a window of 1 – 4 h remains comparatively vague, is interesting to note that when CHO provision was given < 1h prior to exercise evidence has presented negative (Foster *et al.* 1978; Hargreaves *et al.* 1987) and neutral (Devlin *et al.* 1986) performance outcomes. The magnitude of glycogen increase resulting from pre-exercise CHO provision was characterised by Chryssanthopoulous *et al.* (2004) who observed that an increase of 10% was achievable in 3h by the provision of 2.5 $g \cdot kg^{-1}$ CHO meal following an overnight fast.

In relation to practical application pre-exercise CHO should come from foods low in fat and fibre with high glycaemic index carbohydrates (Burk *et al.* 1993), often with low-moderate protein content to minimise propensity to gastrointestinal problems and promote gastric emptying (Rehrer *et al.* 1992). In relation to athlete prescription it is essential to suit the timing, amount and food/supplement choice to the athlete ensuring sufficient nutritional training has been undertaken prior to a competitive event to ensure no gastrointestinal distress from a unique or novel digestive challenge. In relation to training, pre-exercise feeding should be planned to facilitate the intensity and duration of training, considering feeding during exercise and recovery strategy (discussed below) to maximise the adaptive response.

Carbohydrate during exercise

Numerous studies have demonstrated the ergogenic effects of CHO feeding during prolonged moderate- to high-intensity exercise (Burgstrom and Hultman, 1967; Febbraio *et al.* 2000; Widrick *et al.* 1993; Sherman *et al.* 1991). Meta-analysis of 88 randomized crossover studies investigating the effect of CHO intake during exercise concluded a range of substantial 6% improvement to moderate 2% improvements (range -2 to 6.5%) in performance metrics. The authors concluded the largest effect (6.5%) occurred when the supplement consisted of 3 – 10% CHO plus protein drink providing ~0.7 $g \cdot kg^{-1}.h^{-1}$ glucose polymers, ~0.2 $g \cdot kg^{-1}.h^{-1}$ fructose and 0.2 $g \cdot kg^{-1}.h^{-1}$ protein (Vanderbogaerde and Hopkins, 2011). It is important to consider 83% of the included studies utilised cycling exercise and measured ether exercise capacity by time-to-exhaustion (mean duration 106 min) or exercise performance using time-trials (mean duration 47 min), thus interpretation of the overall results should be considered in an exercise and duration specific manor. Nonetheless, the bulk of studies to date demonstrate CHO appears to confer improvements in exercise capacity (time to exhaustion) and exercise performance during prolonged exercise (>2h) at low intensity as well as shorter exercise of high intensity (~1h at >75% $\dot{V}O2_{max}$) (Jeukendrup *et al.* 1997).

It is well established that CHO intake before and during exercise improves performance, however initial studies presented controversy regarding the nature of the mechanism underpinning this response. Glycogen sparing resulting from provision and oxidation of exogenous CHO was suggested, however few studies observed decreased glycogen utilization during continuous cycling exercise with CHO intake (Bergstrom and Hultman, 1967; Erickson *et al.* 1987), with several experiments observing equivocal net glycogen breakdown pre- to post-exercise between nutritional conditions (Coyle *et al.* 1997; De Bock *et al.* 2005; Hargreaves and Briggs, 1988). The lack of observed change in the latter studies may be due to methodological factors. The analysis of muscle glycogen content from whole muscle homogenates does not allow the differentiation of glycogen use between fibre types. Given that glycogen synthase and phosphorylase enzymes are differentially expressed within muscle fibres, with type I exhibiting lower phosphorylase and greater synthase activity compared with type II fibres (Dauggard and Richter, 2004), a distinct patter of glycogen use during exercise is logical. Indeed, De Bock *et al.* (2007) were the first to demonstrate muscle glycogen sparing during exercise in type IIa fibres with provision of CHO in line with appropriate nutritional recommendations compared to

fasted exercise; these authors suggested this as the mechanism by which sprint performance is augmented at the end of an endurance exercise bout when CHO is provided. The intensity of exercise plays an important role in the contribution of fibre types to power production in that lower intensities require more type I with increased type II contribution with increasing intensity (Gollnick *et al.* 1974), thus the glycogen use within specific components of skeletal muscle is highly dependent on exercise intensity and this should be taken into consideration when applying research outcomes to prescription of nutrition within specific sporting situations.

The rate of exogenous CHO oxidation during exercise was initially thought maximal at ~1g/min (Leijssen *et al.* 1995), likely mediated by saturation of the sodium-dependent glucose transporter (SGLT1) in the intestine. Thus, addition of fructose was speculated to be able to increase total delivery of CHO into the circulation via its absorption through a different transporter (GLUT-5) (Ferraris and Diamond. 1997). Subsequently many studies have demonstrated rates of oxidation greater than 1 g/min up to 1.75 g/min when multiple transportable CHO's are provided during exercise (Jeukendrup. 2010). Improved performance has been observed with multiple transportable CHO's, the consensus of research indicates that these are best used during endurance events lasting greater than 3h when CHO is ingested at high rates (Jeukendrup. 2010).

Collectively the significant bulk of experimental data produced to date demonstrates a beneficial effect for provision of CHO during endurance exercise. The mechanisms by which CHO conveys performance improvements has yet to be fully characterised. Given the well documented observations that fatigue coincides with muscle glycogen depletion (Burgstrom *et al,* 1967), exogenous carbohydrate consumption during prolonged exercise has consistantly been shown to attenuate the rate of glycogenolysis thereby sparing muscle glycogen and ultimately delaying the onset of fatigue (Bjorkman *et al*. 1984; Tsintzas *et al*. 1998). Of note the sparing of muscle glycogen appears to be fibre type dependent. The duration and intensity of exercise appear to regulate the fibres in which glycogen is spared, in that shorter duration ($\leq$ 1 h) higher intensity ($\geq$ 70% $\dot{V}O2_{max}$) exercise preferentially spares glycogen in type I fibres (Hulston *et al*. 2009), whereas longer duration (~ 3 h) at lower intensity ($\leq$ 60% $\dot{V}O2_{max}$) appears to spare type II muscle fibres only (Tsintzas *et al*. 1995). Thus, the mechanism for glycogen sparing may be exercise specific and localised to specific fibre pools. One immediate benefit from supply of exogenous CHO is maintained euglycemic during exercise. Maintenance of blood glucose concentrations is purported to

convey beneficial effects on performance via provision of exogenous substrate to contracting muscle but also likely delays fatigue induced by central mechanisms (Nybo, 2003), which also appear to be activated by mouth rinsing CHO when in a glycogen depleted state (Kasper *et al*. 2015). These data point to a combination of different systems interacting under the provision of exogenous CHO to increase performance (Cermac and van Loon, 2013).

Carbohydrate post exercise

Muscle glycogen synthesis following exercise follows a biphasic pattern. Initially a period of rapid synthesis occurs independent of insulin, lasting 30 – 60 minutes. This rapid phase of glycogen synthesis is characterised by exercise-induced translocation of GLUT-4 to the cell membrane facilitating glucose uptake from the circulation. Following the initial rapid phase, muscle glycogen synthesis occurs at a much slower rate and this phase can last for several hours (Jentjens and Jeukendrup, 2003).

The rapid phase of glycogen synthesis proceeds without the requirement of insulin and is thus often referred to as the insulin-independent phase (Piehl, 1974). This rapid phase has been suggested to occur only when post-exercise muscle glycogen concentrations are lower than ~120 – 150 mmol/kg dw (Price *et al.* 1994) and CHO is provided immediately after exercise (Ivy *et al.* 1998). Several mechanisms underpin the insulin-independent nature of this rapid phase. GLUT-4 mediated transport of CHO into skeletal muscle to facilitate rapid resynthesis is stimulated by mechanical stress activating p38 MAPK induced by sustained muscle contraction (Geiger *et al.* 2005), in addition AMPK activity further stimulates GLUT-4 membrane translocation, this process is partly regulated by muscle glycogen concentrations per se given the glycogen binding domain found in the β subunit of AMPK (McBride *et al.* 2009).

Parallel with augmented glucose uptake the enzyme activity of glycogen synthase is significantly increased with low glycogen levels. The increased glycogen synthase activity is regulated by the binding of glycogen synthase, glycogen synthase phosphatase and glycogen phosphorylase to glycogen (Ivy and Kuo, 1998). When glycogen concentrations decrease both glycogen synthase phosphatase and glycogen synthases are released, the active phosphatase then catalyses dephosphorylation of glycogen synthase converting it to its active form increasing the rate of glycogen synthesis (Ivy and Kuo, 1998). The slow phase, or insulin dependent phase is characterised by skeletal muscle displaying an

increased sensitivity to insulin that can last for several hours depending on level of glycogen depletion and provision of CHO (Cartee *et al.* 1989). The precise mechanism underpinning the increased insulin sensitivity is currently unknown, but is likely a cumulative interaction between muscle glycogen concentration, serum factor(s), AMPK and insulin signalling molecules (Jensen and Richter, 2012)

Regarding the amount of CHO required to facilitate maximal rates of glycogen synthesis, early work suggested that provision of 0.7 g/kg/h given immediately after exercise as a single bolus eliciting ~20 mmol/kg dw/h rate of resynthesis was the maximal rate attainable over 4h (Ivy *et al.* 1987). However, later work using smaller but more numerous (30 min interval) provision of CHO feeds following exercise providing 1.2 g/kg/h elicited a much greater synthesis rate of 44.8 mmol/kg dw/h (van Loon *et al.* 2000). Several other studies have observed similar rates of synthesis when providing 1 g/kg/h or greater CHO at 15 – 60 min intervals in the first 0 – 5 h post exercise reporting resynthesis rates of 40 – 50 mmol/kg dw/h (Blom, 1989; Doyle *et al.* 1993; Hickner *et al.* 1997; Jentjens *et al.* 2001; Piehl, 1974; Tarnopolsky *et al.* 1997; van Hall *et al.* 2000). The highest rate of glycogen resynthesis recorded following infusion of CHO while being in a glycogen depleted state was 85 mmol/kg dw/h (Burgstrom and Hultman, 1967). Given the insulemic effects of protein, early studies speculated that the addition of protein may further augment glycogen synthesis. Several well-designed methodologies tested multiple amino acid and protein (whey, wheat, isolate, hydrolysate etc.) combinations with CHO between 0.8 – 1.5 g/kg/h and showed no further benefit to provision of 1.2 g/kg/h CHO alone (Jentjens *et al.* 2001; van Hall *et al.* 2000; van Loon *et al.* 2001; Zawadzki *et al.* 1992). Given CHO alone was most effective at resynthesizing glycogen, the type of CHO ingested and method of delivery are also important factors to consider. CHO of high glycaemic index (GI) has been shown to produce 61% greater rate of resynthesis of glycogen than provision of CHO with low-GI (Burke, 1993). The effect of GI likely mediates its effect via increased gastric emptying and thus concentration in the blood available for absorption by skeletal muscle. Provision of high-GI CHO in liquid versus solid form appears to be equivocal in relation to muscle glycogen synthesis (Reed *et al.* 1989). Liquid provision of CHO using low or high molecular weight monomers and oligomers (concentration of 85 and 350 mosmol.l^{-1} has been shown to be most effective to facilitate glycogen synthesis (Piehl, 1974). Recent work utilising the concept of multiple transportable CHO's suggest this may be a method to further augment glycogen synthesis. Wallis *et al.* (2008) found no benefit of addition of

fructose to glucose for glycogen resynthesis following exercise, however glucose in the glucose-fructose trial was provided at 0.8 g/kg/h, well below the 1.2 g/kg/h recommended. Further work by Trommelen *et al.* (2016) examined glucose, glucose-fructose and glucose-sucrose combinations feeding at 1.5 g/kg/h at 30 min intervals for 5h, this group also noted no additive effect of fructose or sucrose over glucose alone at facilitating glycogen synthesis.

Practically, provision of a mix of fluid and solid CHO is recommended to facilitate rehydration and repletion of glycogen following exercise. The CHO provided should be high-GI and easily palatable providing 1.2 g/kg/h CHO at 30 min intervals for up to 5h if necessary, depending on the duration and intensity of exercise in addition to consideration of the goals of the next training session or proximity of competition.

2.3 Training adaptations to endurance exercise

2.3.1 Overview of adaptations

Physiologists have long held an appreciation for the trainability of skeletal muscle. For example, Astrand (1956) reviewed the physiological limits of human performance, noting that the outstanding feats that could be achieved by athletes were the result of both natural endowment as well as specific exercise training. It was, however, another decade before Holloszy (1967) published seminal work demonstrating that respiratory enzymes in rat skeletal muscle as well as whole-body oxygen consumption could be increased with stringent endurance exercise training. In addition, a plethora of adaptive responses have been observed following endurance exercise training including fibre type shifts towards an oxidative phenotype (Andersen and Heriksson, 1977), angiogenesis and increased capillary to fibre ratio (Andersen and Henriksson, 1977) as well as increased vascular bedding around alveoli. In addition to the increased surface area for gaseous exchange, enhanced cardiac output via increased left ventricular ejection fraction (Ekblom *et al.* 1968) circulates the oxygenated blood at greater rate. Skeletal muscle displays increased capacity for glycogen storage in (Saltin and Rowell, 1980) in addition to increased intramuscular lipid content and oxidation during exercise, as well as reductions in blood lactate accumulation during incremental exercise intensities (Gollinck and Saltin, 1982). Thus, the picture emerges is that increases in exercise capacity or performance are a combination of several tissues and a cumulative result of systemic as well as local adaptations.

Mitochondrial biogenesis underpins skeletal muscle oxidative capacity and is therefore considered the hallmark of intracellular adaptation to endurance exercise (Camera *et al.* 2016). The metabolic adaptations that accompany mitochondrial biogenesis are increased abundance of proteins involved in mitochondrial ATP production, the TCA cycle, mobilization, transport and oxidation of free fatty acids, glycolytic metabolism, antioxidant capacity, glucose transport, glycogen synthesis, and oxygen delivery to and utilisation by skeletal muscle. These training induced increases in metabolic enzyme activity and mitochondrial density result in enhanced respiratory control sensitivity such that a lower [ADP] is required to achieve the same level of oxygen consumption per gram of muscle. This adaptation allows the same cellular rate of oxidative metabolism with less agitation of adenine nucleotides and a lower rate of oxidative phosphorylation per mitochondrion (Holloszy and Coyle, 1984). The effect of this enhanced efficiency of energy production in the mitochondria in addition to the constituents of the skeletal cell results in smaller increases in free [ADP] and greater preservation of [ATP] and [PCr] at the same absolute power output prior to training. These adaptations attenuate the formation of allosteric regulators for glycogenolysis, glycolysis and lactate production after training (Gollinck and Saltin, 1982). Accordingly, the overall capacity for lipid oxidation is markedly increased (Talanian, *et al.* 2010), resulting in an increase in the fraction of ATP provision from oxidative metabolism (resulting from mitochondrial biogenesis) analogous with superior respiratory control sensitivity coupled to decreased flux through glycolytic pathways resulting in reduction of carbohydrate utilization after training. Consequently, decreased carbohydrate utilization during exercise in the trained state is compensated for by a proportional increase in lipid oxidation rates at the same absolute and intensities (Holloszy and Coyle, 1984). Hence, increased endurance performance resulting from training is attributable to enhanced fatigue resistance by virtue of reduced muscle glycogen depletion, enhanced coupling of ATP supply and demand, enhanced transport and β-oxidation of fatty acid sources, thereby producing smaller disturbances to homeostasis (Egan and Zierath, 2013).

2.3.2 Molecular mechanisms regulating training adaptation

Skeletal muscle adaptation to exercise training is a consequence of repeated contraction-induced increases in gene expression that lead to the accumulation of proteins that function to blunt the homeostatic perturbations generated by contraction-induced increases in energy demand and substrate turnover. The development of a specific “exercise

phonotype" is the result of a new, augmented steady-state mRNA and protein levels that stem from the training stimulus (Perry *et al.* 2010), which can be modified by the prevailing energy availability. This section will focus on the molecular regulators of mitochondrial adaptation, while acknowledging the contribution of multiple systems in contributing to enhanced endurance performance.

In accordance with the ~100-fold increase in ATP turnover elicited by endurance exercise, significant perturbations in the intracellular constituents of skeletal muscle bioenergetics and redox reactions are induced triggering respiratory bursts of free radicals in collaboration with calcium oscillations for sustained actin-myosin cross bridge cycling (Egam and Zerath, 2013). The dynamic fluctuation in ratio and subcellular location of these exercise associated energetic by-products activates key enzymes such as AMP-dependent protein kinase (AMPK), p38 mitogen-activate protein kinase (p38 MAPK) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Once activated these kinases converge on nuclear and mitochondrial transcription factors (e.g. p53) and transcriptional co-activators (e.g. PGC-1α), responsible for synthesis of mitochondrial proteins, lipid and DNA constructs required for mitochondrial biogenesis (Egan and Zierath, 2013).

Briefly, increased ADP and AMP resulting from exercise bind to the APMKγ subunit resulting in a conformational change that exposes the amino-terminal kinase domain in the catalytic α subunit. The α subunit can be phosphorylated at Thr172 by activating kinases such as CaMK kinase and liver kinase B-1, resulting in AMPK activation. In addition, the discovery of a glycogen binding domain in the β subunit further supports the exercise induced activation of AMPK (McBride *et al.* 2009). CaMKII autophosphorylates and activates through shifts in intracellular Ca^{2+} concentrations resulting from muscle concentrations. p38MAPK has been shown to be activated by various types of exercise, however the precise mechanism of exercise-induced activation and isoform specificity remain to be fully elucidated. The elevation of cytosolic Ca^{2+} during muscle contraction has been demonstrated to activate p38MAPK likely through a CaMKII mediated mechanism (Drake *et al.* 2015). Following the initial activation of regulatory enzymes following metabolic perturbations, these signalling mechanisms converge on peroxisome proliferator-activate receptor γ coactivator 1α (PGC-1α) (Puigserver and Spiegelamn, 2003) a purported 'master regulator' of mitochondrial biogenesis and p53, a well-known stress sensing gene known for involvement with tumour suppression, genomic stability and recently regulation of mitochondrial respiration (Matoba, *et al.* 2006).

2.3.2.1 PGC-1α

PGC-1α has been termed the 'master regulator' of mitochondrial biogenesis and is implicated as a co-activator for a significant number of nuclear and mitochondrial transcription factors, hence there has been considerable interest in this protein in relation to exercise as discussed here. PGC-1α is a second class co-activator with a basic protein structure consisting of an activation domain with the LXXLL sequence in the N-terminal, a central region associated with repression, and a RNA-binding motif and serine-arginine-rich region in the C-terminal (Puigserver and Spiegelamn, 2003). PGC-1α was discovered via its functional interaction with the transcription factor PPARγ in brown adipose tissue (Puigserver, *et al.* 1998). The gene encoding PGC-1α is located on chromosome 4 in humans, consisting of 798 amino acids it has a predicted molecular weight of 92 kDa (Uguccioni, *et al.* 2010).

PGC-1α is activated by proteins containing histone acetlytransferase binding to its N-terminal when the coactivator is bound to a transcription factor. This binding remodels histones and chromatin and allows for greater access of the transcriptional machinery to DNA for gene transcription (Ugucioni, *et al.* 2010). Additionally, the RNA-binding motif and serine-arginine rich region domains in the C-terminus are characteristics of proteins with mRNA splicing and export activity, thus indicating that PGC-1α acts as a platform to integrate many proteins and processes involved in the production of mature mRNA transcripts (Pugiserver *et al.* 1998). In relation to mitochondrial biogenesis, PGC-1α exerts these effects through direct interaction with, and coactivation of PPARs, ERRs and NRF-1/NRF-2 among other transcription factors (Scarpulla *et al.* 2012). NRF-1/NRF-2 binding sites are located in the promoter regions or multiple nuclear genes encoding mitochondrial proteins including, cytocrome *c*, components of the electron transport chain complexes, heme biosynthesis proteins, mitochondrial import-proteins, and mitochondrial transcription factor A (Tfam) which subsequently binds mitochondrial DNA (mtDNA) (Hood, 2009). In this way, PGC-1α can effectively coordinate both nuclear and mitochondrial genomes in the regulation of mitochondrial biogenesis.

Early work in human exercise trials demonstrated transcription of PGC-1 variants (α, β and PRC) following a single bout of knee extensor exercise, and that this transcriptional response to acute exercise was sustained following training (Mortensen, *et al.* 2007). Seminal work by Burgomaster *et al.* (2008) demonstrated the ability of sprint interval

(SIT) and traditional endurance training to elicit similar increases in PGC-1α protein content despite significantly less time and energy expenditure in SIT. The observed increase correlated with increased mitochondrial enzyme content and augmented lipid oxidation during submaximal exercise (Burgomaster *et al.* 2008). Subsequently, exercise intensity has been shown to be a key factor in determining PGC-1α transcriptional response to exercise. Indeed, when exercise was work matched in a repeated measures design, participants expressed significantly more PGC-1α following work completed at 80% than 40% $\dot{V}O2_{max}$ (Egan *et al.* 2010), however no further beneficial effect has been observed when exercise is undertaken above 100% $\dot{V}O2_{max}$ (Edgett *et al.* 2013).

The accumulation of PGC-1α protein has been shown to result from cumulative repeated transient mRNA bursts, with measurable increases in protein content 24 h following exercise (Perry *et al.* 2010). However, transcription of mRNA from known PGC-1α targets have been observed in parallel with PGC-1α transcription prompting the notion that cellular location in addition to protein content is an essential component of the exercise-PGC-1α interaction. HIT exercise was initially shown to induce nuclear shuttling of PGC-1α in human skeletal muscle (Little *et al.* 2011), subsequent work in rats has shown translocation to both nuclear and mitochondria following exercise (Safdar *et al.* 2011). AMPK activity was subsequently identified as an essential component in this translocation process using both human and murine total knock out models (Smith *et al.* 2013). Of interest, when a muscle specific PGC-1α knock out model was used similar adaptations to endurance exercise training were observed in both exercise capacity and mitochondrial morphology and function (Row *et al.* 2012), demonstrating PGC-1α is not a "sole" master regulator and further eliciting the multifactorial nature of signals integrating to cause mitochondrial biogenesis. Further effects of the regulation of PGC-1α are being explored and recently, a single nucleotide polymorphism (SNP) of the PGC-1α was identified to contribute to slow twitch fibre type accumulation following endurance exercise (Steinbacher *et al.* 2015). Significant evidence exists describing the nutritional regulation of PGC-1α, as discussed in later sections.

2.3.2.2 p53

P53 was first reported and cloned in the mid 1980's, with the protein consisting of 393 amino acids (Matlashewski *et al.* 1984). The role of p53 as a tumor suppressor protein and the effects it has on cell cycle arrest, apoptosis, pro- and antioxidant activity, angiogenesis,

DNA repair, differentiation, fertilisation, aging and senescence is well documented. Whilst these were recognised as primary functions, emerging evidence suggests p53 is also involved in regulating pathways of energy metabolism and mitochondrial biogenesis. Initial evidence for p53 holding a mitochondrial regulatory role is derived from cells lacking p53 reverting to high glucose in aerobic metabolism and having perturbed mitochondrial respiration (Feng *et al.* 2010). Additionally, p53 regulates synthesis of cytochrome *c* oxidase 2 (SCO2), which is required for the assembly of mtDNA-encoded COXII subunit in the COVIV complex (Matoba *et al.* 2006). The importance of p53 in an exercise capacity was first documented following a significant reduction in exercise capacity of p53 null mice (Saleem *et al.* 2009; Park *et al.* 2009).

Exercise *per se* has been shown to facilitate translocation of p53 to both subsarcolemal and intermyofibrillar mitochondria, reducing the nuclear content of p53. This translocation was associated with increased D-loop binding to mtDNA and Tfam association (Saleem *et al*, 2013a). Knocking out p53 was shown to diminish nuclear PGC-1α translocation as well as reducing kinase activity of putative exercise signalling cascades and transcripts mRNA related to mitochondrial biogenesis (Saleem *et al.* 2013b). The knockout of p53 precluded a morphological change in mitochondria in addition to reduction in mitochondrial import protein content, assembly of COXIV complex in intramyofibrillar mitochondria and the expression of proteins regulating mitochondria fusion and fission (Saleem *et al.* 2015). Subsequent to the seminal studies mentioned above, Bartlett *et al.* (2012) were the first to demonstrate the capacity of p53 to become phosphorylated following exercise in human skeletal muscle, an effect augmented by commencing exercise with low muscle glycogen (Bartlett *et al.* 2013). Recently, nuclear translocation of p53 was also demonstrated in human skeletal muscle following exercise (Tachtsis *et al.* 2016).

In the context of exercise, activation and regulation of p53 would appear to be mediated by exercise-nutrition interventions designed to elicit considerable stress on the body. In addition to the work of Bartlett *et al.* (2013) demonstrating the apparent regulatory component of glycogen, exercise intensity has been show essential to elicit increases in mitochondrial respiration rate associated with increase in p53 protein content (Granata *et al.* 2015). Indeed, sprint intervals performed for 30s at 200% peak power elicited greater increases in protein content and mitochondrial respiration than sub-lactate threshold continuous and high intensity intermittent (4min at 90% peak power) work matched training. These authors noted dissociation between changes in respiration and

mitochondrial content of electron transport system components, however, changes in protein content of p53 in addition to PGC-1α and PHF20 correlated with the observed respiration changes. The same group subsequently demonstrated a similar effect with increased total training volume (Granata *et al.* 2016), adding to the notion that increases in p53 activity and protein content require an augmented stress response, ether from nutrition, exercise or combination of both. This logic follows principles of training in that to induce an adaptation, homeostasis needs to be perturbed to force a response minimising the stress placed on the body if the exercise is undertaken again. While the body of literature surrounding exercise and p53 is relatively small, given the number of cellular interactions and regulatory capacity p53 holds, significantly more biomolecular and physiological data is likely to be produced from further examination of this protein in the context of exercise.

2.4 Train smart compete high

While the provision of high CHO before, during and after exercise to promote performance and recovery during competition is an essential part of a well-structured nutritional program (Stellingwerff *et al.* 2012), recent evidence suggests that training with reduced CHO availability augments cellular adaptations to exercise, increasing the potency of the training response leading to enhanced performance. This methodology was initially termed "train-low" and is composed of several nutrition-exercise interactions detailed below.

2.4.1 Physiological and metabolic effects of exercise with low muscle glycogen

Early experiments examining the effects of exercising with low muscle glycogen investigated the metabolic contribution of amino acids to energy production during exercise (Lemon and Mullin, 1980; Haralambie and Berg, 1976). However, these early studies while extremely informative contained an order effect limiting the interpretation of the role of glycogen content *per se*. Blomstrand and Saltin (1999) resolved this via the use of an elegant study design where one leg completed a glycogen depleting exercise in the evening before exercising both legs the next morning, thus providing the same arterial supply to both legs and same hormonal response during exercise. Glycogen breakdown during exercise was 60% lower, and the rate of glucose uptake during exercise was 30% higher in the leg with reduced pre-exercise glycogen content, additionally amino acid efflux from the low glycogen leg was significantly greater indicating differential contributions of substrate to energy production during exercise between high- and low-glycogen legs, and that this shift was caused by low muscle glycogen *per se*.

In addition to differential amino acid metabolism, a significant contribution of lipid oxidation was observed with exercise undertaken with low muscle glycogen. Several studies reported significant increases in FFA concentrations in venous (Widrick *et al.* 1993) and arterial (Pilegaard *et al.* 2002) samples in addition to increased FFA uptake across a – v difference during exercise across the leg (Wojtaszewski *et al.* 2003). While increased circulatory lipid concentrations are apparent while exercising with low muscle glycogen, the concentrations of intramyocellular lipids within skeletal muscle were less well described due to compounding factors of adipose and extracellular lipid contamination of biopsy samples. Utilising proton magnetic resonance spectroscopy (^{1}H-MRI) Johnson *et al.* (2003) demonstrated the capacity for low CHO diets and low muscle glycogen to increase intramyocellular lipid content in skeletal muscle in a relatively short period (48h), and increase the contributions of this sub-fraction to energy production during exercise compared to exercise undertaken following high CHO diet with high muscle glycogen.

Subsequently, the effects on cellular mechanisms directing changes occurring while training with low glycogen were examined. Given the observations that increased glycogen synthase activity and insulin sensitivity following exercise are inversely related to muscle glycogen content (Wojtaszewski *et al.* 2000, 2001), it is clear a high metabolic priority is placed on glycogen resynthesis during recovery from exercise. Based on these observations, Pilegaard *et al.* (2002) observed augmented gene transcription in the early (0 – 5 h) stages of recovery from low intensity (45% $\dot{V}O2_{max}$ for 2h) cycling exercise where one leg commenced exercise with low muscle glycogen compared with a leg that commenced exercise with high muscle. Exercise undertaken with low muscle glycogen displayed elevated mRNA transcription of genes regulating CHO and fat metabolism compared to the leg which started exercise with high muscle glycogen. To understand the regulatory mechanisms underpinning the differential metabolic response of skeletal muscle to exercise with low glycogen, Wojtaszewski *et al.* (2003) observed significantly elevated AMPKα2 activity and ACC^{Ser221} phosphorylation during moderate intensity cycling exercise (70% for 1h) when undertaken with low muscle glycogen. Previous research in rodents demonstrated allosteric regulators of AMPK activity, adenine nucleotide and creatine phosphate content to be similar between muscles with low and normal muscle glycogen (Derave *et al.* 2000; Wojtaszewski *et al.* 2002), implicating a role for glycogen content *per se* on AMPK activity. Indeed, the identification of a glycogen binding domain

on the beta subunit of AMPK provided further support for this regulatory role of glycogen in modulating skeletal muscle metabolism (McBride *et al.* 2009). Further stress signalling mechanisms activated by exercise and metabolic shift were demonstrated to be regulated by the availability of muscle glycogen. For example, the nuclear abundance of phosphorylated p38-MAPK and subsequent IL-6 mRNA transcription was shown to be enhanced following exercise undertaken with low muscle glycogen (Chan *et al.* 2004). The phosphorylation of p38-MAPK has subsequently been demonstrated to require increased circulatory FFAs (Zbinden-Foncea *et al.* 2013), these data and others above demonstrate the complexity and diversity of regulatory mechanisms that bring about the metabolic shift and transcriptional response induced by exercise undertaken with low muscle glycogen.

2.4.2 Exercise in the fasted state

While evidence indicates augmented signalling responses following exercise in a glycogen depleted versus loaded state, research has also shown beneficial metabolic adaptations with exercise in the fasted state. As the name suggests this method involves undertaking exercise, generally shortly after waking before breakfast. The general idea underpinning this modality is the potential for elevated fat oxidation resulting from an overnight fast. Indeed an increased transcription of genes regulating fat metabolism was observed with moderate intensity (75% $\dot{V}O2_{max}$ for 1h) undertaken following an overnight fast (van Proeyen *et al*. 2011), in the counter balanced trial of this study the provision of 6% CHO drink before and at regular intervals throughout the trial dampened the transcriptional response of the same genes to exercise (Cluberton *et al.* 2005). In addition to data from acute interventions, van Proeyen *et al.* (2011) observed that training in a fasted state enhanced citrate synthase and β-HAD enzyme activity and a reduction in glycogen use during 2 h constant load cycling to a greater extent that when training was undertaken with provision of CHO before and during exercise. These observations were accompanied by increases in circulatory FFA availability and oxidation during exercise also seen when exercising with lowered muscle glycogen (Pilegaard *et al.* 2002; Widrick *et al.* 1993; Wojtaszewski *et al.* 2003). Thus, while exercising in a fasted state my not confer the same level of glycogen or energy deficit that intentionally lowering muscle glycogen prior to exercise will, exercising in the fasted state is still able to induce the physiological and metabolic conditions necessary to induce an augmented regulatory response. This response is likely mediated via augmented AMPK activity, which when glucose is provided is suppressed (Civitarese *et al*. 2005) (Table 2.1).

2.4.3 Twice per day model

Modulation of muscle glycogen content via twice per day training has be used previously in acute interventions (Blomstrand and Saltin, 1999), however its application in a chronic training scenario to examine potential performance responses to training with low muscle glycogen was first employed by Hansen *et al*, (2005). This study used fixed intensity knee extensor exercise and elegantly matched total work between bilateral legs over a three-week training period. In this design, one leg trained once per day in a glycogen loaded stat whereas the contralateral leg trained twice every second day where the second session was commenced with reduced muscle glycogen. Training with low glycogen increased time to exhaustion and capacity for glycogen storage compared with the leg that consistently trained with high muscle glycogen. During exercise the contribution of glycogen to energy production was reduced while an enhanced catecholamine concentration was observed, the enzymatic activity of CS was also increased in the leg trained with low glycogen only.

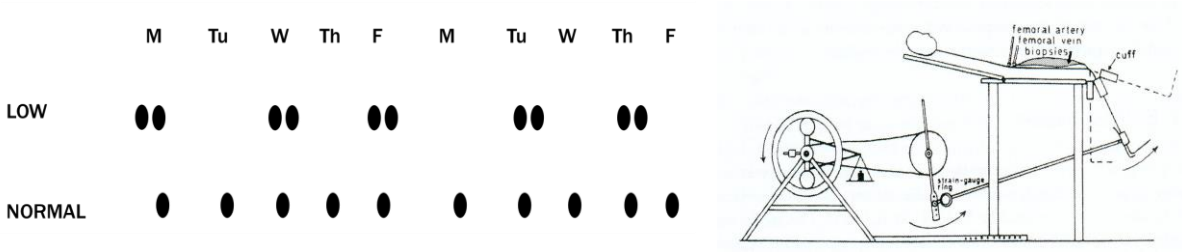

Figure 2.2 – Schematic representation of training routine and leg kicking exercise used by Hansen *et al.* (2005). Black dots represent training sessions. Thus participants in the low CHO group trained twice every other day versus once per day in the normal CHO group.

To address low CHO training from a more practical perspective, Yeo *et al.* (2008) trained two groups of well-trained cyclists for 3 weeks employing a twice per day model using steady state cycling (100 min at 70% $\dot{V}O2_{peak}$) followed by high intensity intermittent exercise (HIT) (8 x 5min maximal work with 1 min recovery) without feeding between sessions. HIT was undertaken with reduced muscle glycogen, whereas the parallel group completed steady state and HIT sessions on different days so that both sessions were completed with high muscle glycogen. In line with previous studies, an increased contribution of fat during steady state exercise was observed in the 'train-low group' in addition to enhanced CS, β-HAD activity and cytochrome c oxidase subunit IV protein content. Despite intensity of HIT sessions being compromised by low muscle glycogen, both groups improved average power output during 60 min TT by ~10%. In a parallel acute study, the same group sought to identify molecular mechanisms underpinning the

differential adaptive response and confirmed that enhanced AMPK mediated signalling is a suggested mechanism (Yeo *et al.* 2009). Despite consistent observations of elevated plasma FFA content and lipid oxidation during exercise following train-low, the specific source of lipids to energy production had not been identified. To answer this question, Hulston *et al.* (2010) assessed fuel utilisation via labelled glucose and palmate infusions, during 60 min steady state cycling exercise before and after 3 weeks of training utilising the same twice per day model of Yeo *et al.* (2008). Following training, the contribution of energy from muscle-derived triglyceride oxidation significantly increased in the group training with low muscle glycogen, this effect was observed in conjunction with increased protein expression of CD36 and β-HAD in skeletal biopsies from low trained participants only. The beneficial effects of low CHO training using the twice per day model are not unique to cycling as indeed, increased SDH activity was reported in both vastus lateralis and lateral head of the gastrocnemius following 6 weeks of running training undertaken with reduced endogenous and exogenous CHO availability (Morton *et al.* 2009) (Table 2.1).

2.4.4 Sleep low

Previous studies had utilised sustained periods of intermittent exercise in the 12 – 18h before an experimental trial followed by fasting or low CHO diet to modulate muscle glycogen between legs within the same subject to study the subsequent shift in metabolism that followed (Blomstrand and Saltin, 1999; Pilegaard *et al.* 2002). This method of "sleeping" with low muscle glycogen and low CHO availability was first examined in the context of training during whole body exercise by Bartlett *et al.* (2013) who employed a low CHO diet and cycling exercise the evening before the main experimental trial thus inducing a period of sleep low. The phosphorylation of the stress response protein p53 was significantly elevated following exercise with low glycogen following a period of sleep-low. In addition to this novel molecular finding, the mRNA content of genes regulating mitochondrial biogenesis (PGC-1α, Tfam) and oxidative phosphorylation (COXIV, PDK-4) were significantly augmented during HIT undertaken following a period of sleep-low (Bartlett *et al.* 2013). Due to the nature of the intervention there was significant energy deficit between high and low CHO conditions hence it is difficult to separate the adaptive responses resulting from systemic and local modulation in energy availability opposed to glycogen content *per se*. To isolate the effect of reduced glycogen Lane *et al.* (2015) employed a sleep low strategy where by participants received 8 g/kg CHO throughout the

day and completed HIT in the evening and then received no further nutrition until completion of a steady state ride the following. In the counter balanced trial participants received 4 g/kg then completed HIT and ingested a further 4 g/kg thus trials were matched for CHO intake but different in temporal spread of nutrition, in this way the trials were energy matched unlike the study of Bartlett *et al*. (2013). This nutrition-exercise interaction of sleeping low brought about augmented phosphorylation of signalling proteins (ACC, AMPK, p38MAPK) known to regulate oxidative adaptations to endurance exercise in the morning following a period of sleeping low. Following the second exercise session an increase in transcription and shift in DNA methylation of selected genes with putative roles in lipid oxidation and transport were observed with sleep low; of interest this nutritional strategy also allowed maintenance of intensity during HIT sessions in the sleep low group. However, no difference in genes regulating mitochondrial biogenesis were observed between groups, the authors stipulate that despite the exercise interventions used the participants still reserved a large amount of muscle glycogen, likely a result of the training status of the participants, thus some of the beneficial effects of training with low glycogen may not have been elicited, this view also points to the potential of a critical glycogen threshold that is necessary to go below before enhanced training response is seen.

Given the capacity for the sleep low strategy employed by Lane *et al.* (2015) to sustain training intensity during HIT sessions, in addition to eliciting augmented lipid transport and oxidation its application in training warranted investigation. Marquet *et al.* (2016) employed this strategy with elite triathletes during a 3-week training block with two groups under the same nutrition conditions employed by Lane *et al.* (2015). Training with the inclusion of sleep low augmented 10 km running performance by -2.9 (± 2.15%) and time to exhaustion during supramaximal cycling by +12.5 (± 19%) both of which were significant improvements over the control condition, in addition the delta efficiency of cycling significantly increased with sleep low over control likely as an artefact of ~1kg decrease in body weight that occurred during the training in sleep low group only (Table 2.1).

2.4.5 Amalgamation of nutritional strategies

All the studies in the sections mentioned above indicate specific signalling, transcriptional, metabolic or performance benefits from their respective nutrition-exercise interactions. However, an optimal strategy has not been developed within this relatively young field of

investigation. Burke *et al.* (2017) recently utilised CHO periodisation during three-week training camp with elite race walkers, where some training sessions were completed with high muscle glycogen and some with low muscle glycogen. While CHO periodisation conferred no additional beneficial effect to race performance versus traditional nutritional recommendations, reductions in body mass and O_2 cost of exercise were noted with periodised CHO intake. These data pave the way for refinement of nutrition-exercise interactions potentially leading to interventions that can bring about performance enhancement.

While considerable research has focused on the adaptive networks regulated by CHO and glycogen availability, very little work around CHO or nutritional periodisation has examined the effect of protein ingestion in these training sessions. Several papers have demonstrated a direct increase in net protein breakdown when exercise was undertaken with low muscle glycogen (Blomstrand and Saltin, 1999; Haworth *et al*. 2010; Lemon and Mullin, 1980), yet how ingestion of amino acids or protein and its effects on signalling or adaptation are not well characterised in a low CHO or endurance exercise context. The next sections will present a brief overview of protein synthesis and review the research to date in provision of protein and CHO during endurance exercise (Table 2.1).

Table 2.1 – Overview current low CHO literature

Reference	Participants	Duration	Exercise Protocol	Molecular Outcomes	Performance Outcomes
Twice per day model					
Hansen et al (2005)	7 untrained men	10 weeks 5 days x week	Knee extensor exercise. One leg trained 50% of sessions with low glycogen (LOW) whist the other trained all sessions with high glycogen (HIGH)	Greater increase in CS activity in LOW condition Increased β-HAD activity only in LOW condition	Improved time to exhaustion knee extensor exercise
Yeo et al (2008)	14 trained cyclists/triathletes	3 weeks 4 x week	100-min steady state (63% PPO) followed by 8 x 5-min intervals at maximal pace either 2h (LOW) or 24h (HIGH) later	Increased CS & β-HAD activity in LOW condition Increased COXIV protein content in LOW condition	Similar improvements (~10%) in 60-min TT for both groups
Morton et al (2009)	30 active men	6 weeks 4 x week	6 x 3-min running at 90% $\dot{V}O2_{max}$ NORM trained once per day whilst LOW+PLA & LOW+GLU trained twice per day (every other day). LOW+GLU ingested CHO before and during every second training session	Greater increase in SDH activity in LOW+PLA compared with LOW+GLU & NORM	Similar improvements in $\dot{V}O2_{max}$ & YoYoIR2 for all groups
Yeo et al (2010)	12 trained cyclists/triathletes	Acute exercise	100-min steady state cycling (63% PPO) followed by 8 x 5-min intervals at maximal pace either 2h (LOW) or 24h (HIGH) later	Greater phosphorylation of AMPK in LOW	N/A
Hulston et al (2010)	14 trained cyclists	3 weeks 6 x week	90-min cycling at 70% $\dot{V}O2_{max}$ followed by (2h apart) HIT (8 x 5-min) in LOW group. HIGH group performed alternate days of either SS or HIT cycling.	β-HAD increased in LOW only Increased fat utilisation in LOW	Similar improvements in 60-min TT for both groups

Cochran (2010)	10 active men	Acute exercise	HIT training (5 x 4-min cycling at 90-95% heart rate reserve) twice per day (separated by 3h). One group consumed CHO (2.3 g·kg) between sessions (HIGH) whereas the other restricted CHO intake (LOW)	Greater phosphorylation of p38MAPK in LOW following PM exercise	N/A
Cochran et al (2014)	18 active men	2 weeks 3 x week	HIT training (5 x 4-min at 90-95% heart rate reserve) twice per day (separated by 3h). One group consumed CHO (2.3 g·kg) between sessions (HIGH) whereas the other restricted CHO intake (LOW)	Similar improvements in CS activity & protein content of both CS and COXIV	Improved 250-kJ TT performance in LOW group only
Fasted training model					
Akerstrom et al (2006)	9 active men	Acute exercise	2h one-legged knee extensor exercise in either a fasted (FAST) or fed (exogenous CHO during) (FED) state.	Reduced AMPKa2 activation in FED	N/A
De Bock et al (2008)	20 active men	6 weeks 3 x week	1 – 2h cycling (75% $\dot{V}O2_{max}$). One group trained in the fasted state (FAST) with the other consuming CHO before and during exercise (FED)	FABPm increased in FAST condition only	N/A
Van Proeyen et al (2010)	20 active men	6 weeks 4 x week	1 – 1.5h cycling (70% $\dot{V}O2_{max}$). One group trained in the fasted state (FAST) with the other consuming CHO before and during exercise (FED)	CS & β-HAD activity increased in FAST condition only	Similar improvements in 1-h TT performance in both groups
Sleep low model					

Marquet et al (2015)	21 triathletes	3 weeks 6 x week	HIT (8 x 5-min cycling at 85% MAP or 6 x 5-min running at individual 10-km intensity) in the evening and LIT (60-min cycling at 65% MAP) the subsequent morning. One group consumed CHO between training sessions (HIGH) whereas the other restricted CHO intake (LOW)	N/A	Improved 10-km TT performance and improved time to exhaustion (150% peak aerobic power) in LOW group only
Marquet et al (2016)	11 trained cyclists	1 week 6 x week	HIT (8 x 5-min cycling at 85% MAP) in the evening and LIT (60 min cycling at 65% MAP) the subsequent morning. One group consumed CHO between training sessions (HIGH) whereas the other restricted CHO intake (LOW)	N/A	Improved 20-km TT performance in LOW group only
Pilegaard et al (2002)	Study a) 6 active men Study b) 6 active men	Acute exercise	1-legged glycogen depleting exercise followed by 2-legged cycling (2-h at 45% $\dot{V}O2_{max}$) the subsequent day (study a) 3-h of 2-legged knee extensor exercise with either normal (NORM) or LOW glycogen (study b)	N/A	Study A: Elevated PDK4, LPL & HKII at rest in LOW only Study A&B: Elevated DK4 & UCP3 post-exercise in LOW only
Wojtaszewski et al (2003)	8 trained men	Acute exercise	60-min cycling at 70% $\dot{V}O2_{peak}$ with either LOW or HIGH muscle glycogen (from exercise/diet manipulation the previous day)	$AMPK\alpha_2$ activated in LOW only Greater phosphorylation of ACC in LOW	N/A
Steinberg et al (2006)	7 active men	Acute exercise	60 min cycling at 70% $\dot{V}O2_{max}$ with either reduced (LOW) or normal (NORM) muscle glycogen	Greater phosphorylation of ACC & $AMPK\alpha_2$ in LOW Increased translocation of $AMPK\alpha_2$ to the nucleus	N/A

Bartlett et al (2012)	8 active men	Acute exercise	High-intensity running (6 x 3-min at 90% $\dot{V}O2_{max}$). LOW performed glycogen-depleting cycling the night before and restricted CHO overnight. HIGH consumed high CHO breakfast & CHO during exercise.	Enhanced phosphorylation of ACC & p53 Enhanced transcriptional activity of PGC-1a in LOW only	N/A
Lane et al (2015)	7 trained cyclists	Acute exercise	Evening bout of high-intensity cycling (8 x 5-min at 80% PPO) followed by 120-min steady state cycling (50% PPO) the subsequent morning. LOW group restricted CHO overnight whereas HIGH group consumed high CHO diet (4 g·kg BM)	Enhanced phosphorylation of ACC post AM exercise Enhanced transcriptional activity of CD36 & FABP3 in LOW group	N/A
Recover low model					
Pilegaard et al (2005)	9 active men	Acute exercise	75-min cycling (75% $\dot{V}O2_{max}$) followed by 24-h recovery with either HIGH or LOW CHO diet	UCP3, LPL & CPT1 remained elevated in LOW for 24h	N/A
Periodised model					
Burke et al (2017)	22 international race walkers	3 weeks 7 x week	3 weeks of intensified training (race walking, resistance training, cross training). Athletes consumed 3 different diets across the training period: a) high CHO b) LCHF c) periodised CHO intake with periods of low CHO training	N/A	Similar improvements in $\dot{V}O2_{peak}$ between all groups Improved 10km race times in high CHO and periodised CHO groups (no change in LCHF) LCHF increased O_2 cost of race walking

2.5 Protein Synthesis

Skeletal muscle comprises 40 – 50% of the body's mass, and displays a high level of plasticity in being able to adapt to increased locomotion and metabolic demands of the environment as well as playing a crucial role in the regulation of whole body homeostasis and health (Atherton and Smith, 2012). Skeletal muscle therefore is highly adaptable dependent on the stimulus imposed and the genetic predisposition of the individual to adaptation (Timmons, 2011). Skeletal muscle mass has the ability to change quite rapidly. Healthy, recreationally active individuals display protein turnover rates of ~1.2% day^{-1} which exists as a dynamic equilibrium: muscle protein break down (MPB) exceeds muscle protein synthesis (MPS) in the fasted state, and MPS exceeds MPB in the fed state (Kumar *et al.* 2009). The changes in equilibrium are evoked by a variety of stimuli including mechanical loads, nutrients, neural activity, cytokines, growth factors and hormones (Sandri, 2008), thus the complexity of the system regulating skeletal muscle protein turnover becomes apparent.

2.5.1 Skeletal muscle protein synthesis

In skeletal muscle, protein synthesis can be induced by insulin like growth factor (IGF1), which in turn activates phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt). Activated PKB/Akt inhibits the protein complex tuberous sclerosis complex 1 – 2 (TSC1 – TSC2), which inactivates the small GTPase protein ras homolog enriched in brain (Rheb). Rheb activates mammalian target of rapmycin complex 1 (mTORC1), which causes an increase in protein translation by phosphorylating the well-known targets p70S6K and 4E-BP1 (Bentzinger *et al.* 2013).

2.5.2 Molecular regulation of protein synthesis

mTOR has emerged as a key regulator of several anabolic processes including protein synthesis, ribosome biogenesis, and mitochondrial biogenesis, as well as catabolic processes such as autophagy (Zoncu *et al.* 2011). It is the centre of a regulatory network that is capable of sensing the energy status, nutritional situation and genotoxic stress levels of the cell, as well as receiving information from membrane-bound receptors for growth factors such as insulin (Weigl, 2012). mTOR exists as two distinct complexes: mTORC1 and mTORC2; mTORC1 is composed of the scaffold protein RAPTOR and is selectively inhibited by the growth regulatory agent rapamycin (Goodman *et al.* 2011), mTORC2

comprises the scaffold protein RICTOR and as a result is unaffected by rapamycin (Weigl, 2012).

mTOR regulates the translational process predominately via two downstream substrates, 4E-BP1 and p70S6K1 (Barr and Esser, 1999). The process of translation is assumed to be regulated at the stages of initiation, elongation, or release from the protein synthesis machinery. Initiation of translation appears to be the most effective site of control because it is the rate limiting step in protein synthesis. Briefly, eukaryotic initiation factor 4E (eIF4E) binds to the 7-methylguanosine cap structure of the 5'end of the mRNA, subsequently the small 40S ribosomal subunit is recurred to the cap structure, where it begins to scan for a start codon. Initiation starts once the eIF4F scaffold structure (composed of eIF4G/A/B) has attached via eIF4E binding eIF4G, combined with the ternary complex (eIF3/Met-tRNA-GTP) (TC) forming the 48S pre-initiation complex (PIC) (Goodman *et al.* 2011; Merrick, 2010 (Figure 2.3). 4E-BP1 binds to eIF4E thus preventing its association with eIF4G, suppressing cap-dependent translation initiation, this inhibition is removed by the phosphorylation of 4E-BP1 by mTORC1 causing 4E-BP1 to dissociate from eIF4E dramatically increasing the rate of protein synthesis. In addition, the activation of mTOR also leads to the phosphorylation, and activation of p70S6K1 which is dissociated from the eIF3 complex. Full activation of p70S6K1 is achieved by its subsequent phosphorylation within its activation loop by PDK1. Once fully active, p70S6K1 can phosphorylate eIF4B and the 40S ribosomal subunit protein S6, stimulating their participation in the 48S-PIC (Holz *et al.* 2005).

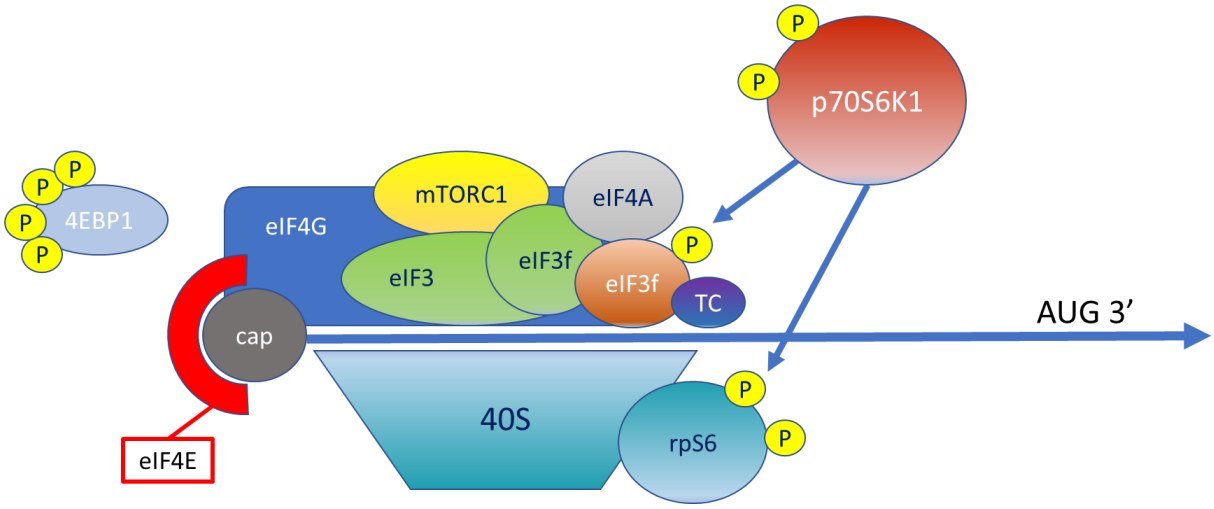

Figure 2.3 – Schematic representation of 48S-PIC. Formation of the 48S-PIC is the first step in translation initiation, the rate limiting step of protein synthesis.

Once initiation has taken place, the mRNA is translated into a peptide chain (Weigl, 2012). The elongation process requires eukaryotic elongation factor 2 (eEF2), which is a GTP-binding protein that mediates the translocation of the ribosome relative to the mRNA after the addition of each amino acid to the peptide chain (Browne and Proud, 2004), and is under inhibitory control of eEF2-kinase (eEF2K). Elongation is the most energy-consuming reaction during protein synthesis and logically not a priority for energy distribution during sustained exercise. Rose *et al.* (2005) identified a Ca^{2+}-calmodulin (CaM)-dependent phosphorylation of eEF2K resulting in a 5- to 7-fold increase in eEF2 inhibition by phosphorylation in working muscles. The Ca^{2+}/CaM binding to eEF2K can be negatively modulated by an mTOR dependent mechanism that, when nutrients are available, could alleviate the Ca^{2+}/Cam dependent inhibition of elongation (Browne and Proud, 2004).

2.5.3 Skeletal muscle protein synthesis during endurance exercise

Several comprehensive studies show a depression in MPS in rodents (Dohm *et al.* 1982; Williamson *et al.* 2006). The suppression of MPS was dependent on modality of exercise, as well as intensity and duration; 1h of swimming produced the smallest decrease in MPS (18%), then 1h of treadmill running (30%) and running to exhaustion producing the largest effect (71%) (Dohm *et al.* 1982). In human participants, early reports suggested a fall in whole body protein synthesis during walking uphill at 50% of maximum O_2 uptake ($\dot{V}O2_{peak}$) (Rennie, *et al.* 1980). More accurate direct measurements of tracer incorporation were made by Bowtell *et al* (1998) who measured a 31% fall in whole body protein synthesis during 2h of treadmill exercise at 60% $\dot{V}O2_{peak}$ following 7 days' diet manipulation to standardise protein intake. However, conflicting evidence also exists in human studies. Carraro *et al* (1990) did not measure a detectable change in MPS during treadmill walking at 40% $\dot{V}O2_{peak}$, the basal values reported may have been uncharacteristically low compared with those on the non-exercise day, indicating a false negative result. Consistent with these observations of reduced MPS, van Hall *et al.* (1999) observed that one-leg knee-extensor exercise in the post-absorptive state leads to an increase in net protein degradation compared to rest, and that this exercise induced increase in net protein degradation is augmented in muscles commencing exercise with low muscle glycogen. This work has recently been supported by Howarth *et al.* (2010) who observed a decrease in net leg protein balance during exercise when undertaken following a 2-day diet with low CHO, compared with rest and high CHO trial. This group attributed the negative

shift in balance in the low CHO condition primarily to an increase in protein degradation, but also to a reduction in synthesis towards the later stages of the exercise.

2.5.4 Protein provision during endurance exercise

Sustained endurance exercise has been shown to alter the balance between MPS and MPB in favour of the later, and this effect is compounded by reduced carbohydrate availability. Given the negative effects of MPB for athletes' performance, several groups have examined MPS responses with different nutrition-exercise interactions. Very few studies have examined the effects of feeding protein alone during endurance exercise. One of the first studies to examine this was conducted by MacLean *et al.* (1994), who administered 77mg/kg of branched-chain amino acids (BCAA) prior to one leg knee extensor exercise at 70% max work capacity for 60 min. This study demonstrated that BCAA supplementation significantly decreased efflux of protein into the intramuscular space and circulation indicating a reduction of net muscle protein degradation, this was accompanied by reductions in 3-methylhistaidine release, a marker of contractile protein degradation. When exercise was undertaken with altered glycogen levels between legs, the provision of BCAA's had the effect of enhancing protein synthesis during the recovery period irrespective of prior glycogen content (Blomstrand and Saltin, 2001). The anabolic response observed during the recovery period, surprisingly, did not seem to be mediated by insulin as arterial insulin concentrations between the two conditions (BCAA and placebo) were similar (Blomstrand and Saltin, 2001).

In an effort to explore the effects of leucine content Pasiakos *et al.* (2011) supplemented 10g of essential amino acids (EAA) in two conditions of, leucine-enriched drinks (3.5 g leucine in 10g EAA) or isonitrogenous EAA drinks (1.87 g leucine in 10g EAA). Participants consumed beverages at the onset of 60 min, 60% $\dot{V}O2_{max}$ cycle ergometry, and every 20 min thereafter until completion of the protocol; the leucine-enriched drink produced a 33% increase in MPS compared to EAA condition, producing a small but positive protein balance after exercise. Whole-body proteolysis was lower with consumption of the leucine-enriched supplement, suggesting that the protein-sparing effect attributed to EAA ingestion during endurance exercise is enhanced by increasing leucine availability. The study does not detail if the participants began exercise following an overnight fast, this data would be useful given the effect of CHO availability on MPS and

MPB during endurance exercise (Howorth, *et al.* 2010). These data point to the potential for provision of leucine rich amino acids to rescue protein balance during exercise.

2.5.5 Carbohydrate and protein feeding during endurance exercise

Carbohydrate ingestion has unequivocally been demonstrated to enhance endurance exercise lasting >1h (Pfeiffer *et al.* 2012), but has little positive effect on anabolic responses or protein kinetics in skeletal muscle (Koopman *et al.* 2007). In contrast, protein intake pre-, during and post-resistive or endurance type exercise has been shown to enhance translation initiation signalling and promote muscle protein synthesis during post-exercise recovery (Moore *et al.* 2009). Indeed, Coffey *et al.* (2011) demonstrated a similar response in that post exercise myofibrillar protein synthesis was enhanced, with equivocal (as percentage of content of muscle) mitochondrial protein synthesis by feeding a protein carbohydrate mix versus carbohydrate alone, before repeated sprint cycle exercise. This work was followed by Hulston *et al.* (2011) who examined ingestion of a carbohydrate, or carbohydrate plus protein solution during 3h cycling at 60% $\dot{V}O2_{max}$, on the rates of leg protein synthesis and breakdown. This group reported consuming protein during endurance exercise increased leg protein synthesis and decreased net leg protein breakdown; however, the fractional synthesis rate of the vastus lateralis was unaffected by protein feeding. The authors reported a positive post-exercise protein balance across the leg with carbohydrate plus protein feeding that was negative with carbohydrate alone. This group concluded that even with protein feeding, a positive protein balance cannot be established in highly active muscles during endurance exercise. In a similar study, Beelen *et al.* (2011) concluded that carbohydrate, or carbohydrate plus protein ingestion during 2h cycle exercise at 55% W_{max} improves whole body protein synthesis and net protein balance but does not further augment mixed muscle protein synthesis rates during exercise. The studies of Hulston *et al.* (2010) and Beelen *et al.* (2011) observed the largest insulin response with carbohydrate plus protein ingestion and a slight positive protein balance during this trial. This observation is likely due to a combination of the stimulatory effects of insulin on the protein synthetic pathway itself, as a combination of increased perfusion of circulating amino acids to the working muscles and insulin stimulating protein synthesis or an artefact of increased energy intake. Collectively these data point to the ability of CHO to further contribute the balancing of fractional synthesis and breakdown during endurance type exercise and may contribute to enhanced synthetic rate during recovery.

2.5.6 Post exercise nutrition

As with the above data demonstrating positive effects of protein intake pre- and during endurance exercise, there are also several studies examining protein intake post-endurance exercise. The addition of protein to carbohydrate given post-endurance exercise has been shown to enhance whole-body and leg protein accretion (Levenhagen *et al.* 2002). Breen *et al* (2011) also measured an increase in myofibrillar MPS with the addition of protein to carbohydrate verses carbohydrate alone, ingested following 90 min cycling at 77% $\dot{V}O2_{max}$. This group, unlike Coffey *et al* (2010), did not observe an increase in mitochondrial MPS rates with carbohydrate + protein verses carbohydrate alone. Given the differences in training status of the two cohorts, the more elite training status in Breen *et al* (2011) may not have undergone stress sufficient to stimulate a synthetic response, or the time frame examined may have been too short for a mitochondrial synthetic response to have been detected. In addition to the indication that protein ingestion pre-, during-, and post-exercise has positive effects on skeletal muscle protein balance during endurance exercise, there is data indicating the addition of protein to carbohydrate verses carbohydrate alone supplemented over a 4.5-week training block, enhances adaptation to aerobic training, with favourable improvements in whole body composition of increased lean and reduced fat mass (Ferguson-Stegall *et al.* 2011).

Several studies have examined the precursor molecular signalling responses to skeletal muscle protein synthesis following nutrient provision. Fujit *et al.* (2007) examined the signalling response of skeletal muscle to carbohydrate + protein provision versus carbohydrate alone at rest. Unsurprisingly, the phosphorylation of mTOR measured at 1h following ingestion was significantly enhanced with protein provision in accordance with enhanced whole-body protein synthetic rate, this was matched by a significant reduction in eEF2 phosphorylation allowing greater elongation and translation rate. Wilkinson *et al.* (2008) also used contralateral resistance and endurance training, to examine the differential signalling effects of acute and chronic exercise in the fed state. This study was the first to show a differential response of exercise modality, in that the resistance trained leg displayed preference towards myofibrillar protein synthesis with greater mTOR phosphorylation immediately after exercise, compared to the endurance trained leg in which mitochondrial protein synthesis was enhanced, with minimal myofibrillar synthesis. Coffey *et al* (2010) reported a significant elevation of mTOR phosphorylation 15 min after exercise versus placebo conditions, returning towards basal at 240 min. Similar data was

subsequently reported by Breen *et al,* (2011) who observed a group x time effect of mTOR phosphorylation following exercise (4 h post) when protein was added to carbohydrate, as reported previously (Fujit *et al.* 2007). The effect of feeding protein alone during exercise has been shown not to produce a discernible change in mTORC1 phosphorylation (Pasiakos *et al.* 2011) despite the addition of extra leucine producing elevated MPS. Although enhanced intracellular signalling does not necessarily correlate with the observed rate of MPS when measured directly (Greenhaff *et al.* 2008), activation of the putative signalling kinases responcible for MPS are still essential for MPS to occure. The differences in signalling observed between studies is likely an artefact of different feeding strategies (during- opposed to post-exercise), the time points at which muscle biopsies were taken, modality of exercise and training status of the participants may also be a deciding factor.

Measuring the effects of physical activity on MPS during energy deficient states is methodologically challenging. As such replication of real world scenarios are difficult to produce due to constraints of traditional tracer methodologies, in being confined to laboratories to sustain constant infusions (Wilkinson *et al.* 2014). Recently one study examined a relatively large cohort (39 adults) over a 21d energy deficit (ED) with the prescription of recommended daily allowance (RDA), 2 x RDA and 3 x RDA protein (Pasiakos, *et al.* 2013). This group demonstrated that consuming protein at RDA caused a greater loss of fat free mass and blunted the anabolic synthetic response to protein containing meal following ED. These findings were concurrent with blunted signalling through p70S6K (Ser^{424}/Thr^{421}), additionally transcription of the mTORC1 inhibitors REDD1 and 2 were enhanced during ED (Pasiakos, *et al.* 2013). The use of healthy active (military personnel) in the above mentioned study, combined with assessment of MPS in the post-absorptive and post-prandial state before and after ED provides one of the most accurate assessments of the effect of energy deficit with high protein availability that has applicable translation to athletes. The data presented above generally points towards the ability of protein to rescue MPS during endurance type exercise when above RDA levels of protein with high leucine content are ingested. No study has however looked at feeding protein before, during and after endurance exercise. In addition, the effect of glycogen availability on protein turnover rates during endurance exercise needs to be examined further.

2.6 Autophagy

A basal level of autophagy is ongoing in all eukaryotic cells and is vital for cellular turnover. Autophagy is the recycling mechanism responsible for the disposal of toxic protein aggregates as well as nansecent organelles. Three different types of autophagy have been described to date and they differ mainly in their mode of substrate delivery to the lysosome. Microautophagy refers to the degradation of substrates in the immediate vicinity of the lysosome through lysosomal membrane invaginations. Chaperone mediated autophagy is a targeted degradation mechanism through which dedicated chaperones recognise selected substrates and directly deliver them to lysosomes for degradation. Macroautophagy (here after termed autophagy) the best characterised of the three, here substrates along with any cytosolic materials surrounding them are engulfed with double membrane vesicles known as autophagosomes which are subsequently delivered to the lysosome for degradation (Vaishten and Hood, 2016)

Increases in autophagy resulting from exercise were first noticed in the 1980's, where increased number and size of autophagic vacuoles was observed in the liver and skeletal muscle of exercised animals (Salminen & Vihko, 1984). However, the functional importance of this system to cellular and skeletal muscle health did not become apparent until more recently. Systemic and internal signalling resulting from endurance exercise naturally precludes the activation of autophagy when you consider muscle contraction constitutes a form of energetic stress. Alterations in molecular messengers such as calcium, AMP, NAD^+, and redox networks activate downstream signalling cascades that converge on acute regulators of autophagic activity via post-translational modifications and longer term nuclear transcriptional remodelling processes. Autophagy induction following exercise has subsequently been documented in various tissues including liver, heart, pancreas, adipose and brain (He *et al.* 2012). Autophagy appears to consist of several key steps, induction, nucleation and expansion, cargo selection, fusion and finally degradation (Ding and Yin, 2012). Each stage is mediated by distinct molecular markers that are regulated by numerous upstream effectors such as energy status of the cell and nutrient availability as well as specific disease pathologies (Dodson *et al.* 2013). Here, autophagy in the context of response to exercise and as contributor to adaptation resulting from endurance exercise will be discussed with specific focus on the targeted degradation of mitochondria by autophagy.

2.6.1 Acute molecular regulators of autophagic process

The induction of the autophagy process is mediated by Atg proteins, several of which are transcriptionally regulated by FOXO3 (Zaho *et al.* 2008). For autophagosome biogenesis and maturation, the Unc-51-like kinase (ULK1)/Atg13/FIP200/Atg101 and Beclin-1/Vps34 complexes integrate regulatory responses from several stress signals induced by exercise such as AMPK, mTOR and FOXO's (Vainshten and Hood, 2016). Beclin-1 is phosphorylated by ULK1 at Ser14 and is necessary for Vps34 activation and full autophagic induction (Petherick *et al.* 2015). Nucleation and expansion of the membrane appears to be mediated via a Beclin-1 complex which can be antagonised by Bcl-2. Indeed, seminal work by He et al (2012) revealed exercise-induced autophagy engages Bcl-2-Beclin complex via phosphorylation of Beclin-1 allowing its emancipation and subsequent full autophagosome nucleation (He *et al.* 2012).

Currently it is unclear where material involved in membrane formation is procured from within the cell; mitochondria, endoplasmic reticulum, plasma membrane, and even the nuclear envelope have all been suggested in membrane donation (Juhasz and Neufeld. 2006). It is likely the selection of membrane material is the outcome of inductive signals specific to the identified target for degradation, although this requires considerable further investigation (Vainshten & Hood, 2016). Expansion and sealing of the phagophore (autophagosome precursor) is mediated by two ubiquitin-like conjugation systems and are involved in the ultimate conjugation of microtube-associated protein light chain 3 (LC3), and its close relatives GABARAP and GATE16 to phosphatidylethanolamine (PE) (Dodson *et al.* 2013). LC3 exists in its inactive form free in the cytosol and must first be cleaved by the protease Atg4, giving rise to LC3-I prior to conjugation to PE. Lipid conjugation converts LC3-I to the LC3-II form that is attached to either side of the growing phagophore membrane (Sandri. 2010). Cargo selection occurs in parallel with the expansion of the autophagosome and, contrary to original ideas is not random, but appears to be regulated by substrate specific receptors such as p62 (Kilonsky *et al.* 2011). p62 contains multiple protein-protein interaction domains, this protein interacts with LC3-II allowing entry of previously ubiquitinated cargo into the autophagosome. Ubiquitin independent targeting of cellular components via organelle-specific autophagy receptors have been identified (Dodson *et al.* 2013).

Mitophagy, the targeted removal of long lived or dysfunctional mitochondria, is achieved via interaction of receptors such as BNIP3 that interact directly with LC3-II on the phagophore (Kubli *et al*. 2012). Additionally, two Parkinson's disease factors have been implicated in exercise induced mitophagy, the E3 ligase Parkin and PINK1 (Pickrell and Youle. 2015). These factors interact under conditions of heightened oxidative stress and/or loss of membrane potential, at which point PINK1 is stabilized on the mitochondrial membrane recruiting Parkin to ubiquitiated targets thus signalling autophagosome formation (Pickrell and Youle. 2012). Once the autophagosome is complete, it transits along microtubule tracks to the lysosome where it fuses to form an autolysosome (Sandri, 2013), although very little is known about this process in response to exercise. The autolysosome subsequently degrades the content of the prior autophagosome via hydrolases inside generated by the lysosome (Dodson *et al.* 2012). Amino acids derived from the degradation process can efflux from the proteolysis within the lysosomal lumen and subsequently be used for biosynthesis of a variety of cellular components, as well as acting as a retrograde signal to activate mTOR and suppress autophagosome activity.

Of interest, two key regulators of mitochondrial biogenesis and exercise adaptation (p53 and PGC-1α) appear to play central roles in regulation of exercise induced autophagy. p53 has been implicated in the transcriptional regulation of autophagy-lysosome system following nuclear translocation. Moreover a global genomic profiling of the p53 transcriptome revealed a large number of autophagy genes regulating various steps throughout the autophagic process are direct targets of p53 (Broz *et al.* 2013). In addition to the transcriptional regulation of autophagy, p53 has been documented to suppress the activity of mTOR (negative regulator of autophagy activity) via several upstream regulators (Feng *et al.* 2005). Specifically, p53 mediated induction of Sestrins 1 and 2 have been documented to regulate autophagy through the modulation of AMPK-mTOR axis (Maiuri *et al.* 2009). Naturally, the regulation of autophagy by p53 is complex and highly dependent on the subcellular location. For instance cytosolic p53 appears to arrest autophagy, in direct opposition to its nuclear status (Rosenfeldt *et al.* 2013). The transcriptional co-activator PGC-1α works to orchestrate the nuclear and mitochondrial genomes during mitochondrial biogenesis (see above). Recent work by Vainshtein *et al.* (2015) demonstrated that PGC-1α is required for normal autophagy and mitophagy kinetics, via tracking localization of key mitophagy markers during exercise as well as the induction of LC3 and p62 genes following endurance exercise. These authors also suggest

a potential mitochondrial regulatory loop where degradation signalled by Parkin may be balanced via targeted degradation of ZNF746 (Paris) by Parkin this releasing the Paris-mediated repression of PGC-1α transcription (Valnshten *et al.* 2015). These data suggest the occurrence of complex regulatory systems that govern the synthesis and degradation of mitochondrial protein, and regulate autophagic function.

2.6.2 Autophagy in skeletal muscle – exercise implications

Recently it has become apparent that autophagy forms an essential part of the adaptive response of skeletal muscle to exercise. Although autophagy appears to have no acute effect on exercise performance *per se* (Verso *et al.* 2014), it appears that the cumulative effects of autophagy are required for adaptation to skeletal muscle. Grumati *et al*, (2011) have demonstrated that collagen-VI null (compromised autophagy) mice demonstrated an exacerbation of the dystrophic phenotype with acute and chronic exercise, suggesting that functional autophagy is required for successful adaptive response to exercise. Further evidence from Lira *et al.* (2013) demonstrated enhanced autophagic flux in parallel with mitochondrial biogenesis in response to chronic exercise, conversely mice lacking critical autophagy protein, Atg6, were unable to increase mitochondrial content or stimulate angiogenesis in skeletal muscle. In accordance with autophagic contribution to the exercised phenotype, He *et al.* (2012) noted that mice with BCL2 mutation presented with altered glucose metabolism and homeostasis, including decreased sarcolemma relocalization of GLUTE 4 during acute exercise. Recently, LoVerso *et al.* (2014) generated tamoxifen inducible muscle specific *atg7* knockout mice, and demonstrated the importance of autophagy in preserving muscle function following damaging exercise (downhill running). The work by LoVerso *et al.* (2014) evidenced the inability mitochondria to sustain membrane potential when autophagy was knocked out in skeletal muscle, interestingly this effect was greater in females than males. Additionally, this group identified that acute autophagy induction did not affect performance or glucose homeostasis thus indicating that alterations in exercise capacity in autohagy-deficent animals is likely the result of secondary aggregates and degeneration, rather than a primary deficiency in autophagy capacity per se (LoVerso *et al.* 2014).

Initial investigations in human skeletal muscle were conducted following an ultra-endurance 24h treadmill run (149.8km ± 16.3 mean distance) which induced significant increases in LC3BII protein content and ATG12 conjugated to ATG5, in addition to

increase in phosphorylation of mitochondrial fission marker DRP (Jamart *et al.* 2012) indicating the removal and degradation of mitochondria under severe exercise stress. The same group observed significant increases in the expression of ATG4b, ATG12, Gabarapl1 and LC3b mRNA following 200 km ultra-endurance run (28h 3min ± 2h mean time to completion), were observed (Jamart *et al.* 2012). The responses observed by this group are difficult to interpret given the extreme nature of the exercise protocol involved. For example blood glucose was reduced below 2 mmol/l at hour 24 in the initial study suggesting a severe reduction in muscle and liver glycogen indicative of significantly enhanced stress responses both within the circulation and skeletal muscle.

To establish the signals regulating the response of autophagy to exercise, Moller *et al.* (2015) employed 1 h cycle exercise in the fed and fasted states at 50% $\dot{V}O2_{peak}$. Following exercise, ULK1 phosphorylation at Ser^{555} was increased concurrently with decreased lipidation of LC3b which correlated positively with AMPKα Thr^{127} phosphorylation. These signalling events appeared independently of being fed or fasted, suggesting induction of autophagy is related to exercise and not nutritional factors. In support, Schwalm *et al.* (2015) employed low and high intensity exercise in both fed and fasted state noting nutrition independent exercise-mediated phosphorylation of ULK1 although at a different site Ser^{317}, as well as reduction in LC3bII protein level and LC3bII/LC3bI ratio with exercise. Of note, p62 protein content decreased only 1h after high intensity exercise and this indicator of autophagic flux again correlated with elevated AMPKα Thr^{172} phosphorylation observed only following high intensity exercise. The authors conclude that in agreement with Moller *et al.* (2015), exercise induced autophagy is independent of nutritional status and further suggest that exercise intensity is an underlying mediator of this system. The above-mentioned studies present interesting observations around the nature of autophagy induction, yet the nature of energy deficit induced resulting from low intensity exercise undertaken in the fasted state is comparatively small. The level of energetic stress or specific nutritional status of skeletal muscle that may tangibly increase the activity of autophagy has not been well characterised, as discussed below.

2.6.3 Nutrient status and autophagy

Early work by Kim *et al.* (2011) identified glucose starvation of cultured cells induced AMPK mediated activation of ULK1 and that this pathway could be blocked by mTORC1 activation subsequently preventing ULK1-AMPK interaction. Further studies observed a

similar response in mice, where markers of autophagy induction and transcriptional responses indicative of autophagic flux were elevated when low intensity exercised was undertaken in a fasted versus fed state and these measures coincided with reduced p70S6K phosphorylation (Jamart *et al.* 2013). In contrast to these studies, Zheng *et al.* (2015) observed that mice fasted for 24h re-activated mTOR-dependent signalling molecules (Akt, p70S6K) reducing LC3 content within skeletal muscle after high intensity exercise. These measures contrast with results from the above-mentioned studies where autophagy induction is independent of fasted versus fed status (Moller *et al.* 2015; Schwalm *et al.* 2015) and, appears to be induced by higher intensity exercise in human skeletal muscle (Fritzen *et al.* 2016). Several studies have observed attenuated or no autophagic response to exercise (Kim *et al.* 2011; Saleem *et al.* 2013), making concise conclusions about the effect of nutrition and exercise on autophagy induction difficult. In mice, it would appear the fibre type measured is likely to be an important consideration in experimental model. Further autophagic responses in human skeletal muscle need to be better characterised to elicit solid conclusions.

Given that autophagic induction by AMPK-ULK1 axis can be blocked by mTOR, it is noteworthy that amino acid starvation has been shown to activate ULK1 and autophagy in an AMPK independent manner (Kim *et al.* 2011). Mitophagy markers BNIP3 and Parkin were also elevated to a greater extent following exercise in the fasted state, via a mechanism where mTOR appears to play a more prominent role than AMPK in autophagy induction (Jamart *et al.* 2013). Future studies are required to examine the effect of amino acid provision on the timing and extent of autophagy activation during exercise (Sanchez *et al.* 2014). The role of nutritional status of skeletal muscle in addition to the availability of exogenous substrate (CHO, protein etc.) in relation to activity of autophagy has many compounding factors. In addition, exercise adds further level of complexity as dependent on the duration and intensity will alter the response of the autophagic machinery. Clearly autophagy is an essential component of exercise adaptation, thus characterising this response in nutrition-exercise specific interactions will allow greater understanding of the contributions this system makes to adaptation.

2.7 Conclusion

The body of knowledge around low carbohydrate training has expanded significantly in recent years. The shift in circulatory metabolites and substrate oxidation observed during

exercise under conditions of low exogenous or endogenous CHO availability has consistently demonstrated a translational effect at a molecular level within skeletal muscle (Yeo *et al.* 2008) and more recently improvements in performance (Marquet *et al.* 2016). Nonetheless characterisation of this response has predominantly focused on putative signalling cascades regulating mitochondrial biogenesis during work matched exercise interventions (Bartlett *et al.* 2015). Indeed, given the emerging role autophagy plays within the adaptive response to exercise (He *et al*. 2012), and the augmented autophagic activity seen under energetic stress (Kim *et al*. 2011) this area warrents investigation in a low CHO context.

Further more, application of real world nutrition practices often undertaken by elite athletes has not been characterised from a physiological or molecular standpoint. Indeed, given that athletes incorporate training sessions with low CHO availability but often ingest protein (Walsh, 2007); in addition to recent data pointing to the beneficial effects of leucine rich protein feeding during endurance exercise (Pasiakos *et al.* 2011) warrants further investigation of these practices. Nutritional interventions such as low CHO training have been shown to alter the molecular response to exercise, yet an unexplored component of the adaptive response to training is autophagy. The chapters in this thesis attempt to resolve some of the questions posed from reviewing the current literature, and expand the mechanistic and practical understanding of training with low CHO to augment adaptation and performance.

Chapter 3 - General Methodology

General Methodology

3.1 *Location of testing and ethical approval*

Exercise and biochemical analysis were carried out in the physiology and biochemical laboratories respectively at the Research Institute for Sport and Exercise Sciences Liverpool John Moores University. Ethical approval was granted from the local ethics committee at Liverpool John Moores University. Analysis of skeletal muscle cell signalling activity was conducted at the University of Stirling. Analysis of plasma amino acid content was conducted at the University of Nottingham.

3.2 Subject characteristics

All the participants that participated in each study were young healthy and recreationally active males (see Table 3.1). None of the participants had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during any study. Participants were asked to maintain habitual activity levels during each study, and refrain from additional exercise, caffeine and alcohol for at least 24 hours prior to any testing sessions.

Table 3.1 – Subject characteristics.

	Study 1	Study 2	Study 3
Age (years)	29 ± 11	24 ± 3	25 ± 3
Height (m)	1.80 ± 0.09	1.78 ± 0.1	1.75 ± 0.1
Body mass (kg)	79.5 ± 9.8	79.6 ± 4	74.4 ± 6.7
$\dot{V}O2_{max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	53.0 ± 6.1	53.6 ± 7	56.5 ± 3.8

3.3 Cardio-respiratory measures

3.3.1 Respiratory gasses during exercise

Participants were required to wear a neoprene face mask for measurement of respiratory gases during assessment of physical fitness and specific sections of exercise testing.

Breath-by-breath measurements were obtained throughout exercise using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US).

3.3.2 Assessment of substrate utilisation

Carbohydrate and fat oxidation rates were estimated via indirect calorimetry (Medgraphics, Minnesota, US) during exercise in all studies using the equations of Jeukendrup and Wallis (2005) assuming negligible contribution of protein oxidation for all calculations. Oxidation of 1g of carbohydrate was assumed energetically equivalent to 17 KJ (80% glycogen, 20% glucose), while oxidation of 1g of fat was assumed energetically equivalent to 40.8 KJ. Oxygen and carbon dioxide are expressed in litres throughout the calculation.

$$\text{Carbohydrate oxidation (g/min)} = (4.21 \times CO_2) - (2.962 \times O_2)$$

$$\text{Fat oxidation (g/min)} = (1.695 \times O_2) - (1.701 \times CO_2)$$

3.3.3 Assessment of peak oxygen uptake ($\dot{V}O_{2max}$) and peak aerobic power

Peak oxygen consumption ($\dot{V}O2_{peak}$) and peak aerobic power output (PPO) were determined during an incremental cycle test performed on an electromagnetically braked cycle ergometer (SRM, Julich, Germany). The participants completed a 10 min warm up at 100 W at a self-selected cadence, before commencing the test. The test began at 100 W and consisted of 2 min stages with 30 W increments in power until volitional exhaustion. Termination of the test occurred upon volitional exhaustion despite strong verbal encouragement. Breath-by-breath measurements were obtained throughout exercise using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US) calibrated with known internal reference gases. $\dot{V}O2_{peak}$ was stated as being achieved by the following end-point criteria: 1) heart rate within 10 beats.min^{-1} of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

3.4 Psycho-physiological measures

3.4.1 Ratings of perceived exertion

Participants reported ratings of perceived exertion (see Table 3.2) during exercise according to a 15-point Likert scale devised by Borg (1970).

Table 3.2 – Ratings of perceived exertion.

Rating	Description
6	No Exertion At All
7	Extreamly Light
8	
9	Very Light
10	
11	
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Extreamly Hard
20	Maximal Exertion

3.4.2 Ratings of perceived enjoyment

Participants reported ratings of perceived enjoyment immediately after exercise in study 1 according to the Physical Activity Enjoyment Scale (PACES) questionnaire validated by Kendzierski and DeCarlo (1991). According to the scale, the higher the score the more enjoyable the subject found the exercise session. An illustration of the PACES enjoyment questionnaire is presented in Table 3.3.

Table 3.3 – Ratings of perceived enjoyment of exercise.

*	I enjoy it	1	2	3	4	5	6	7	I hate it
	I feel bored	1	2	3	4	5	6	7	I feel interested
	I dislike it	1	2	3	4	5	6	7	I like it
*	I find it pleasurable	1	2	3	4	5	6	7	I find it unpleasurable
*	I am very absorbed in this activity	1	2	3	4	5	6	7	I am not at all absorbed in this activity
	It's no fun at all	1	2	3	4	5	6	7	It's a lot of fun
*	I find it energising	1	2	3	4	5	6	7	I find it tiring
	It makes me depressed	1	2	3	4	5	6	7	It makes me happy
*	It's very pleasant	1	2	3	4	5	6	7	It's very unpleasant
*	I feel good physically while doing it	1	2	3	4	5	6	7	I feel bad physically while doing it
*	It's very invigorating	1	2	3	4	5	6	7	It's not at all invigorating
	I am very frustrated by it	1	2	3	4	5	6	7	I am not at all frustrated by it
*	It's very gratifying	1	2	3	4	5	6	7	It's not at all gratifying
*	It's very exhilarating	1	2	3	4	5	6	7	It's not at all exhilarating
	It's not at all stimulating	1	2	3	4	5	6	7	It's very stimulating
*	It gives me a strong sense of accomplishment	1	2	3	4	5	6	7	It doesn't give me a strong sense of accomplishment
*	It's very refreshing	1	2	3	4	5	6	7	It's not at all refreshing
	I felt as though I would rather be doing something else	1	2	3	4	5	6	7	I felt as though there is nothing else I would rather be doing

3.5 Procurement, storage and analysis of muscle and blood samples

Blood samples were drawn from a superficial vein in the anticubital crease of the forearm using ether standard venepuncture techniques or via an indwelling cannula (NHS supply chain, Alferton, UK). Samples were collected into serum separation tubes (SST), K_2EDTA or lithium heparin vacutainers (BD Biosciences, UK). K_2EDTA and lithium heparin tubes were stored on ice while SST vacutainers were stored at room temperature, both for 1 h before centrifugation at 1500 g x 15 minutes at 4˚ C. Following centrifugation, plasma or serum was separated into 4 aliquots and stored at -80˚C for later analysis.

Figure 3.1 – Photograph of subject completing experimental trial with indirect calorimetry for assessment of substrate utilisation and blood sampling to assess serum and plasma metabolites.

3.5.1 Circulating metabolite and insulin analysis

Plasma glucose, lactate, non-esterified fatty acids, glycerol and β-hydroxybutyrate were analysed using a Randox Daytona spectrophotometer and commercially available kits (Randox Laboratories, Ireland). The coefficient of variation for plasma glucose, lactate,

non-esterified fatty acids and glycerol in our laboratory is ≤ 5 %. Insulin concentrations were analysed from serum using commercially available electrochemiluminescence immunoassay (Invitrogen, USA) as per the manufacturer's instructions.

3.5.2 Circulating amino acid analysis

Plasma amino acids were quantified as their *tert*-butyldimethylsilyl (*t*-BDMS) derivatives after the addition of suitable internal standards by Gas Chromatography-Mass Spectrometry: briefly, 200 µl of plasma was deproteinized with 1 ml of 100% ethanol, the supernatant dried under nitrogen at 90ºC, re-dissolved in 500 µl 0.5 M HCl, extracted with 2ml ethyl acetate (to remove lipid fraction), and the aqueous layer was then dried and derivatized (with 100 µl Acetonitrile and 100 µl MTBSTFA) at 90ºC for 90 min. The *t*-BDMS BCAA derivatives were separated on RTX5-ms (15m x 0.25 id, 0.25 µ film thickness) capillary column, initial column temp 70ºC, then ramped at 12ºC/min to 280ºC, injector temperature was 240ºC, Helium carrier gas was 1.2 ml/min. Selected ion monitoring of the (M-57) fragment was performed for each amino analysed, and the area under the peak determined relative to the mass isotopomer of the stable isotopically labelled internal standard used for each amino acid e.g. we monitored m/z 302 for Val and Leu and the isotopomer 303 (for 13^C Valine) and 304 (for 1,2 13^C_2 Leucine).

3.5.3 Muscle biopsies

Muscle biopsies were obtained from separate incision sites (2-3 cm apart) from the lateral portion of the vastus lateralis muscle pre-, post- and 3 h after the exercise protocol in study 2 and pre-, post- and 1.5 h after exercise study 3. Biopsies were obtained using a Bard Monopty Disposable Core Biopsy Instrument (12 gauge x 10 cm length, Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained (approximately 50 mg) under local anaesthesia (0.5 % marcaine) and immediately frozen in liquid nitrogen and stored at –80°C for later analysis.

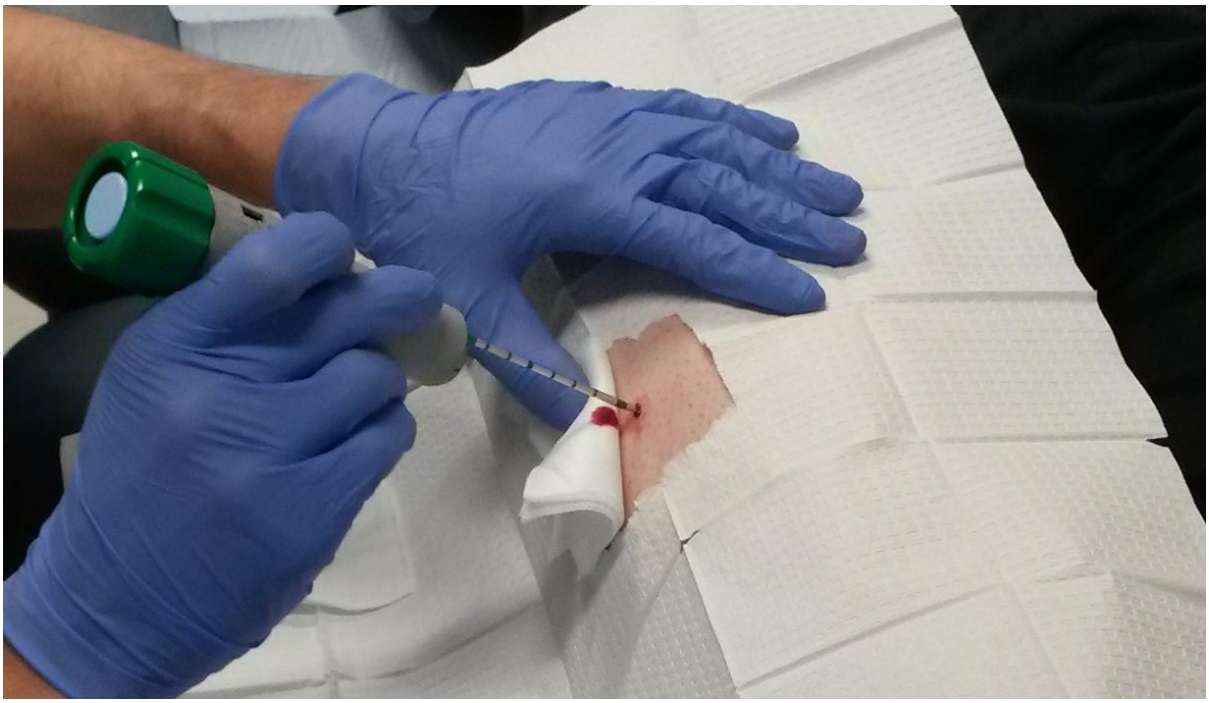

Figure 3.2 – Photograph of skeletal muscle biopsy.

3.5.4 mRNA isolation and QRT-PCR

RNA extraction and analysis: Muscle samples (~ 30mg) were immersed and homogenized in 1ml Trizol (Thermo Fisher Scientific, UK) RNA was extracted according to the manufacturer's instructions. RNA concentration and purity were assessed by UV spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Rosklide, Denmark). 70 ng RNA was used for each PCR reaction.

Primer design: Primer sequences were identified using Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Sequence homology searches ensured specificity, all primers had no potential unintended targets following a blast search. The primers were ideally designed to yield products spanning exon-exon boundaries to prevent any amplification of gDNA. Three or more GC bases in the last five bases at the 3' end of the primer were avoided. Secondary structure interactions (hairpins, self-dimer and cross dimer) within the primer were avoided. All primers were between 16 and 25 bp, and amplified a product of between 72 – 199 bp (Table 3.4). All primers were purchased from Sigma (Suffolk, UK).

Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): rt-qRT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by rotogene software (Hercules, CA, USA). rt-qTR-PCR was performed as follows: hold 50°C for 10 min (reverse transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial denaturation step) and PCR steps of 40 cycles; 95°C for 10s (denaturation), 60°C for 30s (annealing and extension). Upon completion, dissociation/melting curve analysis were performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). Following initial screening of suitable housekeeping genes, GAPDH showed the most stable C_t values across all RT-PCR runs, participants and regardless of experimental condition (25.5 ± 1.01); and was selected as the reference gene in all RT-PCR assays. The relative gene expression levels were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation where the relative expression was calculated as $2^{-\Delta\Delta ct}$ and where C_t represents the threshold cycle. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH) within same subject and condition and to a calibrator of pre-exercise.

Table 3.4 – Primer design.

Gene	Forward sequence 5 - 3	Reverse sequence 5 - 3	NM number	Product length (bp)
PPARGC1A (PGC-1α)	TGCATGAGTGTGTGCTCTGT	CAGCACACTCGATGTCACTC	NM_013261.4	133
P53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA	NM_001126114.2	141
PARK2 (Parkin)	TCCCAGTGGAGGTCGATTCT	GGAACCCCCTGTCGCTTAG	NM_013988.2	73
BECN1 (Beclin1)	ATCTCGAGAAGGTCCAGGCT	TCTGGGCATAACGCATCTGG	NM_001314000.1	144
ATG12	TGAACCACAAAGAAAATCAACTTGC	AGTCTCTTGCCACAAGCATCA	NM_001277783.1	93
ATG4B	TCGCTGTGGGGTTTTTCTGT	AGAATCTAGGGACAGGTTCAGGA	NM_013325.4	164
SQSTM1 (p62)	ATGGTGCACCCCAATGTGAT	CACAAGTCGTAGTCTGGGGA	NM_001142299.1	92
COXIV	CCCGGCATTTTACGACGTTC	TGGTAGCCAACATTCTGCCG	NM_001861.4	94
Tfam	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA	NM_003201.2	135
SIRT1	CGGAAACAATACCTCCACCT	CACATGAAACAGACACCCCA	NM_012238.4	186
FBXO32 (Atrogin1)	TGAGCGACCTCAGCAGTTAC	TGCAATATCCATGGCGCTCT	NM_058229.3	195
TRIM63 (MuRF1)	GACCAAGGAGAACAGTCACCA	GGCAGTTTCCACCAGCTTTG	NM_032588.3	199

3.5.5 Muscle glycogen concentration

Muscle glycogen concentration was determined according to the method described by Van Loon *et al.* (2000). Approximately 3-5 mg of freeze dried sample was powdered, dissected free of all visible non-muscle tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3-4 hours at 100°C. After cooling to room temperature, samples were neutralized by the addition of 250 µl 0.12 mol.L^{-1} Tris/2.1 mol.L^{-1} KOH saturated with KCl. Following centrifugation, 200 µl of the supernatant was analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol·kg^{-1} dry weight (dw) and intra-assay coefficients of variation was <5%.

3.5.6 [γ^{-32}P] ATP Kinase Assay

Human muscle tissue was homogenized by dounce homogenisation on ice in RIPA buffer [(50 mM TrisHCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (v/v) TritonX-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% β-mecertoehtanol, 1 mM Na3(OV)4 and 1 Complete (Roche) inhibitor tablet]. Debris was removed by centrifugation at 14,800 RPM for 45 minutes at 4°C. Following homogenisation protein was aliquoted for PKB/P70 S6 (300ug) and AMPK α1/α2 (100ug) kinase activity analysis and snap frozen and stored at -80°C.

All kinase assays (KA) were carried out by immune precipitation (IP) ether for 2h at 4°C or overnight at 4°C in homogenisation buffer {AMPK [50 mM Tris.HCL pH 7.25, 150 mM NaCL, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM dithiotheritol, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluride, 5 µg/ml soybean tryppsin inhibitor, 1% (vol/vol) Triton X-100] and p70S6K/pan PKB [50 mM Tris-HCL pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (vol/vol) Triton X-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% β-mercaptoethanol, 1 mM $Na_3(OV)_4$ and 1 Complete (Roshe) protease inhibitor tablet per 10 ml]}. Protein G sepharose (2.5 µl per IP) was used to precipitate the immune complexes. Immune complexes were washed twice in assay-specific high-salt washes (homogenization buffers as above with 0.5 M NaCL added) followed by one wash in assay-specific assay buffer (see below). Prior to carrying out the activity assay the immune-bead-complex was suspended in a total of 10 µl of assay buffer for p70S6k1 and pan PKB assays, and 20 µl of assay buffer for AMPK assays. All assays were carried out

in a 50 µl reaction. Assays were started every 20s by the addition of a hot assay mix, which consisted of assay buffer [PKB/p70S6K1 (50 mM Tris.HCL pH 7.4, 0.03% Brij35, and 0.1% β-mercaptoethanol), AMPK (50 mM HEPES pH 7.4, 1 mM DTT, and 0.02% Brij35)], ATP-$MgCL_2$ (100 µM ATP + 10 mM $MgCL_2$ for p70S6K1/panPKB, and 200 µM ATP + 50 mM $MgCL_2$ for AMPK), $^{32}\gamma$-ATP [specific activities as follows; panAMPK (0.25×10^6 cpm/mmol), panPKB (0.5×10^6 cpm/mol), p70S6K (1×10^6 cpm/mmol)] and finally synthetic peptide substrates [Crosstide for panPKB (GRPRTSSFAEG at 30 µM), S6tide for p70S6K (KRRRLASLR at 30 µM), and AMARA for AMPK (AMAEEAASAAALARRR at 200 µM)]. Assays were stopped at 20s intervals by spotting onto squares of p81 chromatography paper (Whatman; GE healthcare, UK) and immersing in 75 mM phosphoric acid and once in acetone. There were then dried and immersed in Gold Star LT Quanta scintillation fluid (Meridian Biotechanologies, Chesterfield, UK) and counted in a Packard 2200CA TriCarb scintillation counter (United Technologies). Assay results were quantified in $nmol.min^{-1}.min^{-1}$ (U/mg). Blanks for background subtractions were carried out with immunoprecipitation kinase with no peptide included in the assay reaction.

3.5.7 SDS-PAGE and Western Blotting

Approximately 20 – 30 mg of frozen muscle was round to powder and homogenised in 120µl of ice cols lysis buffer (25 mM Tris/HCL (ph7.4), 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-Pyrophosphatase, 1 mM Na_3VO_4, 0.27 M sucrose, 1% Triton x-100, 0.1% 2-mercaptoethanol) and supplemented with a protease inhibitor tablet (Complete mini, Roshe Applied Sciences, West Susses, UK). Homogenates were centrifuged at 14000g for 10min at 4°c and the supernatant was collected. The protein content of the supernatant was determined using bicinchoninic assay (Signa, Dorset UK). Each sample was diluted with and equal volume for 2 x Laemmli buffer (National Diagnostics, USA) and boiled for 5 min at 100°c. For each blot a standard control was loaded along with 15 µg of protein from each sample and then separated in Tris-glycine running buffer (10 x Tris/Glycine, Bio-Rad, UK) using pre-cast, 6% stacking and 20% separating gels (Bio-Rad, UK). Gels were transferred semi-dry onto nitrocellulose membranes (Geneflow Ltd. Staffordshire, UK) for 2h at 200 V and 45 mA per get in transfer buffers (10% TRIS/glycine (Sigma), 20% methanol, 70% ddH_2O) After transfer, membranes were blocked for 1h at room temperature in Tris-buffered sailine (TBST: 0.19 M Tris pH 7.6, 1.3 M NaCL, 0.1 % Tween-20) with 5% non-fat milk. The membranes

were then washed for 3 x 5 min in TBST before being incubated overnight at 4°c with total protein content antibodies for LC3b and p62 (Cell Signalling, UK) at concentrations of 1:1000 in 1 x TBST. The next morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with anti-species horseradish peroxidase-conjugated secondary antibody (Bio-Rad, UK) for 1h at room temperature. After a further 3 x 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA) for 5 min. Membranes were visualised using a Bio-Rad Chemi-doc system, and band densities were determined using ImageLab image-analysis software. Samples from each subject for all nutritional conditions were run on the same gel and statistical analysis conducted on raw densitometry data. Data has been expressed as fold change normalised to pre-exercise values.

Chapter 4

Leucine enriched protein feeding does not impair free fatty acid availability or exercise-induced lipid oxidation: beneficial implications for acute training in carbohydrate restricted states

The aim of this chapter was to test the hypothesis that leucine-enriched protein feeding does not impair circulating FFA availability or whole body lipid oxidation during exercise This study was presented orally at the European College of Sports Science (ECSS) Amsterdam, Nederland 2014.The full manuscript was published in Amino Acids November 2014. Additionally, the data presented in this chapter has informed the development of a novel commercially available whey protein based gel (SiS Whey 20).

4.1 Abstract

Given that the enhanced mitochondrial adaptations observed when training in carbohydrate (CHO) restricted states are potentially regulated through free fatty acid (FFA) mediated signalling and that leucine rich protein elevates muscle protein synthesis, the present study aimed to test the hypothesis that leucine enriched protein feeding enhances circulating leucine concentration but does not impair FFA availability nor whole body lipid oxidation during exercise. Nine males cycled for 2 h at 70% $\dot{V}O2_{peak}$ when fasted (PLACEBO) or having consumed a whey protein solution (WHEY) or a leucine enriched whey protein gel (GEL), administered as 22g 1 hour pre-exercise, 11 g/h during and 22g thirty minutes post-exercise. Total leucine administration was 14.4 g and 6.3 in GEL and WHEY, respectively. Mean plasma leucine concentrations were elevated in GEL (P= 0.001) compared with WHEY and PLACEBO (375 ± 100, 272 ± 51, 146 ± 14 $\mu mol.L^{-1}$ respectively). No differences (P= 0.153) in plasma FFA (WHEY 0.53 ± 0.30, GEL 0.45 ± 0.25, PLACEBO 0.65 ± 0.30, $mmol.L^{-1}$) or whole body lipid oxidation during exercise (WHEY 0.37 ± 0.26, GEL 0.36 ± 0.24, PLACEBO 0.34 ± 0.24 g/min) were apparent between trials, despite elevated (P= 0.001) insulin in WHEY and GEL compared with PLACEBO (38 ± 16, 35 ± 16, 22 ± 11 $pmol.L^{-1}$ respectively). I conclude that leucine enriched protein feeding does not impair FFA availability nor whole body lipid oxidation during exercise, thus having practical applications for athletes who deliberately train in CHO restricted states to promote mitochondrial related adaptations.

Keywords: mitochondria, endurance training, lipolysis, whey

4.2 Introduction

Traditional nutritional approaches for endurance training have typically promoted high CHO availability before, during and after training sessions in order to ensure high daily training intensities and volumes as well as promoting recovery (Cermak and Van Loon, 2013; Hawley *et al.* 1997). However, during the last decade, we and others have consistently observed a potent effect of *reduced* CHO availability (i.e. fasted and/or glycogen depleted training) in modulating training-induced adaptations in skeletal muscle (Hawley and Morton, 2013).

Despite the emergence of the train-low paradigm, its practical application in athletic populations is not without limitations, most notably a potential reduction in absolute training intensities (Yeo *et al.* 2008) as well as increased skeletal muscle protein oxidation and breakdown (Lemon and Mullin, 1980; Howarth *et al.* 2010). An obvious solution to compensate for the latter is to consume high quality protein in close proximity to the exercise stimulus given that protein provision before (Coffey *et al.* 2011), during (Hulston *et al.* 2011) and after (Breen *et al.* 2011; Howarth *et al.* 2010) endurance type exercise protocols reduces skeletal muscle protein breakdown and enhances post-exercise skeletal muscle protein synthesis. In this regard, Pasiakos *et al.* (2011) also observed that enhanced concentration of leucine (3.5 g in 10 g EAA) in an essential amino acid mixture consumed during two hours of endurance exercise significantly enhanced post-exercise muscle protein synthesis rates when compared with the same total intake of essential amino acids containing reduced leucine concentration (1.87 g in 10 g EAA). I also observed that consuming protein before, during and after acute exercise undertaken in a glycogen depleted state did not impair the activation of the AMPK- PGC-1α pathway and positively affected molecular regulators of protein synthesis (e.g. eEF2 phosphorylation status), thus demonstrating that high amino acid availability still permits activation of key cell signalling cascades thought to regulate the training-low response (Taylor *et al.* 2013). When taken together, such data suggest that reduced CHO but high protein availability (especially leucine rich protein) may therefore be a strategic approach to stimulate training-induced oxidative adaptations of skeletal muscle.

It is noteworthy, however, that the provision of high protein availability could hinder additional pathways that may be involved in regulation of the training response associated with training-low. Indeed, our group observed that the elevated insulin induced by amino

acid ingestion (albeit administered as a casein hydrolysate solution) attenuated circulating free fatty acid (FFA) availability and whole body rates of lipid oxidation (Taylor *et al.* 2013). This effect may be problematic given that FFA may also act as signalling intermediates involved in regulating training adaptation in addition to providing substrate for oxidation (Zbinden-Foncea *et al.* 2013; Philp *et al.* 2013; Fyffe, *et al.* 2006). Any feeding strategy intended to achieve high protein availability (in an attempt to reduce protein breakdown and promote protein synthesis) during acute train-low sessions should therefore be simultaneously administered with the goal of minimising reductions in lipolysis such that FFA mediated cell signalling can still occur.

Accordingly, the aim of the current chapter was to therefore test the hypothesis that leucine enriched protein feeding enhances circulating leucine availability but does not impair FFA availability and lipid oxidation during exercise. To this end, I utilised two protein feeding strategies consisting of a traditional whey protein solution but also a novel leucine enriched protein gel. The use of the latter is considered particularly advantageous for endurance athletes given that gels are a highly practical approach to feeding when in locomotion (Pfeiffer *et al.* , 2010, 2012; Lee *et al.* 2014). When taken together, I envisage this data will be of immediate practical application for those athletes who deliberately train in CHO restricted states to promote mitochondrial related adaptations.

4.3 Methodology

4.3.1 Participants: Nine males (age 29 ± 10 years, height 179.7 ± 8.6 cm, body mass 79.4 ± 9.8 kg, $\dot{V}O2_{peak}$ 53.0 ± 6.1 $mL \cdot kg^{-1} \cdot min^{-1}$ and peak power 334 ± 38 W) volunteered to participate in the study after providing informed written consent. Participants were recreational and competitive cyclists and tri-athletes who trained between 3 – 7 hours per week and had been cycling regularly for > 1 year. The study was approved by the Research Ethics Committee of Liverpool John Moores University.

4.3.2 Experimental protocol*:* Participants arrived at the laboratory in the morning of the trial (08:00) having followed a prescribed diet (5 $g \cdot kg^{-1}$ CHO, 2 $g \cdot kg^{-1}$ Pro, 1 $g \cdot kg^{-1}$ fat) for two days prior and following an overnight fast. After obtaining measures of nude body mass, an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the anticubital vein in the anterior crease of the forearm and a resting blood sample drawn. After the resting blood sample was taken, the cannula was flushed with ~ 5

ml of sterile saline (Kays Medical supplies. Liverpool UK) to keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw.

Having obtained a resting blood sample, participants' ingested ether a placebo gel (non-calorific gelling agent) and 500 ml of water (PLACEBO), a leucine enriched protein gel that is not currently commercially available (GEL) providing 22g protein + 2.4 g leucine (total leucine of 4.8 g) with 500 ml of water, or 22g of commercially available whey protein (total leucine of 2.1 g; REGO Protein, Science in Sport, UK) mixed in 500 ml water (WHEY) and then rested in the laboratory for 60 min prior to the start of exercise. Blood samples were obtained repeatedly at 20 min intervals during the 60 min pre-exercise period and immediately before exercise commenced. The nutritional compositions of the protein supplements are shown in Table 4.1. On the basis that caffeine can partially restore training intensity when in glycogen depleted states (Lane *et al.* 2013) and that beta-hydroxy beta-methylbutyrate (HMB) can reduce muscle protein breakdown (Wilkinson *et al.* 2013), I also chose to fortify the GEL with additional caffeine (100 mg per 22g of protein) and HMB (1 g per 22g of protein). After the 60 min rest period pparticipants then cycled for 120 min at 60% PPO (~70% $\dot{V}O2_{peak}$) on a fully adjustable electromagnetically braked cycle ergometer (SRM, Julich, Germany). Each subject used their own pedals, cleats and shoes during each trial.

During exercise, feeding of 5.5g protein (WHEY or GEL) or PLACEBO, with 125 ml water occurred at 30 min and 60 min, finally 11g protein (WHEY or GEL) or PLACEBO with 250 ml water was provided at 90 min. In this way subjects ingested 22g of protein from ether WHEY or GEL during the trials or no protein during PLACEBO condition, with all trials fluid matched. Blood samples were taken every 20 minutes during exercise and immediately at termination of exercise. Substrate oxidation (g/min) was determined during exercise using CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US) using equations of Jeukendrup and Wallis (2005). Ratings of perceived exertion (RPE) (Borg 1970), gastrointestinal discomfort (GI) (Pfieffer *et al.* 2012) and heart rate (Polar Kempele 610i, Finland) were recorded every 15 min during exercise.

Upon completion of exercise, participants dismounted from the ergometer, towel dried and nude mass was recorded. Thirty minutes after exercise, participants consumed a further 22g of protein or placebo with 500 ml water in the form of WHEY or GEL and and two more blood samples were taken at 1 h and 2 h after completion of exercise. Laboratory

conditions remained constant across all trials (19 - 21℃, 40 – 50% humidity). In this way, participants consumed a total of 66 g protein during the protein trials (i.e. 22g before, 22g during and 22g after exercise) in the form of whey protein (total leucine intake of 6.3 g) or a leucine enriched protein gel (total leucine intake of 14.4 g). Upon exercise completion, participants also rated their perceived ratings of enjoyment as quantified by the Physical Activity Enjoyment Scale (Kenzierski and DiCarlo, 1991).

Table 4.1 – Protein content of gel and whey supplements

	GEL	WHEY
Total protein (g)	22.0	22.0
Additional leucine (g)	2.4	0.0
Total leucine (g)	4.8	2.1
Total BCAA (g)	7.5	4.9
Total EAA (g)	13.1	9.3
Caffeine (mg)	100.0	0.0
HMB (g)	1.0	0.0

4.3.3 Blood analysis: Blood samples were collected and analysed for circulatory metabolites, NEFA, glycerol, glucose and lactate as well as circulatory amino acids as described in section 3.6.1.

4.3.4 Statistical analysis. Statistical analysis was conducted using the Statistical Package for the Social Sciences software program (SPSS, version 18). Changes in physiological, metabolic and perceptual responses (i.e. physiological variables, amino acids, metabolites, substrate oxidation rates, RPE and GI) were analysed using a two-way repeated measures general linear where the within factors were time and condition. Descriptive statistics were produced for all data sets to check for normal distribution indicated by Kolmogorov-Smirnov (accepted if $P>0.05$). Comparison of AUC for insulin, amino acids and metabolites across all time points was analysed according to a one-way repeated measures general linear where the within factor was condition. Where a significant main effect was, pairwise comparisons were analysed according to Bonferoni post-hoc tests in order to locate specific differences. Baseline AUC was taken at lowest X value, calculation was comleted using trapezoidal method. Statistical significance was set at $P < 0.05$ and all data in text, figures, and tables are presented as means ± SD.

4.4 Results

4.4.1 Physiological and perceptual responses during exercise

A comparison of participants' physiological and perceptual responses during the exercise protocol is shown in Table 4.2. Heart rate ($P<0.01$), lipid oxidation ($P<0.01$) and RPE ($P<0.01$) all displayed progressive increases during exercise, whereas CHO oxidation exhibited a significant decline ($P<0.01$). In contrast, GI discomfort displayed no change during exercise ($P=0.14$). However, there was no difference in heart rate ($P=0.84$), oxygen uptake ($P=0.67$), CHO oxidation ($P=0.97$), lipid oxidation ($P=0.90$), RPE ($P=0.11$) and GI discomfort ($P=0.19$) between the PLACEBO, GEL and WHEY trials. Participants also reported a tendency for a higher rating of perceived enjoyment ($P=0.073$) in the GEL (89 ± 5 AU) versus the WHEY (84 ± 5 AU) and PLACEBO (79 ± 5 AU) trials.

4.4.2 Metabolic responses and substrate oxidation during exercise

Exercise induced significant increases in plasma NEFA ($P<0.01$), glycerol ($P<0.01$) and lactate ($P<0.01$) whereas glucose displayed no significant ($P=0.09$) change (Figure 4.1). Serum insulin significantly increased in the pre-exercise period and showed significant declines during exercise ($P<0.01$) in both the WHEY and GEL trials (Figure 4.2). Accordingly, insulin displayed significant differences between conditions ($P<0.01$) where both WHEY ($P<0.01$) and GEL ($P=0.01$) were significantly higher than PLACEBO (Figure 1.2) though no differences were apparent between the WHEY and GEL trials ($P=1.0$). Despite significant changes in insulin in the protein fed trials, plasma NEFA ($P=0.12$), glycerol ($P=0.42$), glucose ($P=0.19$) and lactate ($P=0.06$) were not different between the PLACEBO, WHEY and GEL trials at rest or during exercise. Given that substrate availability was not different between conditions, the exercise-induced decrease in CHO oxidation ($P<0.01$) and increase in lipid oxidation ($P<0.01$) during the 2 h exercise protocol were not different between conditions ($P=0.97$ and 0.90, respectively).

Table 4.2 – Physiological and perceptual responses during exercise. * denotes significant difference from 30 min, P<0.05.

	30	60	90	120
CHO Oxidation (g/min)				
GEL	3.14 ± 0.18	2.70 ± 0.23*	2.25 ± 0.15*	1.98 ± 0.15*
PLACEBO	3.13 ± 0.19	2.61 ± 0.18*	2.38 ± 0.15*	2.20 ± 0.13*
WHEY	3.13 ± 0.24	2.64 ± 0.22*	2.24 ± 0.17*	1.97 ± 0.18*
Fat Oxidation (g/min)				
GEL	0.12 ± 0.08	0.31 ± 0.11*	0.51 ± 0.06*	0.63 ± 0.05*
PLACEBO	0.05 ± 0.07	0.34 ± 0.08*	0.47 ± 0.07*	0.61 ± 0.07*
WHEY	0.11 ± 0.09	0.40 ± 0.08*	0.47 ± 0.07*	0.60 ± 0.07*
HR (b/min)				
GEL	149 ± 4	154 ± 3	158 ± 4*	164 ± 4*
PLACEBO	148 ± 3	153 ± 3	157 ± 4*	164 ± 4*
WHEY	152 ± 5	156 ± 3	157 ± 4*	163 ± 3*
$\dot{V}O_2$ (%max)				
GEL	67 ± 1.8	68 ± 2.1	69 ± 3.0	67 ± 4.3
PLACEBO	63 ± 1.3	67 ± 1.5	69 ± 1.8	72 ± 2.4
WHEY	66 ± 1.6	71 ± 1.5	67 ± 3.0	68 ± 2.8
RPE (AU)				
GEL	11 ± 1.0	13 ± 0.5*	13 ± 0.6*	14 ± 0.7*
PLACEBO	12 ± 0.5	14 ± 0.5*	15 ± 0.5*	16 ± 0.6*
WHEY	12 ± 0.4	14 ± 0.4*	14 ± 0.7*	15 ± 1.0*
GI (AU)				
GEL	10 ± 0.7	10 ± 0.7	10 ± 1.0	10 ± 1.1
PLACEBO	9 ± 0.1	10 ± 0.8	10 ± 0.9	9 ± 0.8
WHEY	10 ± 0.7	11 ± 0.7	11 ± 0.8	10 ± 0.7

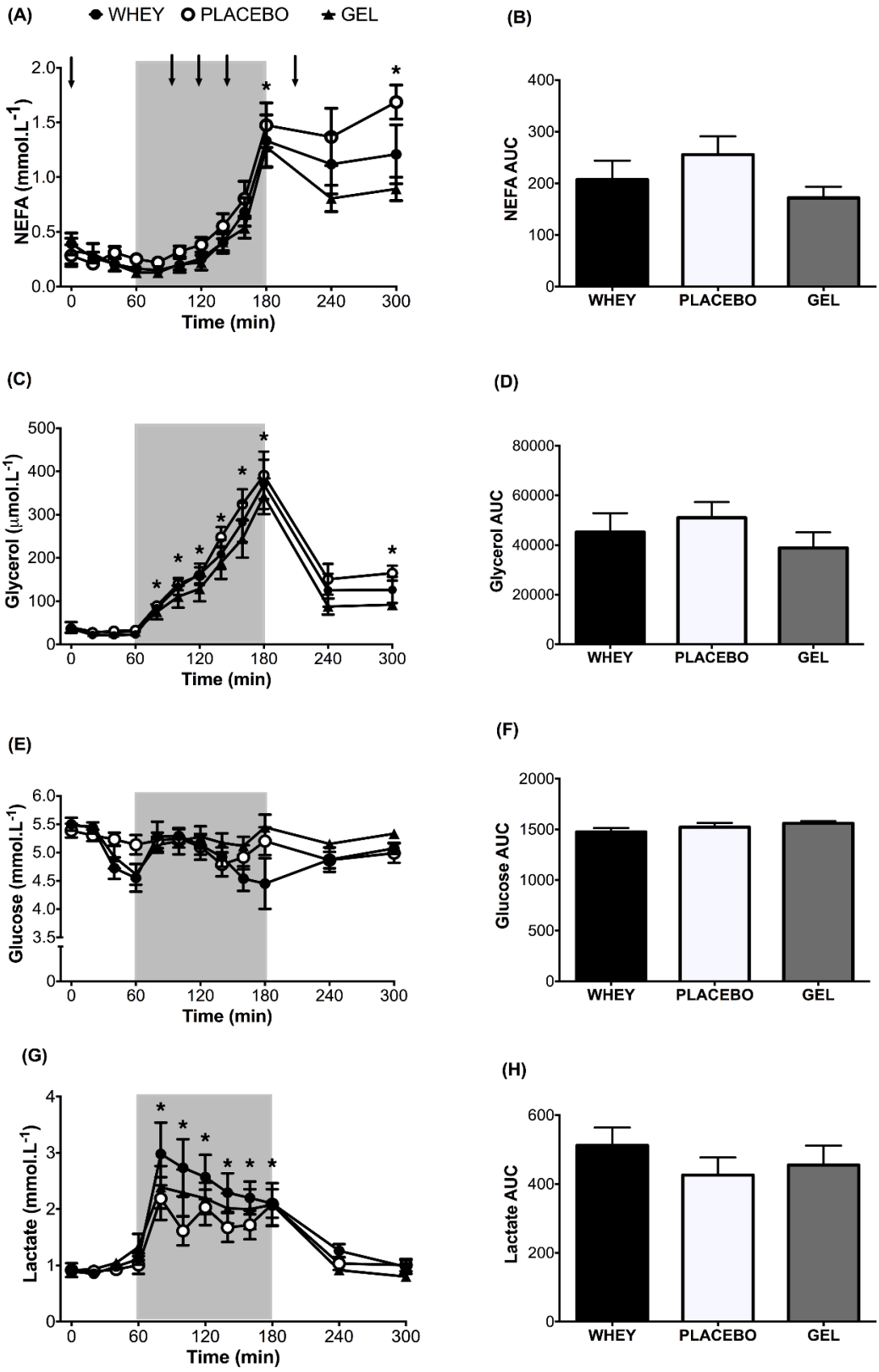

Figure 4.1 - Plasma (A) NEFA, (B) glycerol, (C) glucose and (D) lactate before, during and after exercise in the GEL, WHEY and PLACEBO trials. Arrows represent feeding points. Shaded area represents exercise. * denotes significant difference from baseline values, $P<0.05$.

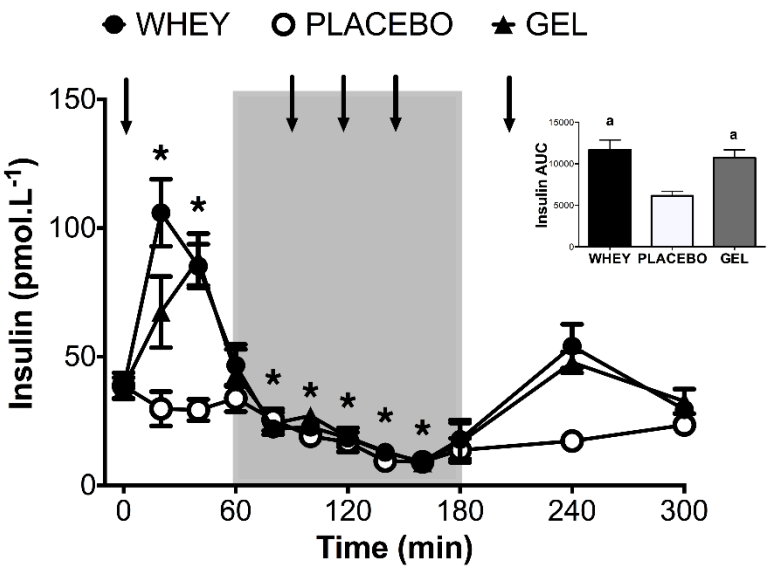

Figure 4.2 – Serum insulin concentrations before, during and after exercise in the GEL, WHEY and PLACEBO trials. Arrows represent feeding points. Shaded area represents exercise. * denotes significant difference from baseline values, P<0.05, a denotes significant difference from PLACEBO, P<0.05.

4.4.3 Plasma amino acid responses

Plasma leucine, BCAAs and EAAs are displayed in Figure 4.3. Feeding-induced increases in plasma leucine (P<0.01) was significantly different between conditions (P<0.01) such that a significant interaction effect was observed (P=0.02). Specifically, plasma leucine was significantly greater in GEL versus both WHEY (P<0.01) and PLACEBO (P<0.01). Similar to leucine, feeding also induced a significant increase (P<0.01) in plasma BCAAs and EAAs for both WHEY and GEL where both a significant effect of condition (P<0.01) and interaction (P<0.01 and P=0.04, respectively) was observed. However, pairwise comparisons revealed there to be no differences in total BCAAs and EAAs between GEL and WHEY (P=1.0 and 0.6, respectively) though both were different from PLACEBO (P<0.01).

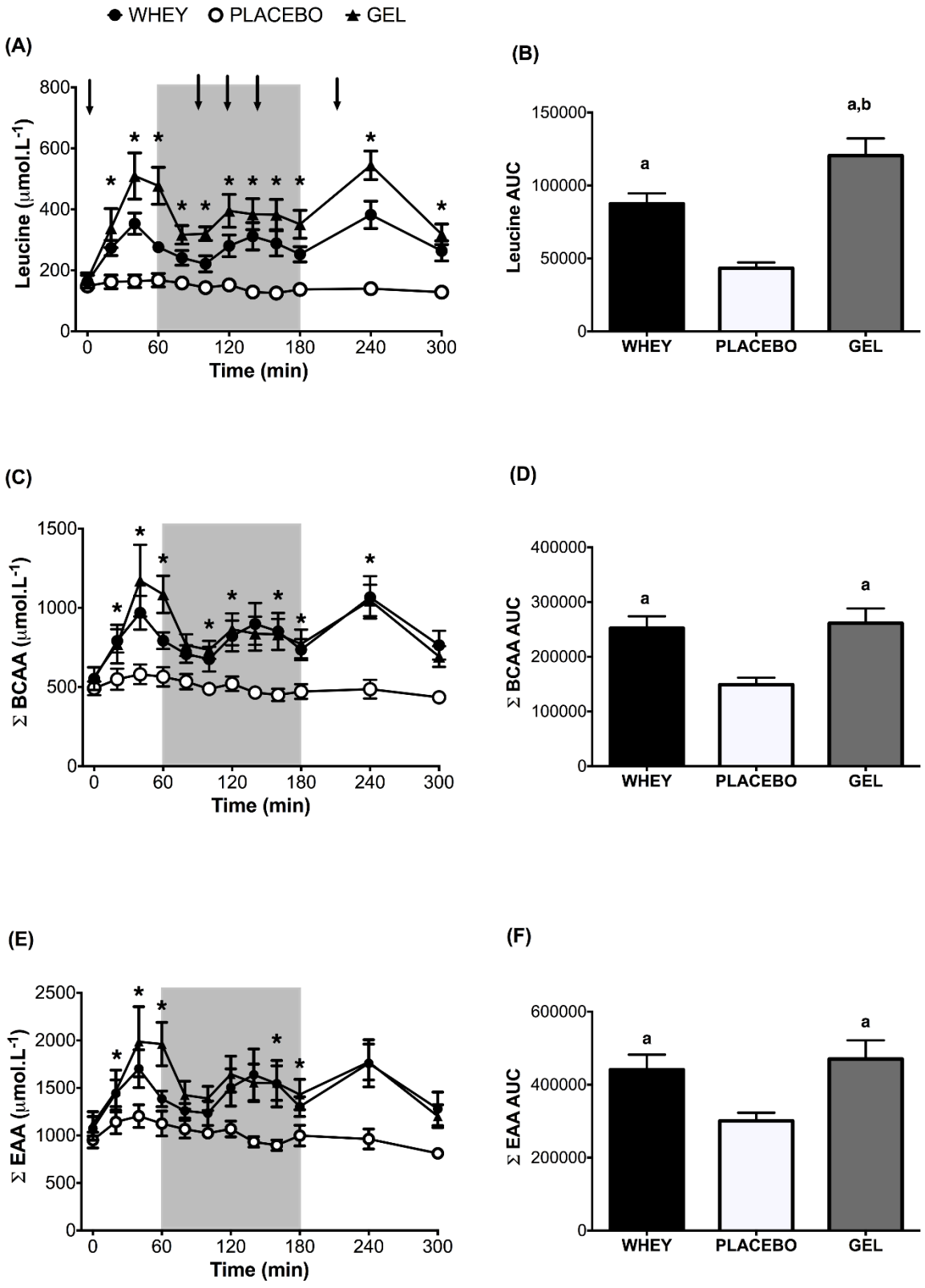

Figure 4.3 – Plasma (A) leucine, (B) BCAAs, and (C) EAAs before, during and after exercise in the GEL, WHEY and PLACEBO trials. Arrows represent feeding points. Shaded area represents exercise. * denotes significant main effect from baseline values, P<0.05, a denotes significant difference from PLACEBO, P<0.05, b denotes significant difference from WHEY, P<0.05.

4.5 Discussion

The aim of the present chapter was to test the hypothesis that leucine enriched protein feeding enhances circulating leucine availability but does not impair FFA availability and lipid oxidation during exercise. To this end, I utilised two protein feeding strategies consisting of a traditional whey protein solution but also a novel leucine enriched protein gel. I provide novel data by demonstrating that provision of protein (in either of the aforementioned forms) before, during and after two hours of endurance exercise undertaken in the CHO restricted state does not impair FFA availability or lipid oxidation. Given that aspects of the enhanced training response observed when training in CHO restricted states are potentially regulated through FFA mediated signalling and also that leucine enriched protein elevates muscle protein synthesis, I therefore consider our data to have practical implications (i.e. feeding strategies) for training in CHO restricted states.

Despite the apparent advantage to carefully scheduling periods of fasted (Van Proeyen *et al.* 2011) and/or glycogen depleted endurance training (Yeo *et al.* 2008; Morton *et al.* 2009), such approaches may be limited in that skeletal muscle protein oxidation and breakdown is increased (Lemon and Mullin, 1980; Howarth *et al.* 2010) and hence, net protein balance becomes negative if amino acids are also not ingested (Hulston *et al.* 2011). If performed chronically (especially in the presence of reduced daily caloric intake), this approach could therefore lead to a loss of skeletal muscle mass (Cabone *et al.* 2013; Mettler *et al.* 2010; Pasiakos *et al.* 2013) and potentially, a de-training effect. To this end, out group recently demonstrated that consuming a casein hydrolysate solution before, during and after glycogen depleted exercise does not impair activation of mitochondrial related signalling pathways (e.g. AMPK- PGC-1α) as well as positively affecting molecular regulators of protein synthesis (e.g. eEF2 phosphorylation status) (Taylor *et al.* 2013). As such, many elite endurance cyclists now perform prolonged morning rides in CHO restricted states but with protein rich breakfasts and additional protein during exercise (typically in the form of whey / casein drinks) in a deliberate attempt to promote oxidative adaptations of skeletal muscle (Walsh, 2014).

In terms of modulating protein synthesis, however, current evidence also suggests that both rapidly digestible but leucine rich proteins are the optimal protein source. For example, Pasiakos *et al.* (2011) observed that enhanced concentration of leucine (3.5 g in 10 g EAA) in an essential amino acid mixture consumed during two hours of endurance exercise

significantly enhanced post-exercise muscle protein synthesis rates when compared with the same total intake of essential amino acids but containing reduced leucine concentration (1.87 g in 10 g EAA). Furthermore, Churchward-Venne *et al.* (2014) demonstrated comparable post-exercise myofibrillar synthesis rates (albeit from a resistance exercise stimulus) from mixed macronutrient drinks containing 25 g whey versus a bolus of 6.25 g whey supplemented with 5 g leucine. Such observations therefore formed the underlying rationale for the present study in terms of studying whey protein (naturally high in leucine) but also, a novel leucine enriched protein gel. Total leucine administration during the data collection period (i.e. 5 hours) was 6.3 g and 14.4 g in WHEY and GEL, respectively. Accordingly, mean plasma leucine levels were higher in GEL versus WHEY and in both feeding strategies, plasma leucinemia increased to levels that would be expected to promote post-exercise muscle protein synthesis (Breen *et al.* 2011; Pasiakos *et al.* 2011). Unfortunately, direct estimates of muscle protein synthesis (and related molecular regulators) were not obtained in the present study nor did I quantify rates of leucine oxidation. Indeed, it is likely that the total leucine delivery in both trials would have resulted in elevated leucine oxidation given that high protein availability potentiates whole body leucine oxidation (Bowtell *et al.* 1998). However, given that exercising in CHO restricted states augments leucine oxidation (Lemon and Mullin, 1980; Wagenmakers *et al.* 1991; Howarth *et al.* 2010), it was a deliberate aim to administer higher exogenous leucine so as to deliver both substrate to promote muscle protein synthesis (Breen *et al.* 2011; Pasiakos *et al.* 2011; Churchward-Venne *et al.* 2013) but yet, also compensate for the higher levels of endogenous leucine oxidation (Lemon and Mullin, 1980; Wagenmakers *et al.* 1991; Howarth *et al.* 2010) that have been shown to reach >50 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ in trained males (Phillips *et al*. 1993).

Importantly, I observed that neither the whey protein solution nor the leucine enriched protein gel attenuated circulating FFA availability or whole body rates of lipid oxidation during exercise, despite elevated serum insulin levels in the pre-exercise period. This finding is especially relevant considering that the enhanced training response observed when training with low CHO availability (particularly the elevated capacity for lipid oxidation during customary exercise) is likely regulated, in part, through FFA mediated signalling. Indeed, the exercise-induced increase in p38MAPK activity is blunted by pharmacological reduction of circulating FFA availability (Zbinden-Foncea *et al.* 2013). Furthermore, exercise undertaken in a glycogen-depleted state increases PPARδ binding to

the CPT1 promoter (Philp *et al.* 2013), the latter considered as a rate limiting enzyme for long chain fatty acid oxidation. The observation of no apparent reduction in lipolysis with protein feeding in the present study disagrees with previous observations from our laboratory where we demonstrated that a casein hydrolysate solution (administered as 20 g 45 min pre-exercise, 10 g during exercise and 20 g post-exercise) attenuated plasma FFA availability and whole body rates of lipid oxidation during exercise. Such discrepancies may be due in part to differences in timing of pre-exercise feeding (i.e. 60 versus 45 minutes before exercise) as well as type of protein consumed (i.e. casein hydrolysate versus predominantly whey isolate proteins). The latter point is particularly relevant given that hydrolysate proteins induce greater insulin responses compared with isolated protein sources (Tang *et al.* 2009; Pennings *et al.* 2011). As such, the effects of protein ingestion on metabolic responses during exercise are likely dependent upon the interplay of a number of important factors including type and timing of protein ingestion. Such factors should therefore be taken into consideration when designing real world feeding strategies.

To achieve the delivery for the leucine enriched protein trial, I chose to develop a novel protein based gel. Indeed, considering that leucine is often not readily soluble in water, I reasoned that a gel format provided a more convenient approach for which to deliver a leucine enriched protein source. Moreover, gels are a highly practical approach to carrying fuel and feeding whilst in locomotion as opposed to conventional approaches of fluid delivery (Pfeiffer *et al.* 2010; Lee *et al.* 2014). Accordingly, gels are a highly utilised form of supplement by endurance athletes (Pfeiffer *et al.* 2012). On the basis that caffeine can partially restore training intensity when in glycogen depleted states (Lane *et al.* 2013) and also that beta-hydroxy beta-methylbutyrate (HMB) can reduce muscle protein breakdown (Wilkinson *et al.* 2013), I also chose to fortify this GEL with additional caffeine (100 mg per 22g of protein) and HMB (1 g per 22g of protein). Although I did not measure parameters related to these products given that the exercise protocol was clamped (i.e. 2 h fixed intensity of 70% $\dot{V}O2_{peak}$) and no indices of protein breakdown were obtained, the observations of no differences in gastrointestinal discomfort between trials supports the notion that multiple ingredients in the gel format were well tolerated. Interestingly, participants' ratings of perceived enjoyment (as quantified by the Physical Activity Enjoyment Scale) also tended to be higher in GEL (89 ± 5) versus both WHEY (84 ± 5) and PLACEBO (79 ± 5). Given that this scale takes into account subjective measurements of mood status related to vigor and arousal, this observation maybe related to the higher

plasma concentrations of leucine observed in the GEL trial and is accordance with the hypothesis that elevated BCAAs (especially in conditions of low CHO availability) may alleviate symptoms of central fatigue (Blomstrand *et al.* 2005; Blomstrand, 2006).

In summary, I provide novel data by demonstrating that neither a whey protein solution nor a leucine enriched protein gel impairs circulating FFA availability or rates of whole body lipid oxidation during two hours of endurance exercise undertaken in a CHO restricted state. Given that the augmented training response observed when training fasted and/or glycogen depleted is potentially regulated through FFA mediated signalling and also that leucine rich protein elevates muscle protein synthesis, I consider this data to have practical implications for training in CHO restricted states.

Chapter 5

Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes

The aim of this chapter was to characterise the effects of reduced carbohydrate (CHO) but high leucine availability on exercise capacity and skeletal muscle cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis and MPS. This work was presented at ECSS Malmo, Sweden 2015, with the full manuscript published in Physiological Reports February 2016.

5.1 Abstract

Using an amalgamation of previously studied "train-low" paradigms, I characteris the effects of reduced carbohydrate (CHO) but high leucine availability on cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis and muscle protein synthesis (MPS). In a repeated measures cross-over design, eleven males completed an exhaustive cycling protocol with high CHO availability before, during and after exercise (HIGH) or alternatively, low CHO but high protein (leucine enriched) availability (LOW+LEU). Muscle glycogen was different ($P<0.05$) pre-exercise (HIGH: 583 ± 158, LOW+LEU: 271 ± 85 mmol·kg^{-1} dw) but decreased ($P<0.05$) to comparable levels at exhaustion (≈ 100 mmol·kg^{-1} dw). Despite differences ($P<0.05$) in exercise capacity (HIGH: 158 ± 29, LOW+LEU: 100 ± 17 min), exercise induced ($P<0.05$) comparable AMPKα2 (3-4-fold) activity, PGC-1α (13-fold), p53 (2-fold), Tfam (1.5-fold), SIRT1 (1.5-fold), Atrogin 1 (2-fold) and MuRF1 (5-fold) gene expression at 3 h post-exercise. Exhaustive exercise suppressed p70S6K activity to comparable levels immediately post-exercise (≈ 20 fmol.min^{-1}.mg^{-1}). Despite elevated leucine availability post-exercise, p70S6K activity remained suppressed ($P<0.05$) 3 h post-exercise in LOW+LEU (28 ± 14 fmol.min^{-1}.mg-1) whereas muscle glycogen re-synthesis (40 mmol·kg^{-1} dw.h^{-1}) was associated with elevated ($P<0.05$) p70S6K activity in HIGH (53 ± 30 fmol.min^{-1}.mg^{-1}). I conclude: 1) CHO restriction before and during exercise induces "work-efficient" mitochondrial related cell signalling but, 2) post-exercise CHO and energy restriction maintains p70S6K activity at basal levels despite feeding leucine enriched protein. These data support the practical concept of "fuelling for the work required" as a potential strategy for which to amalgamate train-low paradigms into periodised training programs.

Keywords: mitochondrial biogenesis, muscle glycogen, train-low

5.2 Introduction

Traditional nutritional strategies for endurance athletes have typically advised high carbohydrate (CHO) availability before, during and after training sessions in order to support high daily training volume and intensities (Burke *et al.* 2011). However, in the last decade accumulating data demonstrate that strategic periods of reduced CHO availability (the so-called "train-low" paradigm) actually augments selected skeletal muscle markers of training adaptation (Hawley and Morton. 2014; Bartlett *et al.* 2015). For example, reducing endogenous and/or exogenous CHO availability during short-term (e.g. 3-10 week) endurance training increases mitochondrial enzyme activity and protein content (Morton *et al.* 2009; Yeo *et al.* 2008; Van Proeyen *et al.* 2011), increases both whole body (Yeo *et al.* 2008) and intramuscular lipid oxidation (Hulston *et al.* 2010) and in some instances, improves exercise capacity and performance (Hansen *et al.* 2005; Cochran *et al.* 2015; Marquet *et al.* 2016). The augmented training responses observed when "training low" are thought to be mediated via the complex regulation of cell signalling pathways with potent roles in modulating an oxidative phenotype. Indeed, when exercise protocols are matched for work done, CHO restriction augments both AMPK (Wojtaszwski *et al.* 2003) and p38MAPK activation (Cochran *et al.* 2010) that ultimately converge on downstream transcription factors and co-activators such as PGC-1α (Psilander *et al.* 2013), p53 (Bartlett *et al.* 2013) and PPARδ (Philp *et al.* 2013). In the context of nutrient-gene interactions, it is therefore apparent that the acute molecular regulation of cell signalling processes provides a theoretical basis for understanding the molecular mechanisms underpinning chronic training adaptations.

The research designs that have been used to study both acute and chronic train-low adaptations thus far, have largely adopted twice per day training protocols (Hansen *et al.* 2005; Yeo *et al.* 2008; Hulston *et al.* 2010), fasted training (Van Proyen *et al.* 2011) and CHO restriction during (Morton *et al.* 2009) and/or post-exercise (Pilegaard *et al.* 2005). More recently, a "sleep-low, train-low" model has also been developed in which athletes perform an evening training session but sleep with reduced post-exercise CHO intake, followed by completion of a fasted training session on the subsequent morning. Using this model, we (Bartlett *et al.* 2013) and others (Lane *et al.* 2015) observed enhanced activation of acute cell signalling pathways and expression of genes with putative roles in regulating training adaptation. Furthermore, when performed chronically as part of a periodised nutrition strategy, this model of CHO restriction also enhanced sub-maximal cycling

efficiency, high-intensity cycling capacity and improved 10 km run time in already well trained triathletes (Marquet *et al.* 2016).

Despite the emergence of the aforementioned train-low paradigms, the optimal approach for which to practically apply with athletic populations is not currently known. Such limitations are most well recognised for the potential reductions in absolute training intensity associated with reduced CHO availability (Widrick *et al.* 1993; Yeo *et al.* 2008; Hulston *et al.* 2010), perturbations to immune function and associated increases in muscle protein degradation (Lemon and Mullin, 1980; Howarth *et al.* 2010), all of which could be detrimental to long term training and athletic performance. Furthermore, in the real world training environments of elite endurance athletes, it is likely that athletes practice an amalgamation of the aforementioned train-low paradigms (either through default of their current training structure or via coach and sport scientist-led practices), as opposed to undertaking one potential strategy in isolation. The complexity of practical train-low models is also further exacerbated by the observations that many endurance athletes (especially cyclists) also practice day-to-day or longer term periods of energy periodisation (as opposed to CHO *per se*) in an attempt to reduce both body mass and fat mass in preparation for key competitive events (Stellingwerff, 2012; Vogt *et al.* 2005). Indeed, the performance improvements observed by Marquet *et al.* (2016) were also associated with a 1 kg reduction in fat mass induced by the periodised sleep-low model. When taken together, such data highlight the requirement to study train-low paradigms that may be more reflective of real world athletic practice (i.e. both CHO and energy restriction) and that are representative of an amalgamation of the train-low protocols previously studied in the research setting.

With this in mind, the aim of this chapter was to examine the effects of high CHO versus low CHO availability on the modulation of those skeletal muscle cell-signalling pathways with putative roles in the regulation of both mitochondrial biogenesis and muscle protein synthesis (MPS). I employed a repeated measures crossover design whereby healthy active males performed an exhaustive cycling based protocol in conditions of high CHO availability (i.e. best nutritional practice of CHO loading and CHO feeding during and after exercise) versus a nutritional protocol representative of both low CHO and energy availability (as achieved via 36 h of reduced CHO intake and omission of CHO intake before, during and after exercise). In an attempt to compensate for the negative effects of energy deficit on muscle protein degradation and synthesis (Pasiakos *et al.* 2010, 2011,

2013; Breen *et al.* 2011; Areta *et al.* 2014), the low CHO protocol was also completed with leucine rich protein availability before, during and after exercise. I specifically hypothesise that reduced CHO availability would impair exercise capacity but nonetheless, would induce comparable or superior mitochondrial related signalling thereby inducing a work-efficient training and nutritional train-low paradigm. I further hypothesised that high leucine availability would up-regulate markers of protein synthesis and reduce markers of muscle protein breakdown.

5.3 Methodology

5.3.1 Participants: Eleven recreationally active and amateur competitive male cyclists (age: 24.0 ± 3.3; height: 1.78 ± 0.1 m; body mass: 79.6 ± 4.0 kg) who trained between 3 – 7 hours per week took part in this study. Mean $\dot{V}O2_{peak}$ and peak power output (PPO) for the cohort was 53.6 ± 7.0 $mL \cdot kg^{-1} \cdot min^{-1}$ and 285 ± 20 W respectively. None of the participants had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during the course of the study. All participants provided informed written consent and all procedures conformed to the standards set by the Declaration of Helsinki (2008). The study was approved by the local Research Ethics Committee of Liverpool John Moores University.

5.3.2 Design: In a repeated measures cross-over design separated by 7-9 days, participants completed two exhaustive exercise trials in conditions of high CHO availability (HIGH) or reduced CHO availability but with leucine enriched protein feeding before, during and after exercise (LOW+LEU). At 36-40 h prior to the main experimental trials, all participants performed a glycogen depletion protocol followed by 36 h of high or low CHO intake so as to manipulate pre-exercise muscle glycogen content prior to the exhaustive exercise protocol. Participants in HIGH then completed the exhaustive exercise protocol in conditions considered as best nutritional practice i.e. pre-exercise meal consisting of both CHO and protein, CHO intake during exercise and both CHO and protein intake post-exercise. In contrast, participants in LOW+LEU commenced the exhaustive exercise protocol with reduced pre-exercise muscle glycogen and only consumed leucine enriched whey protein before, during and after exercise. As such, this trial represented conditions of reduced CHO and absolute energy availability but high protein availability throughout.

Muscle biopsies were obtained from the vastus lateralis immediately before, post- and at 3 h post-exercise. An overview of the experimental protocol is shown in Figure 5.1.

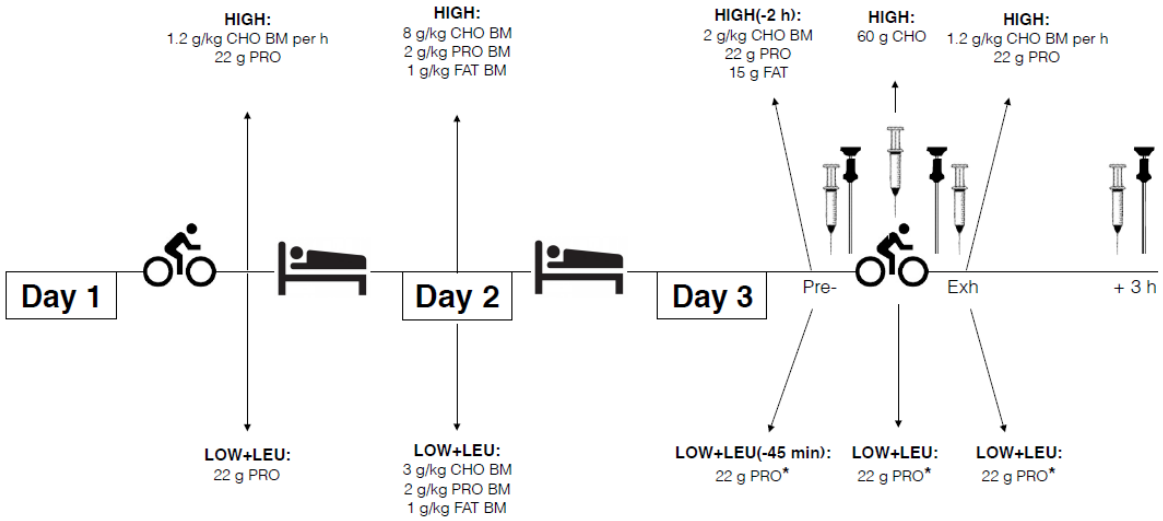

Figure 5.1. Overview of study protocol. On the evening of day 1, participants completed a glycogen depleting protocol followed by 3h of best practice recovery nutrition (HIGH) or sleep-low (LOW+LEU). Throughout the entirety of day 2, participants consumed a high CHO diet (HIGH) or alternatively, a low CHO and low energy dietary protocol (LOW+LEU) that was matched for both protein and fat intake. During the main experimental trial on day 3, participants performed an exhaustive cycling protocol in conditions of best practice nutrition (HIGH) in which high CHO intakes were consumed before, during and after exercise. In contrast, in the LOW+LEU trial, participants consumed leucine-enriched protein only. In this way, the LOW+LEU trial represented 3 days of amalgamation of train-low strategies consisting of sleep-low (day 1), low dietary CHO intake (day 2), and omission of CHO intake before, during and after exercise (day 3). Muscle biopsies were obtained immediately pre-exercise, at the point of exhaustion (Exh) and at 3 h post-exercise. * denotes leucine-enriched protein.

5.3.3 Assessment of maximal oxygen uptake: At 7-10 days prior to the experimental trials, all participants were initially assessed for peak oxygen consumption ($\dot{V}O2_{peak}$) and peak aerobic power (PPO) as determined during an incremental cycle test performed on an electromagnetically braked cycle ergometer described in section 3.4.4 general methodology.

5.3.4 Day 1 – Glycogen depletion protocol. Participants arrived at the laboratory on the evening (19.00) of the first experimental day having avoided alcohol and vigorous physical activity for the previous 24 h. Nude body mass was recorded and a heart rate (HR) monitor (Polar FT1, Finland) fitted. Participants then performed an intermittent glycogen-depleting cycling protocol lasting ~120 min, as described previously by our group (Taylor *et al.*

2013). The activity pattern and total time to exhaustion were recorded, and water was consumed ad libitum throughout exercise. These parameters were repeated exactly during the second experimental condition and all cycling protocols were conducted on a fully adjustable electromagnetically braked cycle ergometer (SRM, Julich, Germany). Following the depletion exercise protocol, participants in HIGH consumed high carbohydrate at a rate of 1.2 $g \cdot kg^{-1}$ body mass (BM) carbohydrate per hour for the first three hours in a mixture of bars and fluids (GO Bars / Go Energy, Science in Sport, Nelson, UK) and 22g of whey protein immediately post-exercise (REGO Protein, Science in Sport, Nelson, UK). In contrast, when participants completed the LOW+LEU trial, they consumed no carbohydrate within the 3 h recovery period but did consume the same bolus of 22g whey protein (REGO Protein, Science in Sport, Nelson, UK) immediately post-exercise.

5.3.5 Day 2 – Nutritional protocols. During day 2, participants in the HIGH trial consumed 8 $g \cdot kg^{-1}$ BM carbohydrate as a mixture of foods and sports supplements designed to maximise muscle glycogen replenishment whereas in the LOW+LEU trial, participants consumed 3 $g \cdot kg^{-1}$ BM carbohydrate in order to minimise muscle glycogen replenishment. In both trials, participants also consumed 2 $g \cdot kg^{-1}$ BM protein and 1 $g \cdot kg^{-1}$ BM fat. In this way, it was expected that participants would commence the main experimental trial on the morning of day 3 with high (HIGH) or low (LOW+LEU) muscle glycogen whilst also having consumed identical protein and fat intake and completed the same exercise loading patterns.

5.3.6 Day 3 - Main experimental trial. On the morning of the Day 3, participants reported to the laboratory in a fasted state and an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the anticubital vein in the anterior crease of the forearm and a resting blood sample drawn. After a resting blood sample was taken, the cannula was flushed with ~ 5 ml of sterile saline (Kays Medical supplies. Liverpool UK) to keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw. Following blood sampling, participants in HIGH received a standardised breakfast containing 2 $g \cdot kg^{-1}$ BM CHO and an absolute dose of 22g whey protein and 15 g fat at 2 hours prior to commencing the exhaustive exercise protocol. Two hours were provided post-prandial to allow sufficient time for digestion prior to commencing exhaustive exercise. In contrast, participants in the LOW+LEU trial consumed a leucine enriched protein drink that is not commercially available (Science in Sport, Nelson, UK) containing a total of 22g protein of which 6.3 g was leucine at 45 min

prior to commencing exercise. The leucine enriched protein was given 45 min prior to exercise (as opposed to 2 hours prior) so as to ensure sufficient circulatory amino acid availability "during" the exercise protocol (Impey *et al.* 2015). Additionally, these timings and dosing strategies are in accordance with nutritional practices of professional road cyclists and as such, it was my aim to examine a real world nutritional protocol (Walsh, 2013).

Participants then completed a prescribed cycling protocol consisting of 4 x 30 seconds high intensity intervals (HIT) at 150% PPO interspersed with 2.5 min active recovery at 40% PPO followed by 45 min steady state (SS) cycling at 50% PPO to examine the effects of altered substrate availability on standardised exercise responses (i.e. RPE, HR, substrate utilisation). During the HIT and steady state component, participants in HIGH ingested a 6% CHO solution providing 20 g of CHO (GO Electrolyte, Science in Sport, Nelson, UK) at 20-minute intervals to provide 60 g in the first 60 minutes of exercise, thereby in accordance with current nutritional recommendations for CHO intake during exercise. Participants in LOW+LEU ingested one third of the leucine enriched protein mix providing 7.3 g of protein at the same 20 minute intervals as the feeding strategy in HIGH, so as to provide 22g protein per hour in an attempt to maintain high circulatory amino acid availability during exercise (Impey *et al.* 2015). Following the steady state cycle, 5 min of active recovery was provided and participants then commenced an exercise capacity test consisting of intermittent "1 min efforts" corresponding to 80% PPO interspersed with 1 min recovery periods at 40% PPO. This intermittent protocol was followed until the participants reached volitional fatigue. Physiological and perceptual measures were recorded at regular intervals throughout exercise and substrate utilisation was assessed during the steady state component of the exercise protocol using online gas analysis (CPX Ultima, Medgraphics, Minnesota, US) and the equations of Jeukendrup and Wallis (2005).

At the point of exhaustion, participants in HIGH received the same 1.2 $g \cdot kg^{-1}$ BM CHO feeding strategy as following the depletion ride and an absolute dose of 22g whey protein immediately after whereas participants in LOW+LEU consumed 22g of the leucine enriched protein only immediately after exercise (Fig 5.1). In this way, participants in HIGH undertook and recovered from exercise having ingested 6.3 $g \cdot kg^{-1}$ BM CHO across the duration of the experimental trial along with a total of 44 g of protein, which contained 4.6 g of leucine. In comparison, participants in LOW+LEU consumed a total of 66 g of protein of which 18.9 g was leucine across the duration of the main experimental trial.

Laboratory conditions remained constant across all experimental trials (19 – 21ºC, 40 – 50% humidity) and an overview of the nutritional feeding protocol is shown in Figure 5.1.

5.3.7 Blood analysis: Blood samples were collected in vacutainers containing K_2 EDTA, lithium heparin or serum separation tubes. Serum and plasma were aliquoted and stored at -80℃ until analysis. Plasma glucose, lactate, NEFA, glycerol, β-hydroxybutyrate and amino acids were analysed as described in section 3.6.1.

5.3.8 Muscle biopsies: Skeletal muscle biopsy samples were collected as described in section 3.6.3.

5.3.9 mRNA extraction and analysis and Reverse transcription quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): Isolation and quantification of mRNA and assessment of mRNA transcription changes were completed as outlined in section 3.6.4.

5.3.10 Muscle glycogen concentration: Muscle glycogen was determined via method outlined in section 3.6.5.

5.3. 11 [γ^{-32}P] ATP Kinase Assay: Thirty mg of muscle tissue was used for the measurement of p70S6K1 and AMPKα1 and α2 activity as previously described (McGlory *et al.* 2014); section 3.6.6.

5.3.12 Statistics: All statistical analyses were performed using Statistical Package for the Social Scientist (SPSS version 21). Descriptive statistics were produced for all data sets to check for normal distribution indicated by Kolmogorov-Smirnov (accepted if P>0.05).. Changes in exercise capacity were analysed using Student t – test. Changes in physiological and molecular responses between conditions (i.e. muscle glycogen, circulatory metabolites, amino acids, mRNA transcription and activity of signalling molecules) were analysed using two way repeated measures general linear model, where the within factors were time and condition. If Mauchley's test of sphericity indicated a minimum level of violation, as assessed by a Greenhouse Geisser epsilon (ε) of ≥ 0.75, data were corrected using the Huynh-Feldt ε. If Mauchley's test of sphericity was violated data were corrected using Greenhouse Geisser ε. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferoni post hoc tests in order to locate specific differences. An alpha value of $P < 0.05$ was utilized for all tests and all data in text, figures and tables are presented as mean ± SD.

5.4 Results

5.4.1 Skeletal muscle glycogen content and exercise capacity

Muscle glycogen content was significantly higher pre-exercise (P=0.003) in the HIGH trial compared with the LOW+LEU trial (Figure 5.2A). Exhaustive exercise also significantly reduced (P<0.001) muscle glycogen stores to comparable levels (<120 mmol·kg^{-1} dw) with no difference observed between conditions (P=0.278). As expected, post-exercise CHO feeding significantly increased (P=0.003) muscle glycogen in the HIGH trial to approximately 200 mmol·kg^{-1} dw whereas no glycogen re-synthesis was observed in the LOW+LEU trial. In accordance with distinct differences in CHO availability, exercise capacity was significantly greater (P<0.001) in the HIGH trial (158 ± 28 min) compared with the LOW+LEU trial (100 ± 17 min), an effect that was apparent in ten of the eleven participants (range: 4 to 113 min, 95% CI differences: 38 to 79 min) (Figure 5.2B).

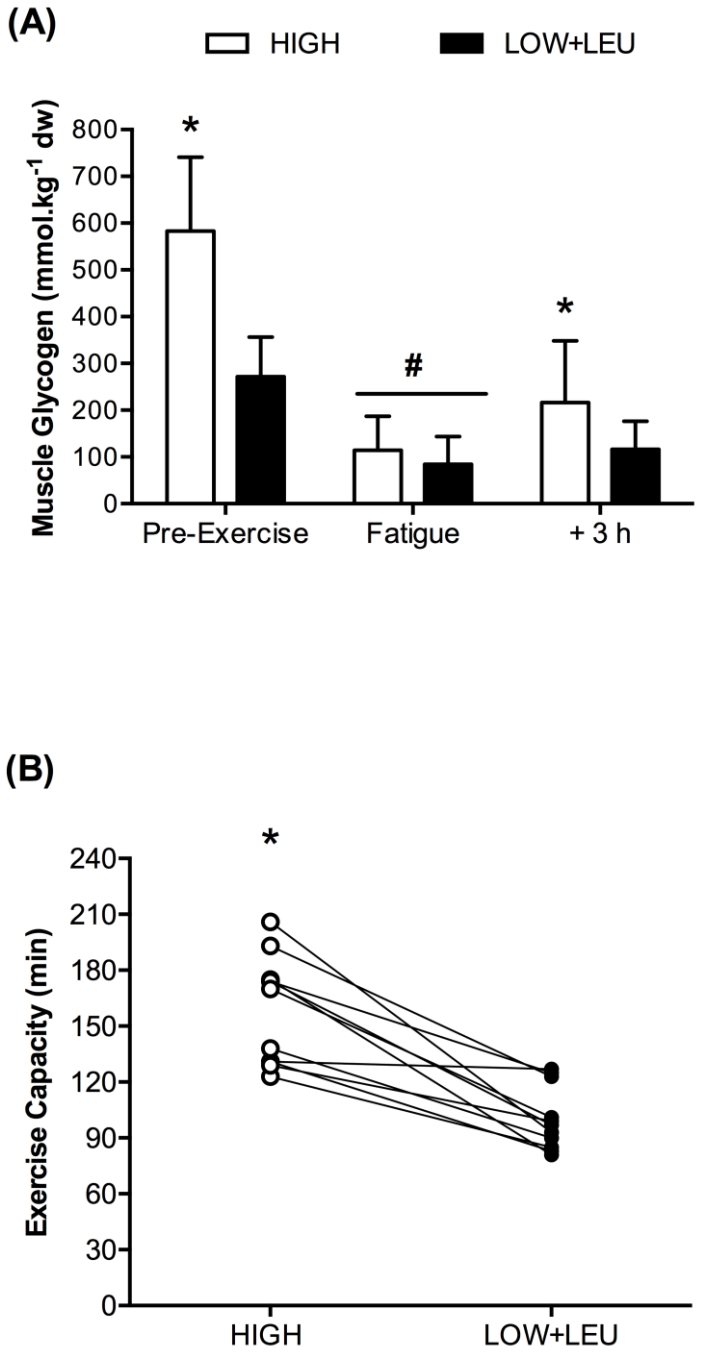

Figure 5.2 – (A) Skeletal muscle glycogen content, (B) Exercise capacity (reflective of set work protocol plus time to exhaustion). *P<0.05, significant main effect of condition, #P<0.05, significant main effect of exercise.

5.4.2 Physiological and metabolic responses to exercise

Subject's heart rate (P=0.458) and plasma lactate (P=0.929) during exercise did not display any significant differences between trials (Figure 5.3A and B, respectively). However, in

accordance with differences in CHO availability, plasma glucose was significantly lower (P=0.007) in the LOW+LEU trial when compared with the HIGH trial (Figure 5.3C). In contrast, plasma NEFA (P<0.001), glycerol (P<0.001) and β-OHB (P<0.001) were all significantly elevated during exercise in the LOW+LEU trial compared with the HIGH trial (Figure 5.3D, E and F, respectively). As a result of such differences in substrate availability, the pattern of fuel use during the steady state component of the exercise protocol was different such that participants in LOW+LEU oxidised less CHO (P=0.004) and more lipid (P=0.007) when compared with the HIGH trial (Figure 5.3G and H, respectively).

5.4.3 Regulation of mitochondrial biogenesis related cell signalling

The exhaustive exercise protocol did not increase AMPKα1 activity in either the HIGH or LOW + LEU trial (data not shown). In contrast, exhaustive exercise significantly increased AMPKα2 activity by approximately 4-fold immediately post-exercise (P=0.001) though no difference was observed between conditions (Figure 5.4A). Similarly, PGC-1α mRNA (P=0.007) was significantly increased to comparable levels between trials at 3 h post-exercise with no differences between conditions (Figure 5.4B). p53 (P=0.01), SIRT1 (P=0.007) and Tfam (P=0.029) mRNA were significantly elevated pre-exercise in the LOW+LEU condition compared with the HIGH trial (Figure 5.4C, D and F respectively). Acute exercise also significantly increased p53 (P=0.013), Tfam (P=0.038) and SIRT1 (P=0.013) mRNA to comparable levels at 3 h post-exercise such that no differences were apparent between conditions. In contrast, neither nutrient availability nor the exhaustive exercise protocol affected COXIV mRNA levels (Figure 5.4E).

5.4.4 Plasma amino acid levels

Plasma leucine (P<0.001), BCAA (P<0.001) and EAA (P<0.001) concentrations were all significantly elevated (Figure 5.5A, B and C, respectively) before, during and after exercise in the LOW+LEU trial compared with the HIGH trial.

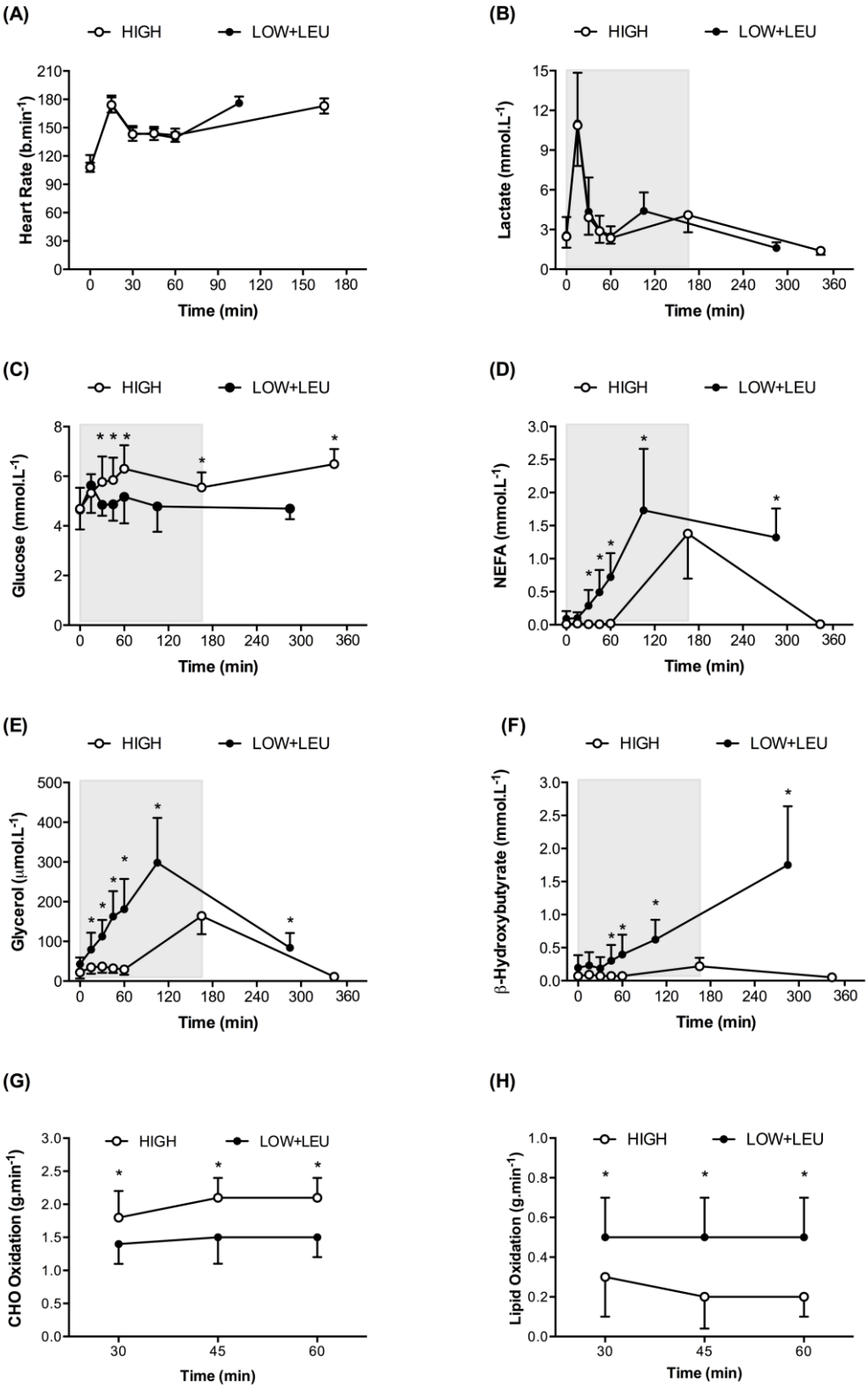

Figure 5.3 – (A) Heart rate responses during exercise and plasma, (B) Lactate, (C) Glucose, (D) NEFA, (E) Glycerol, (F) β-hydroxybutyrate, before, during and after exercise. (G) Carbohydrate oxidation and (H) Lipid oxidation during exercise. *P<0.05, significant main effect of condition. Shaded area represents exercise duration.

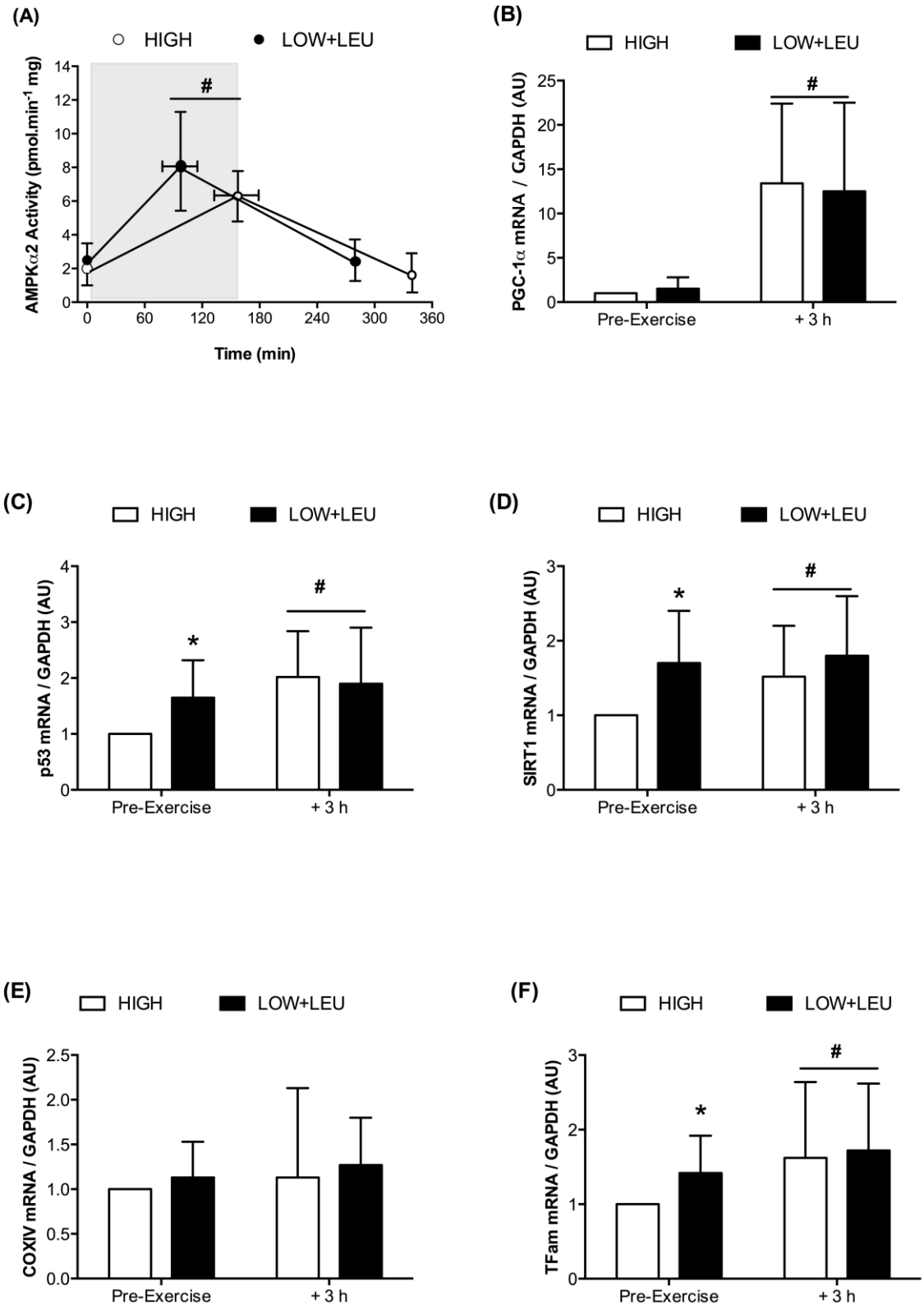

Figure 5.4 – (A) AMPKα2 activity pre-, post- and 3h post-exercise. Shaded area represents exercise duration. (B) PGC-1α, (C) p53, (D) SIRT1, (E) COXIV, and (F) Tfam mRNA content pre- and 3 h post-exercise. Shaded area represents exercise duration. *P<0.05, significant main effect of condition. #P<0.05, significant main effect of exercise.

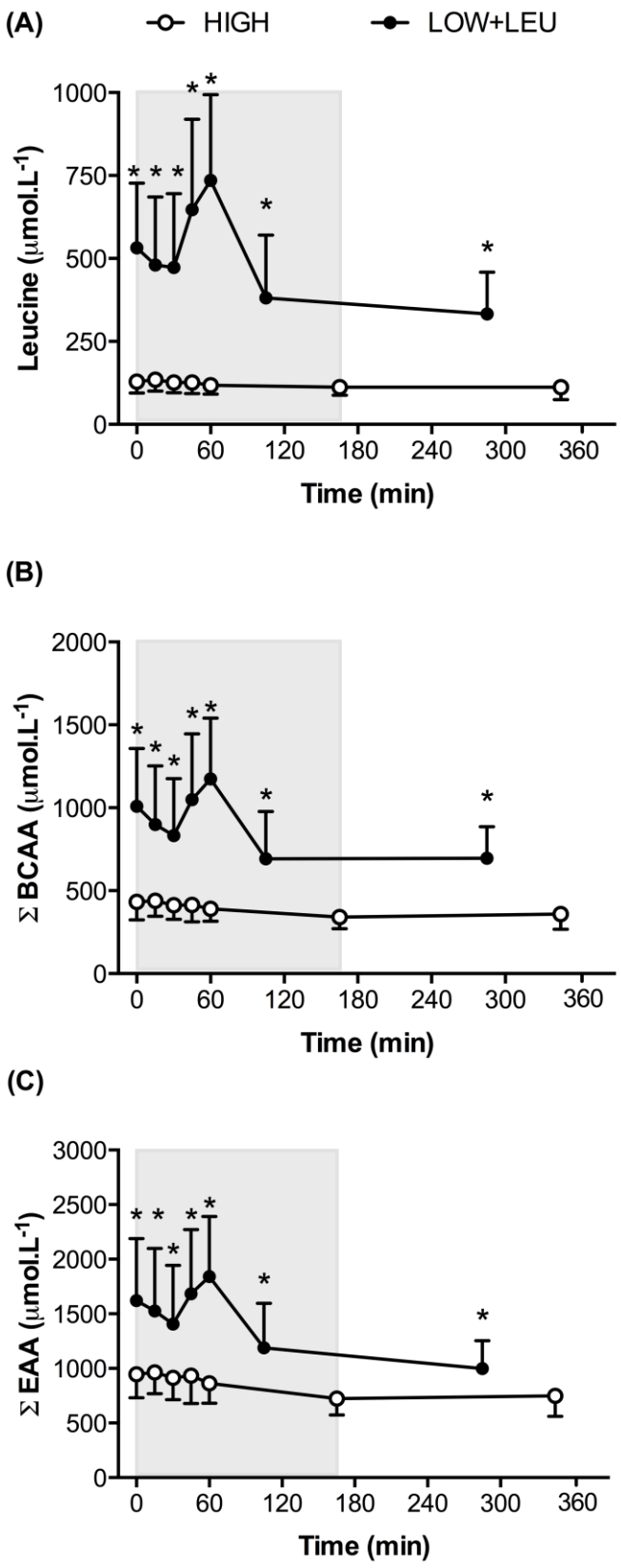

Figure 5.5 – (A) Leucine, (B) BCAAs, and (C) EAAs before, during and after exercise. Shaded area represents exercise duration. *P<0.05, significant main effect of condition.

5.4.5 Regulation of protein synthesis and breakdown related cell signalling

p70S6K activity was significantly elevated (P=0.03) immediately prior to exercise in the LOW+LEU trial compared with the HIGH condition (Figure 5.6A). Following exhaustive exercise, p70S6K activity was suppressed (P<0.001) to comparable levels in both the LOW+LEU and HIGH conditions. In contrast to pre-exercise status, p70S6K activity at 3 h post-exercise was significantly higher (P=0.04) in the HIGH trial compared with the LOW+LEU trial. PKB activity tended to increase (P=0.056) immediately post-exercise in both groups (Figure 5.6B). However, at 3h after exercise, PKB activity was significantly greater in the HIGH trial compared with the LOW+LEU condition (P=0.021). Consistent with the feeding of CHO before and after exercise, plasma insulin levels were significantly higher immediately pre- and 3 h post-exercise (P<0.05) in the HIGH trial compared with the LOW+LEU trial (Figure 5.6C) whereas exhaustive exercise reduced insulin levels (P=0.001) to comparable levels between trials. Atrogin1 mRNA expression was higher immediately (P=0.009) before exercise in the LOW+LEU trial compared with the HIGH trial whereas pre-exercise nutrient status had no effect on MuRF1 mRNA content (Fig 5.7A and B, respectively). Exhaustive exercise also increased (P<0.05) the expression of both genes at 3 h post-exercise to comparable levels in the LOW+LEU and HIGH trials.

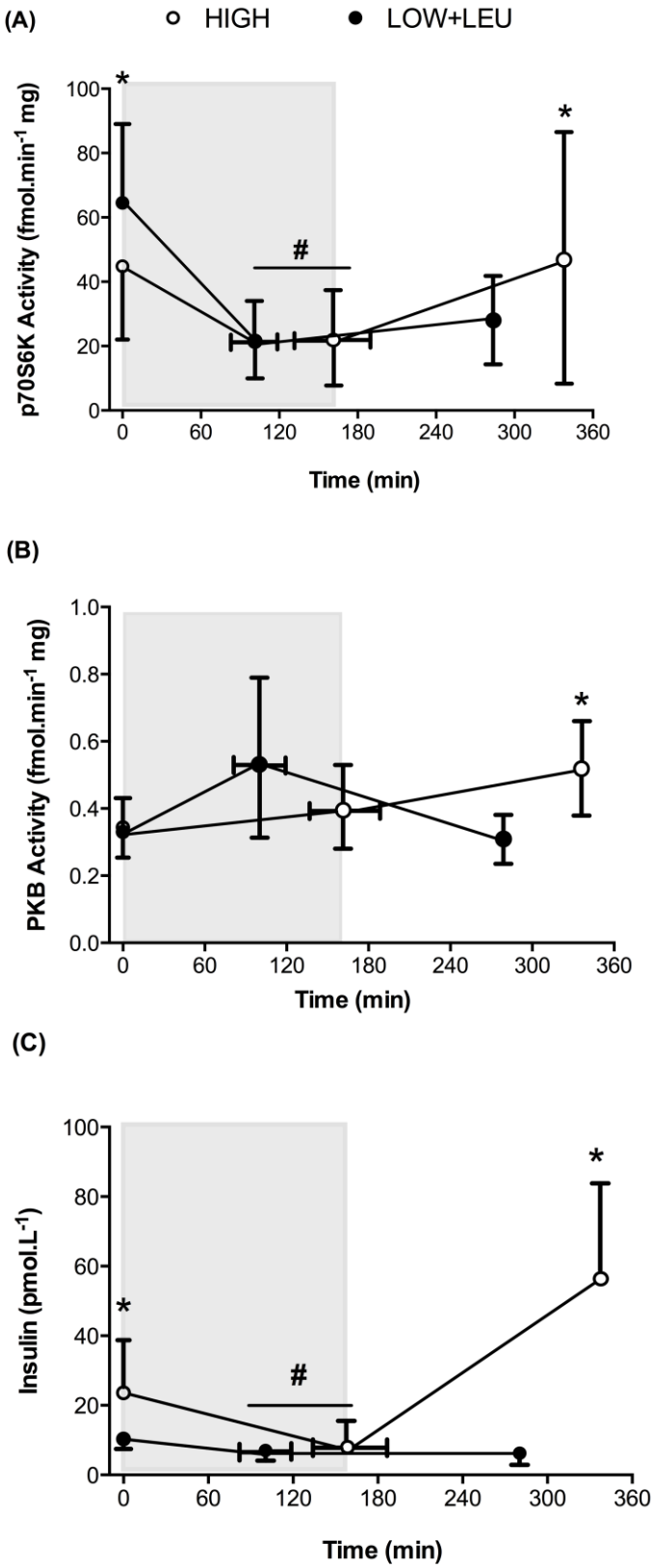

Figure 5.6 – (A) p70S6K, (B) PKB, and (C) Serum insulin pre-, post-, and 3 h post-exercise. Shaded area represents exercise duration. *P<0.05, significant main effect of condition, #P<0.05, significant main effect of exercise.

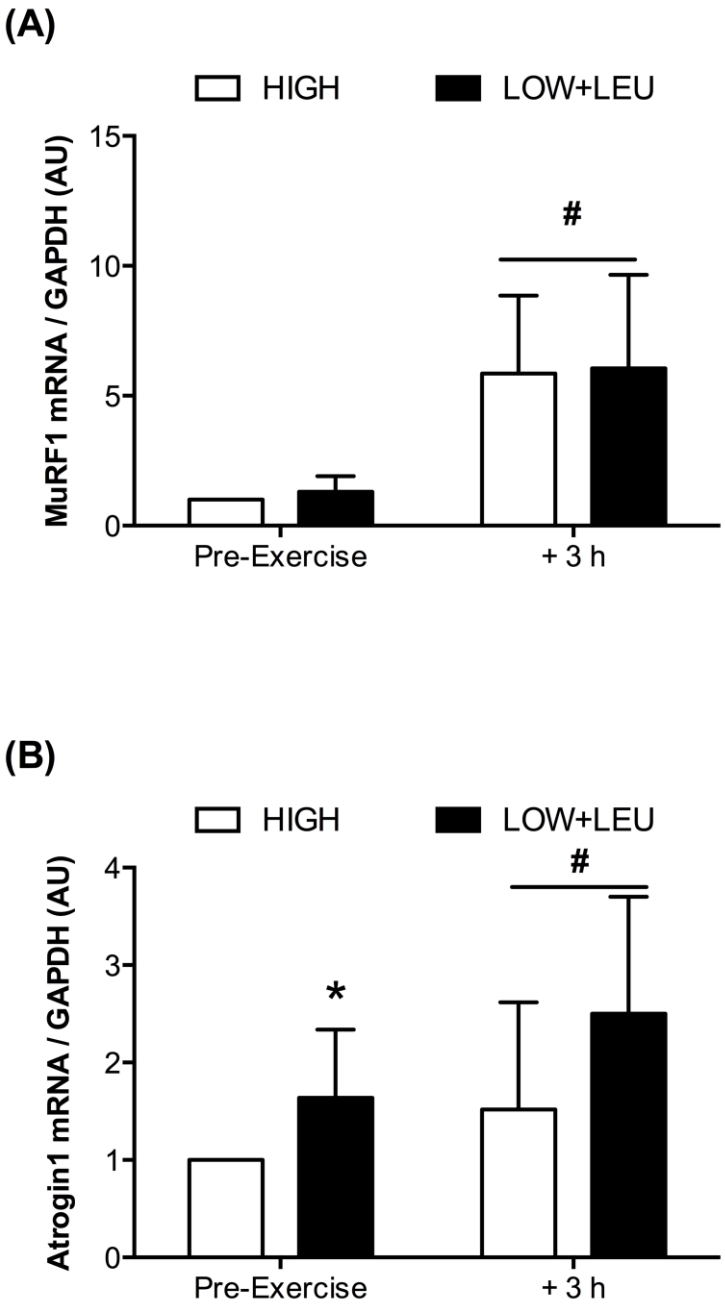

Figure 5.7 – (A) MuRF1 and (B) Atrogin1 mRNA content pre- and 3 h post-exercise. *P<0.05, significant main effect of condition, #P<0.05, significant main effect of exercise.

5.5 Discussion

The aim of the present study was to assess the effects of high CHO versus low CHO (but high leucine) availability on the modulation of those skeletal muscle cell-signalling pathways with putative roles in the regulation of both mitochondrial biogenesis and muscle protein synthesis. I adopted a train-low model that represents an amalgamation of previously studied train-low paradigms (incorporating both CHO and energy restriction) and that I consider representative of the real-world practices often adopted by elite level endurance athletes. Confirming the hypothesis, I provide novel data by demonstrating that although reduced CHO availability impaired exercise capacity, the CHO restriction protocol induced comparable AMPK-PGC-1α signalling, thus inducing work-efficient signalling responses, given that the low CHO group completed 60min less total work to elicit the same AMPK- PGC-1α signalling responce. In contrast, refraining from CHO intake in the post-exercise period (despite the intake of leucine rich protein) maintains p70S6K activity at basal levels. When taken together, these data have practical implications by suggesting that: 1) CHO restriction before and during exercise induces work-efficient cell signalling related to mitochondrial biogenesis but furthermore, 2) complete CHO and energy restriction in the post-exercise period impairs the activation of key signalling proteins regulating skeletal muscle modelling processes. As such, these data suggest that although athletes may benefit from carefully scheduled periods of reduced CHO availability before and during exercise so as to enhance mitochondrial related cell signalling, they should consume *both* CHO and protein post-exercise so as to restore the activation of MPS-related signalling. Furthermore, this data lend support for a potential fuel for the work required train-low paradigm in that athletes could strategically reduce CHO availability prior to completing pre-determined training workloads that can be readily performed with reduced CHO availability, thereby inducing a work-efficient approach to training. Alternatively, when the goals of the training session are to complete the highest workload possible over more prolonged durations, then adequate CHO should be provided in the 24 h period prior to and during the specific training session.

To achieve this model of CHO restriction, I employed a protocol consisting of an amalgamation of previously studied train-low models. For example, having completed an initial glycogen depletion protocol on the evening of day 1, participants adhered to a dietary protocol consisting of reduced CHO and energy intake for the subsequent 36 h prior to arriving on the laboratory on the morning of day 3 for the main experimental trial.

This initial approach is thus representative of an acute sleep-low model (i.e. CHO restriction in the initial hours following completion of the depletion protocol on day 1) followed by consumption of a low CHO diet for the entirety of day 2. This approach was successful in inducing marked differences in pre-exercise muscle glycogen content (≈ 600 versus 300 mmol·kg^{-1} dw in HIGH and LOW+LEU, respectively) on the morning of the main experimental trial commenced on day 3 (Figure 5.2). Consistent with the effects of post-exercise CHO restriction on regulation of gene expression (Pilegaard *et al.* 2005; Bartlett *et al.* 2013; Lane *et al.* 2015), I also observed marked effects on the expression of genes associated with regulatory roles in mitochondrial biogenesis. Indeed, participants arrived in the laboratory on the morning of day 3 with higher pre-exercise mRNA content of p53, SIRT1 and Tfam in the LOW+LEU trial (see Figure 5.4), all of which are thought to be regulators of mitochondrial biogenesis (Bartlett *et al.* 2014, 2015; Gurd *et al.* 2010; Saleem *et al.* 2013). The observation of enhanced p53 mRNA following post-exercise CHO restriction is consistent with previous data from our group demonstrating that the exercise-induced phosphorylation of p53^{Ser15} is enhanced with CHO restriction before, during and after exercise (Bartlett *et al.* 2013). On this basis, it is possible that sustained p53 activation in recovery from the glycogen depletion exercise protocol in conjunction with no CHO intake in the LOW+LEU trial may be a common signalling axis regulating the aforementioned gene expression responses. Indeed, p53 is known to auto-regulate its own expression (Deffie *et al.* 1993) as well as both basal and exercise induced Tfam mRNA levels (Saleem *et al.* 2013). Furthermore, previous work in cell culture models of nutrient deprivation has shown transcriptional regulation of SIRT1 is mediated through p53 binding to the SIRT1 promoter (Nemoto *et al.* 2004).

In relation to the main experimental trial on day 3, participants also refrained from CHO intake before, during and after the exercise protocol (but consumed leucine rich protein), thereby representative of an amalgamation of fasted train-low protocols (Van Proyen *et al.* 2011) and protein only training sessions (Taylor *et al.* 2013; Impey *et al.* 2015). As expected, I observed distinct differences in substrate availability and fuel utilisation during the steady-state exercise protocol, as evidenced by β-OHB levels comparable with nutritional ketosis (Cox & Clarke, 2014), as well as greater levels of circulating NEFA, glycerol and lipid oxidation in the LOW+LEU trial compared with the HIGH trial. Consistent with the well-documented effects of both endogenous and exogenous CHO availability on exercise performance (Hawley *et al.* 1997; Stellingwerff and Cox, 2014), I

also observed reduced exercise capacity in the LOW+LEU trial compared with the HIGH trial, an effect that was evident in ten participants. However, one subject showed no difference in exercise capacity between LOW+LEU and HIGH conditions. This response is difficult to explain but may be due to subject's preferences towards training in a fasted versus fed state, or may be an artefact of motivation during HIGH condition. Such data therefore reiterate the obvious necessity for high CHO availability before and during those training sessions in which prolonged high-intensity workloads are required to be completed.

From a biochemical perspective, it is well-accepted that reduced muscle glycogen stores induce greater skeletal muscle cell signalling when compared with "matched-work" exercise protocols (i.e. completion of identical duration and intensity) undertaken with high glycogen stores (Bartlett *et al.* 2015). For example, $AMPK^{Thr172}$ phosphorylation (Yeo *et al.* 2010; Lane *et al.* 2015), ACC^{Ser79} phosphorylation (Bartlett *et al.* 2013), AMPK-α2 activity (Wojtaszewski *et al.* 2003) and the nuclear abundance of AMPK-α2 protein content (Steinberg *et al.* 2006) are all up-regulated to a greater extent when acute exercise is completed with reduced pre-exercise muscle glycogen stores, an effect likely mediated via the presence of a glycogen binding domain on the β-subunit of the AMPK heterotrimer (McBride *et al.* 2009). Furthermore, exogenous CHO feeding during exercise attenuates AMPK activity only when muscle glycogen sparing has occurred (Akerstrom *et al.* 2006). The present data extend these findings as I show for the first time comparable cell signalling effects despite the completion of significantly less work completed. Indeed, although I observed no effects of exercise on AMPK-α1 activity (as reported by others, Fujii *et al.* 2000), I observed similar increases in AMPK-α2 activity and PGC-1α mRNA expression at comparable levels of absolute glycogen, despite mean differences of 60 minutes less work completed in LOW+LEU vs. HIGH conditions. Such data therefore support the "glycogen threshold" hypotheses (Philp *et al.* 2012) surmising that a critical absolute level of glycogen must be exceeded in order for significant AMPK signalling to occur during prolonged endurance exercise protocols.

Given the effects of reduced CHO and energy deficit on muscle protein degradation and protein synthesis (Pasiakos *et al.* 2010, 2013; Areta *et al.* 2014; Lemon and Mullin, 1980; Howarth *et al.* 2010), I also chose to feed leucine enriched whey protein before, during and after the main experimental trial on day 3. In accordance with the role of leucine availability in modulating MPS (Churchward-Venne *et al.* 2014; Karlsson *et al.* 2006), I

observed higher pre-exercise p70S6K activity in the LOW+LEU trial when compared with the HIGH trial, though I acknowledge that differences in timing of pre-exercise feeding between trials may also have contributed to this finding. The effects of acute endurance exercise on regulation of p70S6K are not well established (and are typically limited to measures of phosphorylation status) with some studies reporting increases (Masher *et al.* 2007, 2011) and others, no change (Coffey *et al.* 2006; Vissing *et al.* 2013). Nevertheless, consistent with the notion that exercise suppresses MPS during exercise (Rose & Richter, 2009; Rennie, *et al.* 1980), I observed significant reductions in p70S6K activity immediately post-exercise to almost identical levels between trials. To my knowledge, I am only the second researcher to directly quantify p70S6K activity in response to endurance type exercise protocols and the data conflict with Apro *et al.* (2015) who observed no change in response to 5 x 4 min cycling at 85% $\dot{V}O2_{max}$. Such discrepancies between studies are likely most related to the exhaustive and energy restricting nature of the exercise protocol. Whilst it is difficult to directly compare the total energy expenditure between this study and the data of Apro *et al.* (2015), the exercise interventions used here elicited considerably lower muscle glycogen concentrations of ~100 mmol·kg^{-1}dw vs 350 mmol·kg^{-1}dw in the study of Apro et al (2015). The mechanism(s) behind suppressed p70S6K activity following exhaustive exercise likely involve a large number of signalling mechanisms and regulators within skeletal muscle. Indeed, AMPK mediated inhibition of mTOR through TSC2 (Sanchez *et al.* 2012) or via interaction with v-ATPase-Ragulator at the late endosomal/lysosme surface (Zhang *et al.* 2014), as well as p53-REDD1 mediated inhibition of mTOR (Feng & Levine, 2010; Keller *et al.* 2013) are all potential candidates. Further studies are now required to test these hypotheses in human skeletal muscle.

In relation to post-exercise feeding, I observed that the co-ingestion of carbohydrate and whey protein feeding was sufficient to rescue p70S6K activity in the HIGH trial whereas p70S6K remained suppressed in the LOW+LEU trial despite the intake of "leucine-enriched" whey protein feeding. Whilst it is difficult to readily ascertain the precise mechanism(s) underpinning these data, it is noteworthy that I did observe increased upstream signalling of PKB (Akt) in the HIGH condition, an effect that may be simply related to insulin mediated activation given the repeat 90 g intakes of CHO immediately post and at 1 and 2 h post-exercise. In addition, the potential reduced PKB mediated activation of p70S6K may be due to the presence of low muscle glycogen *per se* given previous data demonstrating that the post-exercise (albeit in resistance exercise) activation

of Akt phosphorylation is suppressed (independent of post-exercise feeding) when muscle glycogen levels are comparable to that observed in the present study i.e. <150 mmol·kg^{-1} dw (Creer *et al.* 2005). Alternatively, the reduced activation of p70S6K in the LOW+LEU trial could be due to high circulating NEFA concentrations given that high fat availability can impair MPS. Indeed, infusion of Intralipid and heparin to elevate circulating NEFA concentrations (to comparable levels seen here) attenuates MPS in human skeletal muscle in response to ingesting 21g amino acids under euglycemic hyperinsulemic clamp conditions (Stephens *et al.* 2015). Furthermore, Kimball *et al.* (2015) also reported that high fat feeding impairs MPS in rat liver in a manor associated with reduced p70S6K phosphorylation (but not PKB phosphorylation).

The functional relevance of such divergent responses cannot be ascertained from the present study given that I did not directly quantify MPS. Indeed, previous data have suggested that low muscle glycogen availability (during resistance exercise protocols) does not have any measurable effect on post-exercise MPS (Camera *et al.* 2012) whereas the recovery from endurance exercise when glycogen levels remain low results in negative protein balance (Howarth, *et al.* 2010). As noted previously, however, the experimental protocol adopted here was a deliberate manipulation of both CHO and energy availability. Indeed, consistent with the effects of acute energy deficit on skeletal muscle proteolysis (Carbone *et al.* 2013), I also observed increased resting mRNA expression of Atrogin 1 in the LOW+LEU trial when compared with the HIGH trial. Furthermore, given that acute energy deficit also impairs MPS in a manor associated with reduced PKB (Pasiakos *et al.* 2010) and p70S6K phosphorylation (Pasiakos *et al.* 2013) the divergent signalling responses observed here may indeed manifest as functional reductions in MPS and negative protein balance. From a practical perspective, the data suggest that whilst there may be benefits of restricting CHO intake in the post-exercise period in terms of enhancing mitochondrial signalling, it is also necessary to consume sufficient CHO intake in the immediate post-exercise period so as to replenish muscle glycogen to sufficient levels *per se* and/or obtain upstream signalling effects associated with feeding, the result of which could maintain the activity of those signalling proteins with putative roles in regulating MPS and skeletal muscle remodelling.

It is, of course, beyond the scope of the present paper to offer definitive guidelines on how best to periodise CHO restriction into an overall athletic training programme. Nevertheless, in accordance with the work of Lane *et al.* (2015), I consider the signalling responses

observed herein to offer further mechanistic support for the performance improvements observed by Marquet *et al.* (2016) whilst adopting the 3-week sleep low training paradigm. In essence, the theme that emerges appears to be the concept of both "day-to-day" and "meal-by-meal" CHO periodization in accordance with the upcoming training workloads that have been prescribed. In practice, this approach of forward planning could represent an amalgamation of train-low paradigms and is perhaps best communicated by the principle of "fuel for the work required". Careful day-to-day periodization (as opposed to chronic periods of CHO restriction) is likely to maintain metabolic flexibility and still allow for the completion of high-intensity and prolonged duration workloads on heavy training days e.g. interval type workouts undertaken above lactate threshold. Intuitively, train-low sessions may be best left to those training sessions that are not CHO dependent and in which the intensity and duration of the session is not likely to be compromised by reduced CHO availability e.g. steady-state type training sessions performed at intensities below the lactate threshold.

In summary, I have utilised an amalgamation of previously studied train-low paradigms (considered representative of real world athletic practice) to demonstrate for the first time that CHO restriction before and during exhaustive exercise induces work-efficient cell signalling related to mitochondrial biogenesis, in that the low CHO group was able to achieve the same level of AMPK- PGC-1α signalling response while completing 60min less work. However, in the absence of CHO feeding and absolute energy intake in the 3 h post-exercise period, p70S6K activity remains suppressed despite consuming leucine rich protein immediately post-exercise.

When taken together, these data allude to a potential muscle glycogen threshold hypothesis, surmising that reduced pre-exercise muscle glycogen may facilitate a greater portion of training being completed within an optimal window, within which enhanced activation of the pathways regulating mitochondrial biogenesis is seen. Importantly this hypothesis also suggests that keeping glycogen and energy intake at critically low levels post-exercise may impair the regulation muscle protein synthesis related signalling. Furthermore, this data lend support for a potential fuel for the work required train-low paradigm, in that athletes could strategically reduce CHO availability prior to completing pre-determined training workloads that can be readily performed with reduced CHO availability, thereby inducing the work-efficient approach to training detailed above. Alternatively, when the goals of the training session are to complete the highest workload

possible over more prolonged durations, then adequate CHO should be provided in the hours prior to and during the specific training session to provide sufficient fuel to sustain the required intensity and duration of that specific training session. In this way, the fuel for the work required paradigm suggests that in the same way training is periodised, carbohydrate should also be periodised to maximise the potential for training adaptations to occur, given the observed work efficiency seen here. Thus, the concept of fuel for the work required suggests that nutrition strategies before, during and after training should reflect the desired outcome of each specific training session, as such this requires considered forward planning for both training and nutrition to facilitate the desired outcome.

Chapter 6

Co-ingestion of whey protein and carbohydrate reactivates p70S6K1 post-exercise in recovery from an acute train-low endurance training session

The aim of this chapter was to examine the role of protein composition in regulating skeletal muscle cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis, MPS and autophagy when in recovery from a train-low endurance training session. This work was presented at ISENC 2016, Newcastle, UK; currently under review for publication.

6.1 Abstract

The purpose of this chapter is to examined the effects of whey versus collagen protein on regulation of post-exercise skeletal muscle remodelling processes (associated with mitochondrial biogenesis, protein synthesis and autophagy) when in recovery from a train-low endurance training session. In a repeated measures cross-over design, eight males completed a 75-min non-exhaustive cycling protocol. Protein provision consisted of 22g of collagen (COLLAGEN) or whey (WHEY) protein 45 min prior to exercise, 22g during exercise and 22g immediately post-exercise. Carbohydrate (CHO) was also consumed immediately post- and at 1 h post-exercise in both trials (1.2 g/kg body mass). Exercise decreased ($P<0.05$) muscle glycogen content by comparable levels from pre-to post-exercise in both trials (≈ 350 to 150 mmol·kg^{-1} dw). Exercise induced ($P<0.05$) comparable increases in PGC-1α (8-fold), ATG12 (1.3-fold) and Parkin mRNA content (1.3-fold) at 1.5 h post-exercise, as well as similar reductions in LC3bI, II and p62 protein content ($P < 0.05$). Exercise suppressed ($P<0.05$) p70S6K activity to comparable levels immediately post-exercise (≈ 25 fmol.min^{-1}.mg^{-1}). Nonetheless, post-exercise feeding increased p70S6K activity at 1.5 h post-exercise ($P<0.05$), the magnitude of which was greater ($P <0.05$) in WHEY (180 ± 105 fmol.min^{-1}.mg^{-1}) versus COLLAGEN (73 ± 42 fmol.min^{-1}.mg^{-1}, respectively). When consumed with post-exercise CHO ntake, whey protein augments post-exercise p70S6K1 activity to a greater extent than compared with collagen. We also observed that protein composition does not modulate markers of mitochondrial biogenesis, autophagy and mitophagy when in recover from an acute train-low endurance training session.

Keywords: autophagy, mitophagy, parkin, glycogen

6.2 Introduction

The concept of deliberately training with reduced carbohydrate (CHO) availability to enhance endurance-training adaptations the train-low model is gaining increased popularity amongst both academic and athletic circles (Yeo *et al.* 2008; Marquet *et al.* 2016). During the last decade, our group and others have consistently demonstrated that both reduced endogenous and exogenous CHO availability augments training-induced increases in whole body and intramuscular lipid metabolism (Hulston *et al.* 2010), oxidative enzyme activity and protein content (Yeo *et al.* 2008) and moreover, improves exercise performance (Cochran *et al.* 2015; Marquet *et al.* 2016). Such adaptations are likely mediated by increased activation of regulatory cell signalling pathways (e.g. activation of upstream cell signalling kinases and downstream transcriptional co-activators and transcription factors) to induce a co-ordinated up-regulation of both the nuclear and mitochondrial genomes (Wojtaszewski *et al.* 2003; Bartlett *et al.* 2013; Philp *et al.* 2013). From a practical perspective, I have previously suggested the fuel for the work required paradigm whereby CHO availability is periodised in both a day-to-day and meal-by-meal manner in accordance with the upcoming workload of the prescribed training session(s) (Impey *et al.* 2016). The fuel for the work required concept suggests that given the potential, from a molecular signalling perspective, to improve the efficiency with which training elicits adaptive responses the nutritional programming before, during and after each training should be specific to the duration, intensity and desired outcome of each training session.

When incorporating train-low sessions into an athletic training programme, it is also suggested that athletes should consume increased protein before (MacLean *et al.* 1994; Coffey *et al.* 2011), during (Hulston *et al.* 2011) and/or after (Rowlands *et al.* 2015) each training session. Indeed, such a feeding strategy is facilitative of a positive net protein balance and activates the molecular machinery (i.e. the Akt-mTOR-p70S6K signalling axis) required to stimulate post-exercise remodelling processes including muscle protein synthesis (Breen *et al.* 2011). Furthermore, increased amino acid availability prior to and during train-low sessions does not impair circulating free fatty acid availability (Impey *et al.* 2015) nor the key signalling pathway (i.e. the AMPK-PGC-1α pathway) that is thought to mediate many of the beneficial adaptations associated with train-low protocols (Taylor *et al.* 2013). It is noteworthy, however, that maintaining post-exercise glycogen and energy availability at the critically low levels associated with exhaustion impairs the post-exercise

activation of p70S6K activity, despite consuming leucine enriched whey protein (Impey *et al.* 2016). On this basis, I therefore suggested the presence of a muscle "glycogen threshold" i.e. a metabolic window of absolute pre- and post-exercise glycogen concentrations that should not be exceeded to activate the cell signalling pathways that mediate both mitochondrial biogenesis and muscle protein synthesis. To this end, such data suggest that whilst beneficial oxidative adaptations may be achieved with reduced pre-exercise CHO availability, the post-exercise meal should contain a combination of both protein and CHO to provide the necessary substrate and energy to stimulate glycogen synthesis and post-exercise muscle protein synthesis.

In addition to signalling relating to mechanisms of protein synthesis, transcription of markers of bulk protein breakdown such as MuRF1 and Atrogin1 have been shown to be elevated during train low sessions (Impey *et al.* 2016). Indeed, while bulk degradation processes are important to degrade malfunctioned proteins and restore energy balance during energetic stress, recent data has pointed to the specific targeted degradation of cellular constituents, termed autophagy, as an essential part of the adaptive response to exercise training (He *et al*. 2012; Lira *et al*. 2013). Mitochondrial targeted degradation (termed mitophagy) has recently been identified to occur in response to exercise however little is known about this response in human skeletal muscle or if nutritional status affects this adaptive response (Vainshten *et al.* 2015; Vainshten and Hood, 2016).

To further explore this concept, the primary aim of the present chapter was to test the role of post-exercise protein composition when ingested with CHO in activating post-exercise remodelling processes when in recovery from completion of a train-low training session. To this end, I adopted an experimental design whereby recreationally active male cyclists completed a non-exhaustive session in which glycogen remained within the chosen theoretical glycogen threshold window e.g. pre-and post-exercise concentrations of <350 and >150 mmol·kg dw^{-1}, respectively. Furthermore, given the emerging evidence that both autophagy and mitophagy are key processes involved in training adaptation (He *et al.* 2012; Masiero *et al.* 2009; Vainshten and Hood, 2016), I also assessed the effects of protein composition on both gene expression and protein markers of both processes.

6.3 Methodology

6.3.1 Participants: Eight competitive and recreational male cyclists (age: 25 ± 3 years; height: 175 ± 0.1 cm; body mass: 74.4 ± 6.7 kg) who trained between 3 – 10 hours per week took part in this study. Mean $\dot{V}O2_{peak}$ and peak power output (PPO) for the cohort was 56.5 ± 3.8 $mL \cdot kg^{-1} \cdot min^{-1}$ and 327 ± 26 W respectively. None of the participants had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during the course of the study. Participants volunteered to participate in the study after providing informed written consent. All procedures conformed to the standards set by the Declaration of Helsinki (2008) and the study was approved by the local Research Ethics Committee of Liverpool John Moores University.

6.3.2 Design: In a repeated measures, randomised and cross-over design separated by 7-9 days, participants completed two non-exhaustive acute exercise trials in conditions of reduced CHO availability with whey (WHEY) or collagen (COLLAGEN) provision before, during and after exercise. At 36-40 h prior to the main experimental trials, all participants performed a glycogen depletion protocol followed by 36 h of low CHO and energy intake (as replicated from Impey *et al.* 2016) to manipulate pre-exercise muscle glycogen content prior to the main experimental exercise protocol undertaken on Day 3. In accordance with the reduced CHO availability protocol studied previously (Impey *et al.* 2016), participants refrained from CHO intake on the morning of the main experimental trial as well as during exercise, but consumed CHO at a rate of 1.2 g/kg body mass immediately post-exercise and at 1 h post-exercise. This approach was designed to allow participants to commence and undertake the exercise protocol with reduced endogenous (i.e. <350 mmol/kg dw muscle glycogen) and exogenous CHO availability (i.e. no pre-exercise meal or CHO feeding during exercise). The provision of CHO in the post-exercise period was in an attempt to restore muscle glycogen and energy availability to remain within the chosen glycogen threshold i.e. >150 mmol/kg dw. During completion of the WHEY trial, participants consumed 22g of whey protein (in beverage form) at 45 minutes prior to exercise, 22g during exercise and a further 22g immediately post-exercise. During completion of the COLLAGEN trial, participants consumed 22g of collagen protein (in the form of a gel) at identical time-points and in fluid matched conditions. As such, both trials represented deliberate conditions of reduced CHO and absolute energy availability (to mimic an amalgamation of train-low protocols, Impey *et al.* 2016) but with high protein availability in the form of whey (WHEY) or collagen (COLLAGEN)

throughout. Muscle biopsies were obtained from the vastus lateralis immediately before, post- and at 1.5 h post-exercise. An overview of the experimental protocol is shown in Figure 6.1.

6.3.3 Assessment of maximal oxygen uptake: Participants were initially assessed for peak oxygen consumption ($\dot{V}O2_{peak}$) and peak aerobic power (PPO) as determined during an incremental cycle test performed on an electromagnetically braked cycle ergometer as previously described (Impey *et al.* 2015); section 3.4.4.

6.3.4 Experimental Protocol:

Day 1 and 2: Participants arrived at the laboratory on the evening (17.00) of the first experimental day having avoided alcohol and vigorous physical activity for the previous 24 h. Nude body mass was recorded and a heart rate (HR) monitor (Polar FT1, Finland) fitted. Participants then performed an intermittent glycogen-depleting cycling protocol lasting ~120 min. This protocol and all subsequent cycling protocols were conducted on a fully adjustable electromagnetically braked cycle ergometer (Load Excalibur, Nederland). The exercise protocol used to deplete muscle glycogen has previously been used in our laboratory (Taylor *et al.* 2013; Bartlett *et al.* 2013; Impey *et al.* 2016). This protocol was chosen to maximally deplete muscle glycogen in both type I and type II muscle fibres. The activity pattern and total time to exhaustion were recorded, and water was consumed ad libitum throughout exercise. These parameters were repeated exactly during the second experimental condition. Participants then consumed a diet low in carbohydrate (3 $g \cdot kg^{-1}$ BM) but high in protein (2 $g \cdot kg^{-1}$ bw) over the next 36 h to minimise muscle glycogen replenishment to ~300 – 350 $mmol \cdot kg^{-1}$ dw on the morning of the main experimental trial (Impey *et al.* 2016).

Day 3: On the morning of the Day 3, participants reported to the laboratory in a fasted state and an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the anticubital vein in the anterior crease of the forearm and a resting blood sample drawn. After a resting blood sample was taken, the cannula was flushed with ~ 5 ml of sterile saline (Kays Medical supplies. Liverpool UK) to keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw. Participants then received 22g of protein from one of two supplement sources 45 minutes prior to beginning exercise in a randomised crossover design. Protein was provided via ether a commercially available collagen gel (Muscle Pharm, USA) (COLLAGEN) or whey isolate solution

(WHEY) (Science in Sport, Nelson, UK). Fluid intake was matched in both conditions to 500 ml at this time-point. Participants then rested for 45 minutes prior to commencing exercise. Protein was given 45 min prior to exercise (as opposed to 2 hours prior) to ensure sufficient circulatory amino acid availability "during" the exercise protocol (Impey *et al*. 2015). Additionally, these timings and dosing strategies are in accordance with nutritional practices of professional road cyclists and as such, it was an aim to examine a real world nutritional protocol (Vogt *et al*. 2005). Following a 5 min warm up at 150 W, participants then completed a prescribed cycling protocol consisting of 4 x 30 seconds high intensity intervals at 200% PPO interspersed with 2.5 min active recovery at 40% PPO, followed by 45 min steady state cycling at 60% PPO and finally, 3 x 3min intervals at 90% PPO. During the HIT and steady state component, participants ingested 7.3g of protein every 20 min to provide 22g of protein per hour. Physiological and perceptual measures were recorded at regular intervals throughout exercise (e.g. heart rate, RPE) and substrate utilisation was assessed during the steady state component of the exercise protocol using online gas analysis (CPX Ultima, Medgraphics, Minnesota, US) and the equations of Jeukendrup and Wallis (2005). Following completion of the training session, participants consumed 22g of COLLAGEN or WHEY protein immediately post-exercise as well as $1.2 g \cdot kg^{-1}$ BM carbohydrate in the form of sports drinks (Science in Sport, Nelson, UK) and snacks (Jaffacakes, UK) immediately and 60 minutes post-exercise so as to maximise muscle glycogen resynthesis. Laboratory conditions remained constant across all experimental trials (19 – 21ºC, 40 – 50% humidity) and an overview of the nutritional feeding protocol is shown in Figure 6.1.

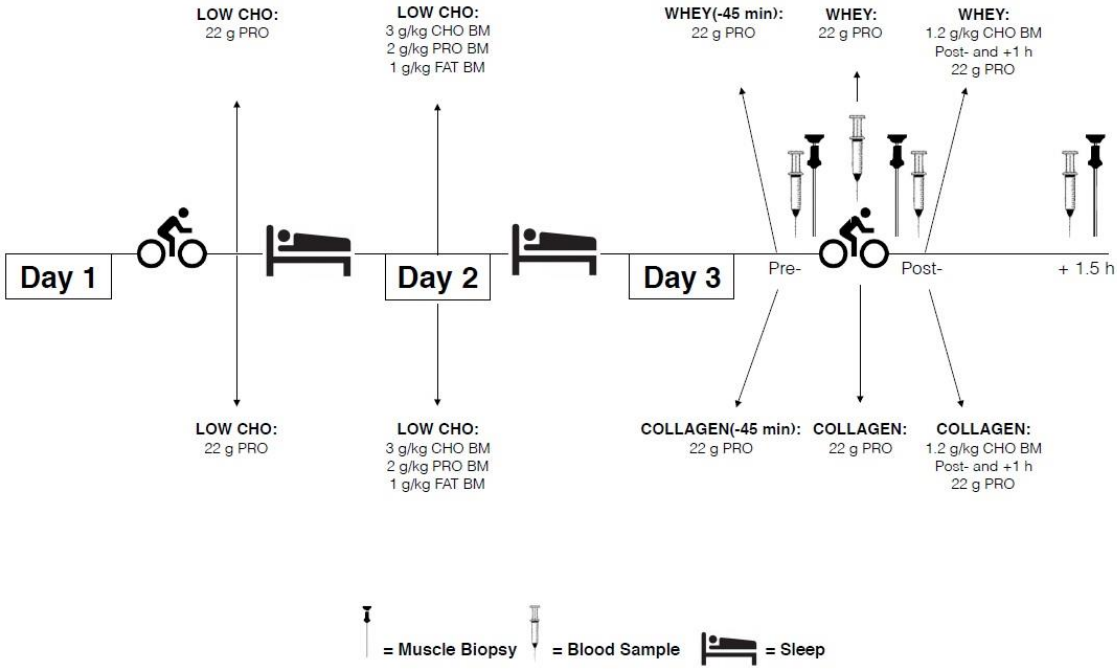

Figure 6.1. Schematic representation of the experimental design. On the evening of day 1 participants completed a glycogen depleting protocol followed by 22g of whey protein. Throughout the entirety of day 2 participants consumed a low CHO and low energy dietary protocol that was matched for both protein and fat intake. During the main experimental trial on day 3 participants ingested 22g of protein before commencing a set work protocol, participants consumed a further 22g during exercise and a final 22g immediately after exercise. Protein was provided as collagen gel (COLLAGEN) or as a protein beverage (WHEY). In addition to protein post-exercise, 1.2 g·kg bw CHO was provided immediately and 1h post-exercise to facilitate glycogen resynthesise. Muscle biopsies were obtained immediately pre-exercise, post-exercise and 1.5 h post-exercise.

6.3.5 Muscle biopsies: Skeletal muscle biopsies were obtained from the vastus lateralis immediately before exercise, immediately after exercise and at 90 minutes post-completion of the exercise protocol. Muscle biopsies were obtained as described in section 3.6.3.

6.3.6 Blood analysis: Blood samples were collected in vacutainers containing K_2 EDTA, lithium heparin or serum separation tubes, and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4℃. Serum and plasma were aliquoted and stored at -80℃ until analysis. Plasma glucose, lactate, FFA, glycerol, β-hydroxybutyrate and amino acids were analysed as described in section 3.6.1.

6.3.7 RNA extraction and analysis and Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): Quantification of mRNA content and assessment of mRNA transcription were performed as described in 3.6.4.

6.3.8 Muscle glycogen concentration: Muscle glycogen concentration was determined according to section 3.6.5.

6.3.9 [γ^{-32}P] ATP Kinase Assay: 30 mg muscle tissue was used for the measurement of p70S6K1 and PKB (Akt) activity as previously described (McGlory *et al.* 2014); section 3.6.6.

6.3.10 SDS-PAGE and Western Blotting: Approximately 20 – 30 mg of frozen muscle was probed for LC3 I and II and p62 content. Samples from each subject for all nutritional conditions were run on the same gel and statistical analysis conducted on fold changes relative to pre-exercise values, as described in section 3.6.7.

6.3.11 Statistics: All statistical analyses were performed using Statistical Package for the Social Scientist (SPSS version 21). Descriptive statistics were produced for all data sets to check for normal distribution indicated by Kolmogorov-Smirnov (accepted if P>0.05). Changes in physiological and molecular responses between conditions (i.e. muscle glycogen, circulatory metabolites, amino acids, mRNA, protein content and kinase activity) were analysed using two way repeated measures General Linear Model, where the within factors were time and condition. If Mauchley's test of sphericity indicated a minimum level of violation, as assessed by a Greenhouse Geisser epsilon (ε) of ≥ 0.75, data were corrected using the Huynh-Feldt ε. If Mauchley's test of sphericity was violated, data were corrected using Greenhouse Geisser ε. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferoni post hoc tests in order to locate specific differences. An alpha value of $P < 0.05$ was utilized for all tests and all data in text, figures and tables are presented as mean ± SD.

6.4 Results

6.4.1 Physiological and metabolic responses to exercise

Subject's heart rate (P=0.614) during the steady-state component of the exercise protocol did not display any significant differences between trials (Figure 6.2A). Exercise significantly reduced (P<0.001) muscle glycogen stores to comparable levels (150 mmol·kg^{-1} dw) with no difference (P=0.485) observed between conditions (Figure 6.2B). Plasma NEFA, glycerol and β-OHB all significantly increased during exercise (P<0.001) though plasma glucose did not display any change (P = 0.112) (Figure 6.2 C, D, E and F, respectively). Additionally, the exercise-induced increase in plasma NEFA was suppressed in the WHEY compared with the COLLAGEN trial (P=0.046) whereas no statistical differences were observed between trials for glycerol (P=0.08), β-OHB (P = 0.07) or glucose (P=0.963). Despite differences in NEFA availability during exercise, no differences were observed in either CHO (P=0.64) or lipid oxidation (P=0.75) during the SS component of the exercise protocols (Figure 6.2G and H, respectively).

6.4.2 Regulation of markers of mitochondrial turnover

In accordance with similar glycogen utilisation and metabolic stress between trials, the magnitude of the exercise-induced increase (P<0.01) in PGC-1α mRNA content at 90 min post-exercise was not different (P = 0.370) between trials (Figure 6.3A). In contrast, neither exercise (P = 0.261) nor experimental condition (P = 0.550) affected p53 mRNA content (Figure 6.3B). As an acute marker of mitophagy, the mRNA content of Parkin was significantly increased (P = 0.037) from pre- to post-exercise though no effect of condition was observed (Figure 6.3C). Nonetheless, a second mitophagy marker of Beclin1 mRNA content was not affected by exercise (P = 0.147) or dietary condition (P=0.348) (Figure 6.3D). Collectively, such data suggest that acute exercise up-regulates transcriptional markers of both mitochondrial biogenesis and autophagy, though leucine availability does not affect these responses.

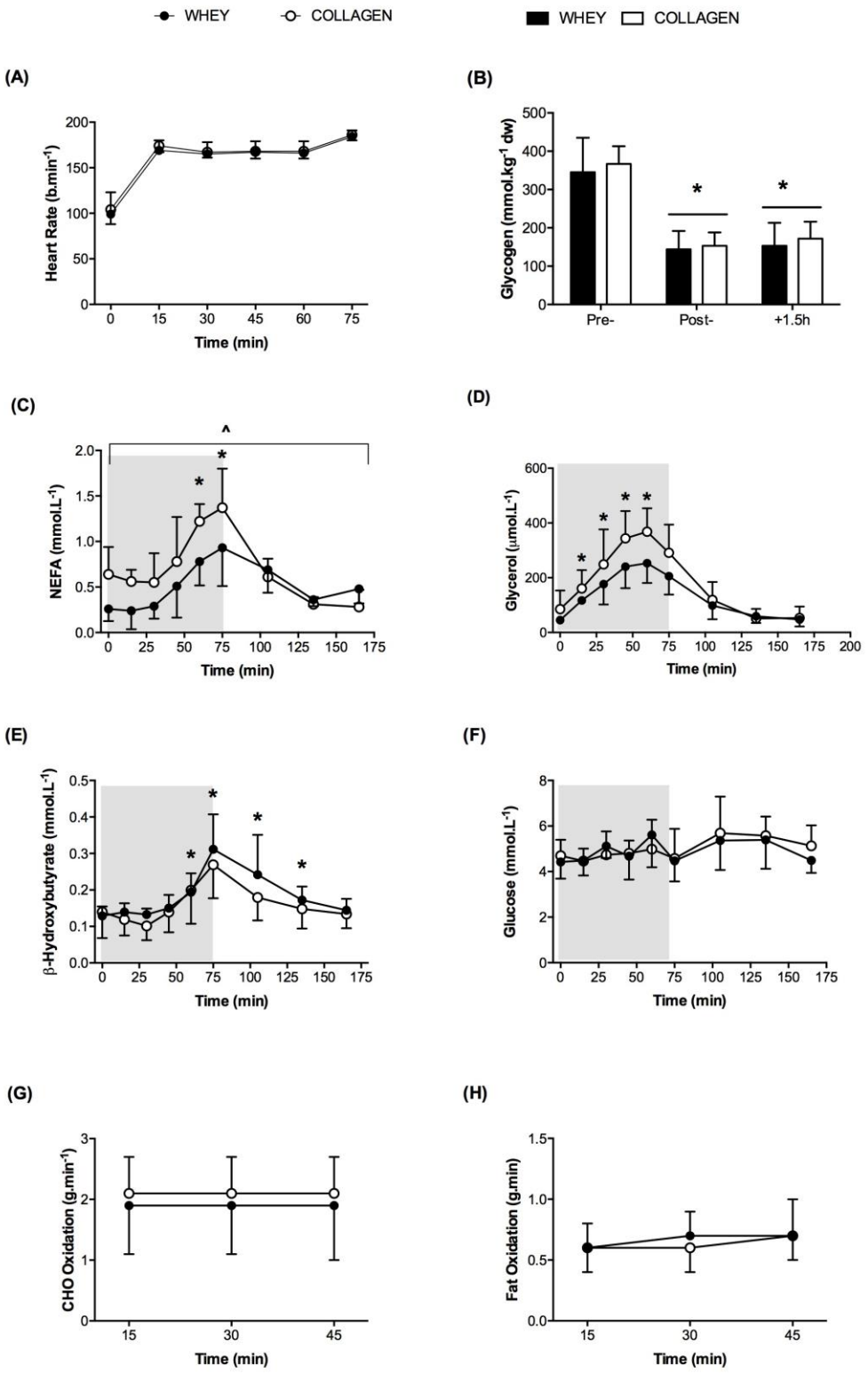

Figure 6.2. (A) Heart rate response during exercise, (B) Muscle glycogen concentrations across experimental trial, Plasma (C) NEFA, (D) glycerol, (E) βOHB and (F) Glucose, (G) CHO and (H) Fat oxidation during steady state component of exercise. *P<0.05 significant difference from pre-exercise, ^P<0.05 significant main effect of condition. Shaded area represents exercise duration.

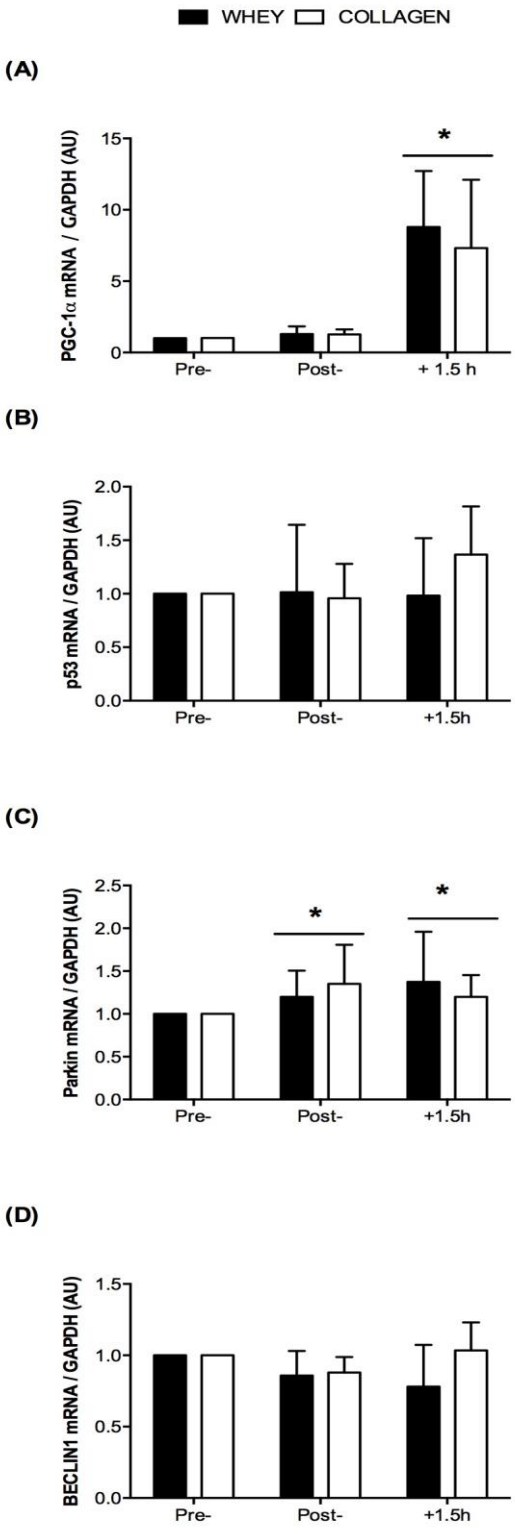

Figure 6.3. Fold change of mRNA content in markers of mitochondrial biogenesis (A) PGC-1α, (B) p53 and mitophagy (C) Parkin, (D) BECLIN1. *P<0.05 significant difference from pre-exercise.

6.4.3 Regulation of protein synthesis related signalling

Plasma leucine, BCAAs and EAAs all displayed significant changes (P=0.043, 0.028 and 0.021, respectively) during the sampling period (Figure 6.4 A, C, E respectively). Additionally, leucine (P=0.02) and BCAA concentrations (P=0.03) were higher in WHEY compared with COLLAGEN whereas EAA approached statistical significance between trials (P=0.06). When expressed as AUC data, only plasma leucine (P=0.025) showed differences between trials (Figure 6.4B) whereas as AUC for BCAA (P=0.135) and EAA (P=0.062) did not achieve significance (Figure 6.4 D and F, respectively).

In accordance with the provision of CHO post-exercise, plasma insulin significantly increased from pre- and post-exercise values (P = 0.034) though the magnitude of change was not different between trials (P = 0.159) (Figure 4G). As such, no difference (P=0.187) was apparent between trials for insulin AUC data (Figure 6.4H)

PKB activity was significantly elevated at 90 min post-exercise (P = 0.003) compared with pre-exercise values, irrespective of nutritional condition (P=0.37) (Figure 6.4I). Exercise suppressed (P=0.015) p70S6K activity to comparable levels immediately post-exercise (≈ 25 fmol.min^{-1}.mg^{-1}). However, post-exercise feeding increased p70S6K activity at 1.5 h post-exercise (P=0.004), the magnitude of which was greater (P=0.046) in WHEY (180 ± 105 fmol.min^{-1}.mg^{-1}) versus COLLAGEN (73 ± 42 fmol.min^{-1}.mg^{-1}) (Figure 6.4J). These data suggest that increased leucine availability from WHEY condition appears to regulate post-exercise p70S6K activity independent of insulin mediated stimulation of PKB.

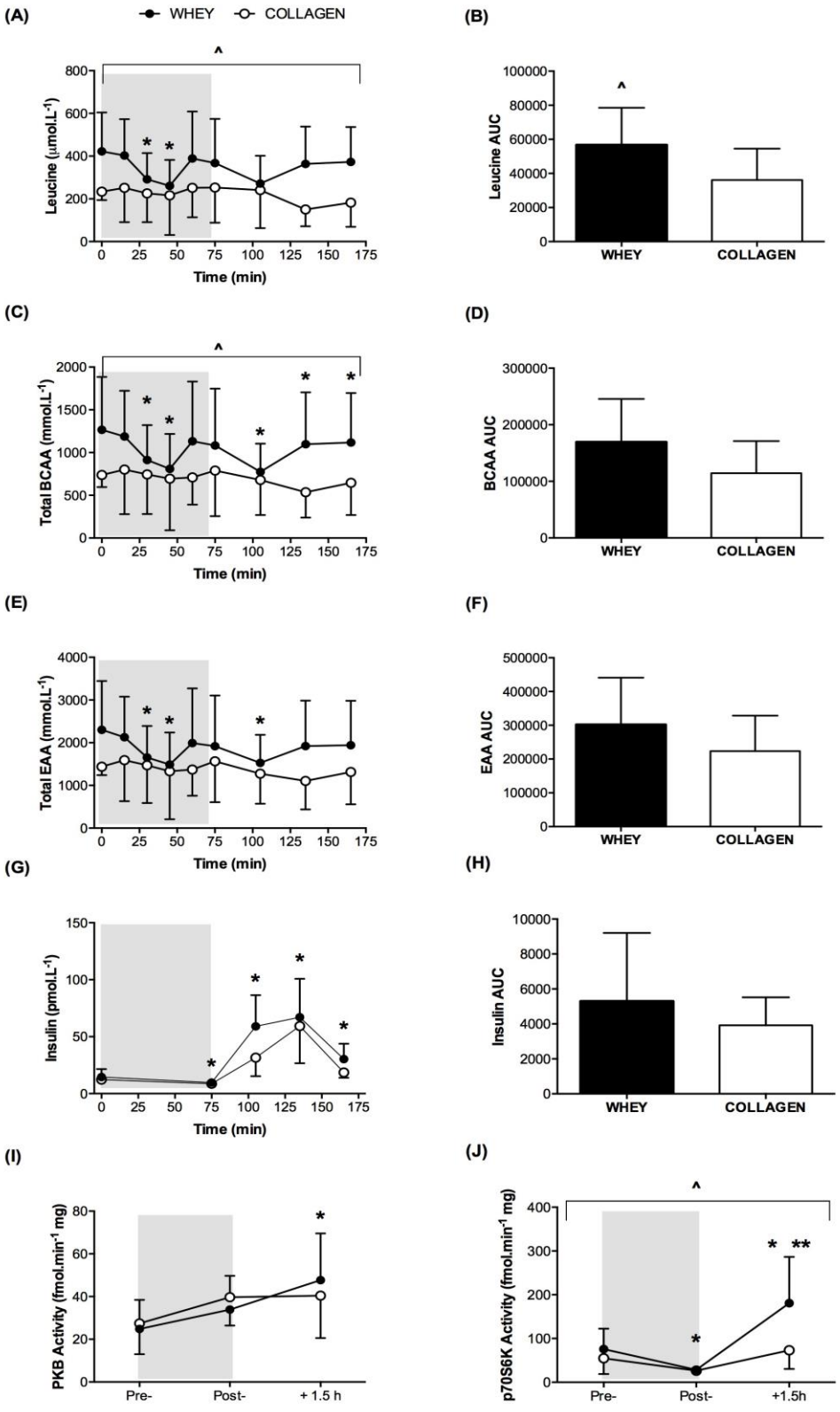

Figure 6.4. Circulatory plasma and total AUC (A, B) Leucine, (C, D) Total BCAA, (E, F) Total EAA and (G, H) Insulin. Kinase activity of (I) PKB and (J) p70S6K. Shaded area represents exercise duration. *P<0.05 significant difference from pre-exercise, **P<0.05 significant difference from post-exercise, ^P<0.05 significant main effect of condition.

6.4.4 Regulation of markers of autophagy

Exercise significantly decreased the protein content of LC3bI, LCBII and p62 ($P<0.001$; $P=0.021$; $P<0.001$, respectively) from pre- to post- exercise (Figure 6.5 A, B, D, respectively) though these changes were not influenced by nutritional condition ($P=0.255$, 0.754, 0.401, respectively). Additionally, the ratio of LC3bII/I displayed a trend ($P = 0.072$) towards increasing at 90 min post exercise (Figure 6.5C) with no differences also observed between conditions ($P=0.312$). The mRNA content of ATG12 was significantly increased ($P = 0.015$) immediately post- and at 90 min post exercise, with no differences ($P=0.56$) between conditions (Figure 6.5E). In contrast, the additional autophagy markers of ATG4b (Figure 3.5F) and p62 mRNA (Figure 6.5G) were not affected by exercise ($P=0.711$; $P=0.261$, respectively) nor condition ($P=0.76$; $P=0.55$, respectively). Representative western blots are shown in Figure 6.5H. Collectively, these data suggest that acute non-exhaustive exercise induces transient changes in autophagy related gene and protein expression, an effect that is not modulated by the composition of protein provision.

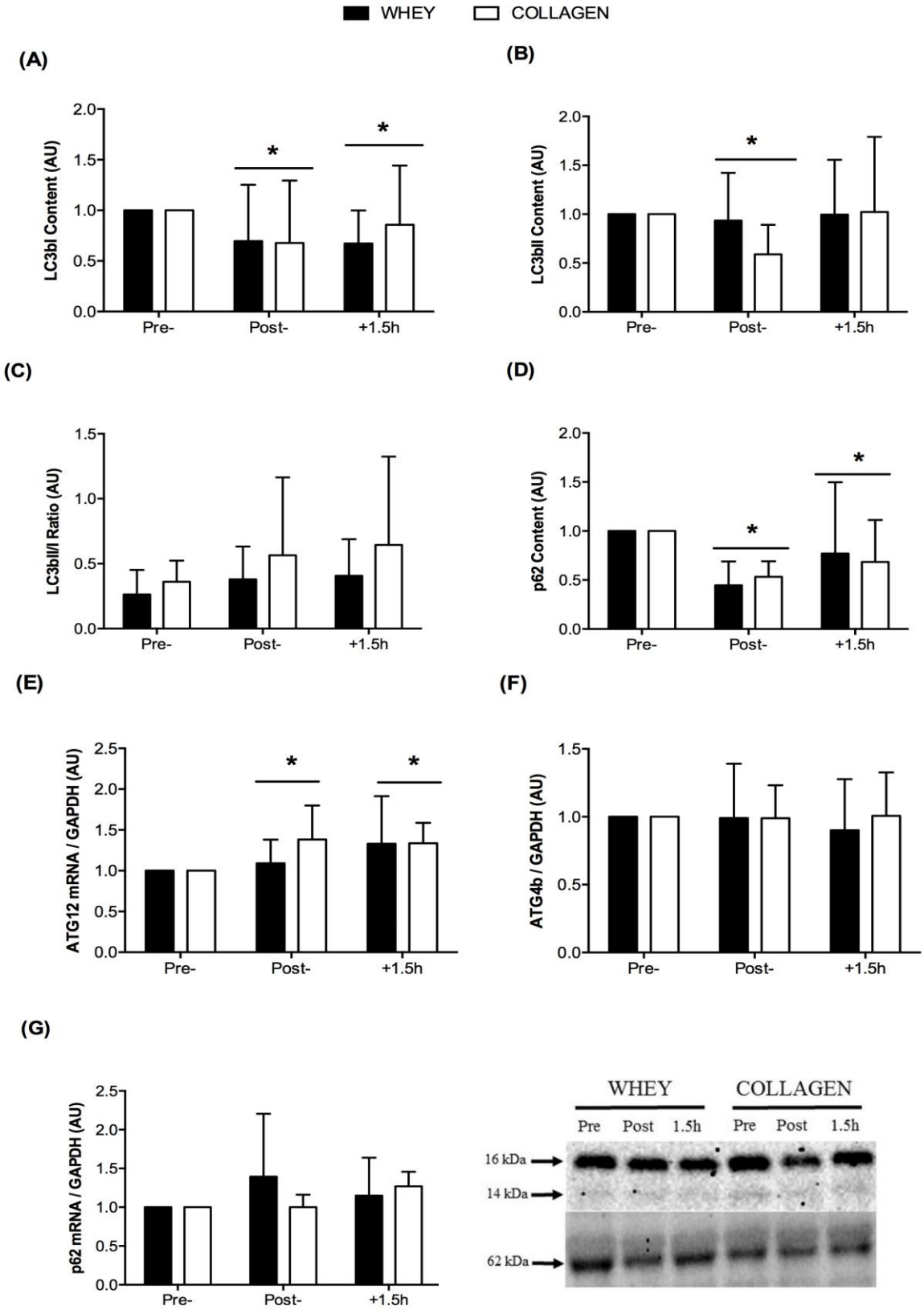

Figure 6.5. Protein content of bulk autophagy markers (A) LC3bI, (B) LC3BII, (D) p62 and (C) LC3bII/I ratio. mRNA expression of (E) ATG12, (F) ATG4b and (G) p62. (H) representative western blots. *P<0.05 significant difference from pre-exercise.

6.5 Discussion

The aim of the present study was to examine the effects of whey versus collagen protein on skeletal muscle adaptations associated with the acute regulation of mitochondrial biogenesis, muscle protein synthesis and autophagy. The rationale was motivated by chapter 5 in that keeping glycogen (and energy) at the critically low levels associated with exhaustion does not permit the re-activation of p70SK61 activity, despite feeding leucine-enriched whey protein (Impey *et al*. 2016). As such, I employed a similar train-low model (Impey *et al*. 2016) but provided CHO in the post-exercise period in accordance with guidelines to promote short-term muscle glycogen re-synthesis (Hulston *et al*. 2011). To manipulate protein availability across the experimental period, I fed a matched dose of whey or collagen protein from commercially available sources. When considered with my recent observations examining CHO (and energy) restriction (Impey *et al*. 2016) as well as post-exercise high fat feeding (Hammond *et al*. 2016), the data suggest that in exercise conditions that reduce glycogen to near depletion, the beneficial effects of whey protein on activation of p70S6K1 activity are especially apparent when co-ingested with sufficient post-exercise CHO intake. I also provide novel data by demonstrating that acute non-exhaustive exercise up-regulates markers of both mitophagy and autophagy, an effect that is not modulated by protein composition.

The rationale for train-low protocols is based on the premise that completing acute training sessions with reduced endogenous and exogenous CHO availability leads to activation of regulatory cell-signalling pathways (e.g. AMPK, p38, p53 and PGC-1) thereby co-ordinating an up-regulation of both the nuclear and mitochondrial genomes (Hawley and Morton, 2014; Bartlett *et al*. 2013). When collectively examining the acute cell signalling studies conducted to date (Bartlett *et al*. 2013; Camera *et al*. 2015; Mathai *et al*. 2008; Pilegaard *et al*. 2002; Psilander *et al*. 2013), such data consistently demonstrate that the augmented signalling responses associated with CHO restriction are particularly evident when absolute pre-exercise muscle glycogen concentrations are ≤350 mmol/kg dw. However, restoring post-exercise glycogen levels to >500 mmol/kg dw can attenuate exercise-induced changes in gene expression (Pilegaard *et al*. 2005), whilst keeping glycogen (and energy) at critically low post-exercise levels (i.e. <100-150 mmol/kg dw) can reduce activation of signalling proteins regulating protein synthesis (Impey *et al*. 2016). Such data therefore suggest the presence of a potential muscle glycogen threshold that is facilitative of promoting training adaptations. To this end, the combination of the

non-exhaustive exercise protocol utilised here and post-exercise CHO feeding was successful in achieving an absolute range of muscle glycogen, remaining in our suggested muscle glycogen threshold (i.e. ≤350 and >150 mmol/kg dw). We acknowledge, however, that this range is likely specific to the training status of the populations examined.

Confirming previous studies (Impey *et al*. 2016; Hammond *et al*. 2016), we observed that acute high-intensity exercise reduces p70S6K1 activity. Such studies further demonstrated that the presence of reduced CHO (and energy availability) and/or high post-exercise fat availability also suppresses the re-activation of p70S6K1, even when leucine enriched whey protein was consumed in the post-exercise period. Based on these studies, I therefore suggested that the apparent suppression of p70S6K1 activity may be due to 1) reduced insulin and PKB signalling or, 2) a direct effect of increased fat availability (Kimball *et al*. 2015) and/or reduced glycogen mediating suppression of mTORC1 complex via energetic stress related mechanisms (Bylund-Fellenius *et al*. 1984). The present data lend support for the latter mechanism for several reasons. First, I observed that the superior whey-induced increase in p70S6K1 activity when compared with collagen feeding was independent of post-exercise insulin and PKB activity (Figure 4G, I). Second, the post-exercise circulating FFA availability observed in our collagen trial was similar to that achieved with both CHO restriction (Impey *et al*. 2016) and post-exercise high fat feeding protocols (Hammond *et al*. 2016), though I acknowledge the limitations with making inferences on muscle FFA uptake on snapshot assessments of circulating FFA per se. Nonetheless, it remains possible that subtle alterations in FFA availability (as caused by prior dietary manipulations) can have associated implications on mTOR related signalling (Stephens *et al*. 2015). I would also suggest that glycogen availability per se at the exercise intensity used here may have a contributory and regulatory effect on components of the mTOR pathway. In this regard, it is possible the enhanced p53 and AMPK activation induced by post-exercise CHO restriction (Bartlett *et al*. 2013; Wojtaszewski *et al*. 2003) exerts a p53-REDD1 and/or AMPK-TSC2 mediated inhibition of mTOR (Feng *et al*. 2010; Keller *et al*. 2013; Rose *et al*. 2009), thereby reducing downstream activation of p70S6K1. Further studies are now required to directly test these hypotheses in human skeletal muscle. When considered with our previous studies (Impey *et al*. 2016; Hammond *et al*. 2016), the present data therefore suggest that in those exercise conditions in which muscle glycogen is near depletion, the beneficial effects of whey protein (i.e. leucine mediated activation of mTOR: Greiwe *et al*. 2001; Han *et al*. 2012) are

especially apparent when co-ingested with post-exercise CHO feeding. From an applied practice perspective, such findings lend further support for the fuel for the work required concept that emphasise both a day-by-day and meal-by-meal approach to nutritional periodization. Taken together, it is therefore suggested that whilst there may be benefits of commencing training with reduced endogenous and exogenous carbohydrate availability, the post-exercise meal should contain a combination of both protein and CHO, the latter to provide the necessary substrate and energy to stimulate both glycogen and muscle protein synthesis, respectively.

The cellular recycling process of autophagy has also emerged as an integral component of exercise induced remodelling processes, functioning to restore energetic balance and preventing the accumulation of damaged organelles or aberrantly folded proteins (Lira *et al*. 2013). Nonetheless, our understanding of how exercise affects autophagic flux is currently limited due to between species (rodent versus humans), nutritional status (fed versus fasted) and differences in exercise protocols (e.g. modality, intensity and duration) between studies (He *et al*. 2012; Lira *et al*. 2013). Furthermore, to the best of my knowledge, no investigators have yet examined the effects of endurance exercise on autophagy whilst also quantifying muscle glycogen availability. On this basis, this data suggest that acute exercise undertaken in conditions of reduced CHO availability induces transient changes in markers of autophagy. In agreement with other researchers, we also observed that non-exhaustive exercise reduces LC3bII (Fritzen *et al*. 2016) and p62 protein content (Schwalm *et al*. 2015) immediately post-exercise. Furthermore, we provide novel data demonstrating exercise up-regulates changes in Parkin mRNA, an effect that may be mediated through upstream PGC-1α signalling (Vainshtein *et al*. 2015). Whilst I acknowledge that we cannot currently make accurate inferences on autophagic flux based on measurement of these makers *per se*, it is noteworthy that none of the above parameters were influenced by protein composition or availability before, during or after exercise. While some studies have demonstrated a suppressive effect of mTOR signalling on autophagy (Kim *et al*. 2011) others have suggested a basal level of mTOR/p70S6K1 activity is required to mediate maintenance level of autophagy (Datan *et al*. 2014). However considerably more work is required in this area to elaborate the precise effects of autophagy in human skeletal muscle under nutrient-exercise stressors.

In summary, I demonstrate that whey protein augments post-exercise p70S6K1 activity to a greater extent than that compared with collagen protein when in recovery from an acute

non-exhaustive train-low endurance training session. When considered in context with our previous train-low studies examining post-exercise CHO (and energy) restriction or high fat feeding, our data suggest that the beneficial effects of whey protein (i.e. leucine mediated activation of mTOR signalling) is especially apparent when consumed with sufficient post-exercise CHO intake and/or when muscle glycogen remains in a potential muscle glycogen threshold. I also observe that protein composition does not modulate markers of mitochondrial biogenesis, autophagy and mitophagy.

Chapter 7 – Synthesis of Findings

7. Synthesis of Findings

This chapter will present an overview of the experimental findings of this thesis in relation to the original aims and objectives outlined in chapter 1. A general discussion is then presented where specific attention is given to how the present data has advanced the understanding of both the mechanistic and practical application of the train-low paradigm. Finally, a review of limitations of the experimental chapters is presented followed by recommendations for future research.

7.1 Achievement of aims and objectives

The primary aim of the present thesis was to examine the effects of reduced CHO but high protein availability on the regulation of molecular pathways associated with modulating mitochondrial biogenesis, MPS and autophagy. On the basis of characterising such molecular responses, a secondary aim was to formulate a novel framework for which to practically apply train-low paradigms.

Aim 1

To test the hypothesis that leucine-enriched protein feeding does not impair circulating FFA availability or whole body lipid oxidation during exercise (Study 1, Chapter 4).

In addition to reduced CHO availability, there is a growing rationale to consume increased protein before, during and after train-low sessions. Whilst such a feeding strategy is advantageous in terms of promoting a positive net muscle protein balance, the provision of amino acids (and associated rise in plasma insulin) may also be disadvantageous owing to the fact that some of the training adaptations arising from train-low may be mediated by increased FFA availability, secondary to the effects of CHO restriction *per se*. Nonetheless, the data presented in this study demonstrate that whey protein or leucine enriched whey protein feeding does not impair FFA availability nor whole body lipid oxidation during exercise, thus suggesting that protein feeding does not hinder a key objective of train-low sessions. Additionally, the leucine enriched protein trial in this study

consisted of a novel delivery of protein in the form of a "protein gel", a delivery matrix that is now commercially available for oral consumption (SiS, Science in Sport, Whey 20)

Aim 2

To characterise the effects of reduced carbohydrate (CHO) but high leucine availability on exercise capacity and skeletal muscle cell-signalling responses associated with exercise-induced regulation of mitochondrial biogenesis and MPS (Study 2, Chapter 5).

When matched for work done, numerous investigators have demonstrated that training in conditions of reduced CHO availability augments many of the acute cell signalling responses that regulate training adaptation (Table 2.1). However, using an experimental protocol consisting of an amalgamation of previously studied train-low protocols, the data presented in this chapter demonstrated that although reduced CHO availably impaired exercise capacity (i.e. less work was completed compared with high CHO availability), comparable cell-signalling responses were observed. As such, these data lend support for the fuel for the work required paradigm in that CHO restriction before and during exercise induces work-efficient mitochondrial related cell signalling (i.e. comparable activation of AMPK-PGC-1α path way can be achieved under low CHO conditions from performing significantly less work/training than when exercise is undertaken with high CHO). Nonetheless, post-exercise CHO and energy restriction impairs the regulation of p70S6K1 related pathways despite feeding leucine enriched protein. Such data therefore suggest that although there are potential metabolic benefits associated with reduced pre-exercise CHO availability, the post-exercise meal should contain sufficient CHO to restore muscle glycogen to sufficient levels and/or provide the necessary energy to support post-exercise remodelling processes, driven from both circulatory (i.e. insulin) and intracellular processes (i.e. glycogen assimilation).

Aim 3

To examine the role of protein composition in regulating skeletal muscle cell-signalling responses associated with exercise-induced regulation of mitochondrial

biogenesis, MPS and autophagy when in recovery from a train-low endurance training session (Study 3, Chapter 6).

Having identified the potential importance of post-exercise CHO intake in Chapter 5, the aim of Chapter 6 was to examine the role of protein composition in regulating post-exercise skeletal muscle remodelling processes when in recovery from a train-low training session. Using the train-low paradigm developed in Chapter 5, the data presented in this study suggest that whey protein intake is indeed facilitative of reactivating p70S6K1 related signalling pathways but only when consumed with sufficient post-exercise CHO intake and/or when muscle glycogen remains in a potential muscle glycogen threshold (e.g. <300 but >150 mmol·kg^{-1} dw). These data also demonstrate that acute exercise induces transient changes in markers of mitophagy and autophagy, an effect independent of leucine availability.

7.2 General discussion of findings

7.2.1. Overview of molecular mechanisms regulating mitochondrial turnover

Mitochondrial biogenesis underpins skeletal muscle oxidative capacity and is therefore considered a hallmark of intracellular adaptation to endurance exercise (Holloszy, 1967). In addition to increased number and size of mitochondria per gram of muscle tissue, recent data also highlight the importance of regulating mitochondrial quality via targeted autophagy to support adaptive oxidative responses to endurance training (He *et al.* 2012). From a functional perspective, the study of the regulation of mitochondrial turnover (biogenesis and degradation) has important implications for health and performance. In relation to exercise performance, training induced increase in metabolic enzyme activity and mitochondrial density/function result in enhanced respiratory control, thus the same cellular rate of oxidative metabolism can be achieved with less agitation of adenine nucleotides and lower rate of oxidative phosphorylation per mitochondrion, cumulatively observed as enhanced efficiency of energy production (Holloszy and Coyle, 1984). These adaptations result in preservation of ATP and PCr during exercise, attenuating the formation of allosteric regulators for glycogenolysis, glycolysis and lactate production, ultimately reducing muscle glycogen oxidation (Gollinck and Saltin, 1982). Physiologically, this manifests as the classic rightward shift in the lactate threshold curve

(Holloszy and Coyle, 1984) thereby increasing the absolute work that can be undertaken before onset of acidosis and fatigue.

The molecular mechanisms underpinning mitochondrial turnover are increasingly being understood. It is now generally recognised that acute muscle contraction induces homeostatic perturbations to cellular energy status to activate several cell signalling kinases such as CaMKII, p38MAPK and AMPK. These kinases converge on nuclear and mitochondrial transcription factors (e.g. p53, Tfam, NRF1/2) and transcriptional co-activators (e.g. PGC-1α), all of which are collectively responsible for synthesis of mitochondrial proteins, lipid and DNA constructs required for mitochondrial biogenesis. In this way, a controlled expression of those proteins involved in the regulation of substrate oxidation, substrate transport and mitochondrial fusion and fission collectively occur in the hours and days following each successive exercise training bout, providing the exercise stimulus is sufficient to perturb homeostasis (Perry *et al.* 2010). In addition to regulating mitochondrial biogenesis, several of these signalling kinases converge in coordination with others to regulate the targeted degradation of cellular constituents termed autophagy (Ding and Yin, 2012). Of particular interest, p53 and PGC-1α appear to play central roles in the regulation of exercise induced mitophagy. Indeed, nuclear translocation of p53 (seen during exercise) appears to regulate bulk auophagic processes (Broz *et al.* 2013) in addition to PGC-1α regulating mitophay through a regulatory feedback loop (Valnshten *et al.* 2015). The ability of exercise to regulate mitochondrial turnover would appear, from a molecular perspective, to represent a balance in the coordination of signalling cascades dependent on a wide variety of factors such as, energy status of the cell, intensity and duration of exercise. An overview of how acute exercise affects key cell signalling processes is shown in Figure 6.1 where specific attention is given to areas informed by the experimental data collected in the present thesis.

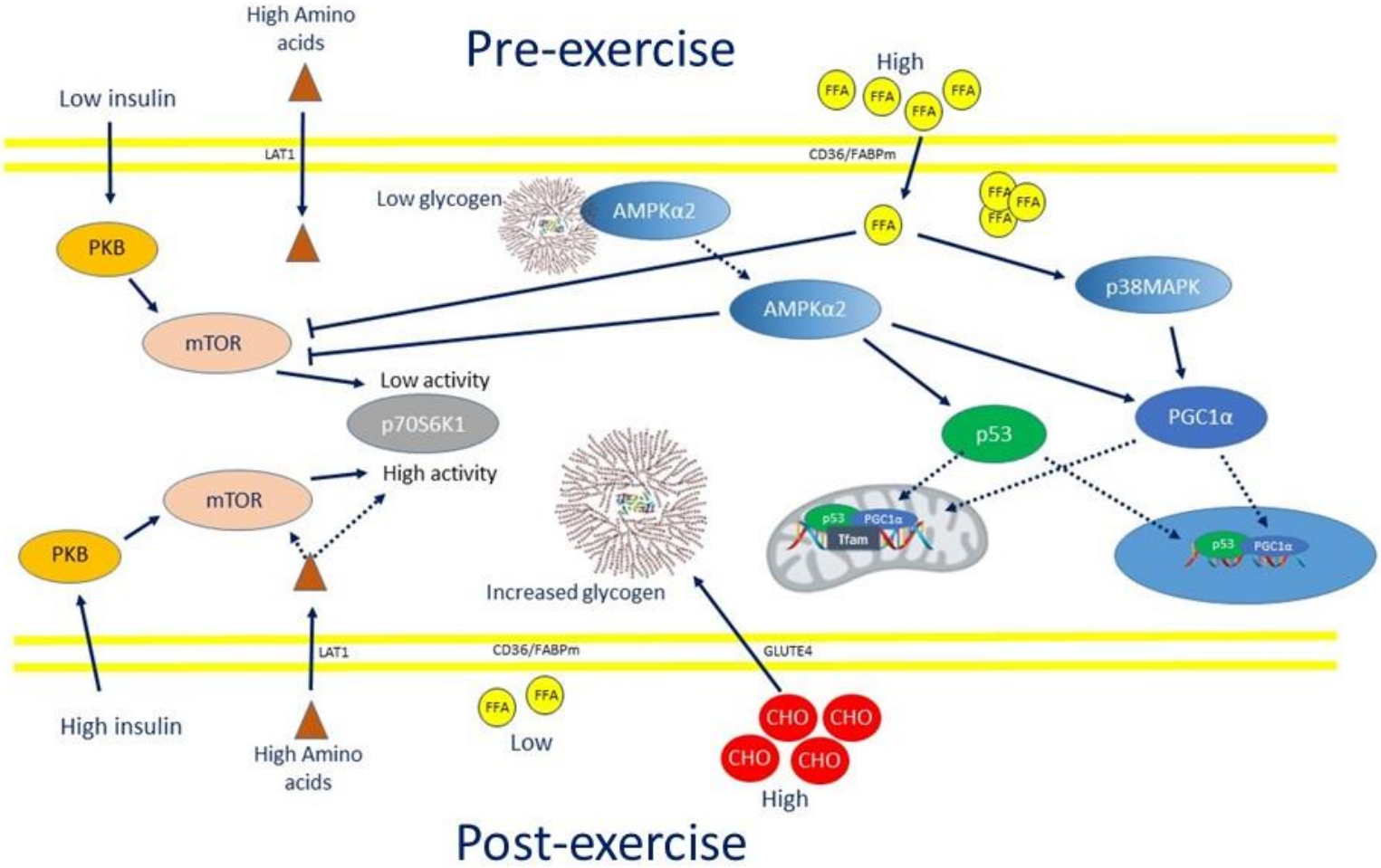

Figure 7.1 – Summary of the alterations in skeletal muscle signalling resulting from employing CHO periodisation following a fuel for the work required paradigm. Pre-exercise low exogenous and endogenous CHO availability with high protein availability, facilitates augmented mitochondrial regulated signalling. Despite high protein availability, when CHO and energy availability remains low, p70S6K1 activity is suppressed during post-exercise recovery period. p70S6K1 suppression is likely a result of elevated AMPKα2 activity in addition to elevated FFA. Post-exercise provision of high CHO and leucine rich protein increases circulatory insulin facilitating increases in PKB-mTOR-p70S6K1 signalling. In addition the provision of leucine rich protein further augment p70S6K1 activity. Solid lines with arrow heads represent active effectors of the origin; solid lines with blunted heads represent repression effectors of the origin; dashed lines represent translocation.

7.2.2. Effects of CHO availability on mitochondrial biogenesis

The ability for nutritional manipulation, particularly of carbohydrates, to augment the activation of signalling process regulating mitochondrial turnover has been an intense area of investigation in the last decade. Early studies initially aimed to understand the shift in metabolism when exercise is undertaken with reduced muscle skeletal muscle glycogen (Lemon and Mullen, 1976). However, at the turn of the millennium, researchers realised the potential to augment the signalling mechanisms regulating mitochondrial biogenesis by undertaking exercise with reduced skeletal muscle glycogen (Pileggard *et al.* 2002).

For example, Wojtaszewski *et al.* (2003) observed significantly elevated AMPKα2 activity and ACC^{Ser221} phosphorylation during 1h cycling exercise undertaken with low muscle glycogen. Previous research in rodents demonstrated that the allosteric regulators of AMPK activity, adenine nucleotide and creatine phosphate content were similar between muscles with low and normal muscle glycogen (Derave *et al.* 2000; Wojtaszewski *et al.* 2002), therefore implicating a role for glycogen content *per se* on AMPK activity. Indeed, a glycogen binding domain regulating activity was subsequently discovered in the β subunit (McBride *et al.* 2009). These seminal studies identified augmented AMPK activity when work-matched exercise was undertaken with reduced muscle glycogen and later studies also demonstrated the role of exogenous CHO feeding during exercise on regulating training adaptation (Akerstrom *et al.* 2006; Morton *et al.* 2009). Additionally, the work presented in this thesis presents the first evidence that a comparable level of AMPK activity can be induced in a work efficient manner when exercise is undertaken with low endogenous and exogenous CHO availability (Impey *et al.* 2016). This observation in line with research from other groups (Table 2.1) points to the potential for a specific range of glycogen concentrations within which intracellular signalling relating to mitochondrial biogenesis is augmented (Figures 7.1, 7.2). Such data lend support for the fuel for the work required model in that beneficial cell signalling responses can be achieved with strategic periods of reduced CHO availability (Chapter 5).

Consistent with augmented kinase activity, several investigations have identified increases in mRNA production from genes known to be regulated by transcription factors and transcriptional co-activators regulating mitochondrial biogenesis. Increased mRNA transcription has been seen using contralateral leg models (Hansen *et al.* 2005; Pilegaard *et*

al. 2002), exercising in the fasted state (Cluberton *et al.* 2005; Russel *et al.* 2005; van Proeyen *et al.* 2011), training twice per day (Pislander *et al.* 2013; Yeo *et al.* 2008) and sleep low models (Bartlett *et al.* 2013; Lane *et al.* 2015). In accordance, here I demonstrate comparable mRNA transcription of PGC-1α when exhaustive exercise was completed having started with high or low CHO despite total exercise duration being ~60 min less in the low CHO condition (Impey *et al.* 2016). Furthermore, using an amalgamation of low CHO methodologies (sleep low, low CHO diet and restriction of exogenous CHO during exercise) I demonstrated a significant increase in mRNA transcription of SIRT1, Tfam and p53 with post-exercise CHO restriction and subsequent low CHO diet following a glycogen depletion exercise protocol. When taken together, it appears therefore manipulation of CHO availability before, during and after exercise can significantly alter the molecular mechanisms underpinning mitochondrial biogenesis, in a time ans work efficient manner.

The incorporation of nutritional periodisation into a structured training program based on the data I present here in addition to previous research, suggests that when high intensity (lactate threshold or above) is the goal of the specific training session then high CHO should be provided to meet the glycolytic nature of that training, yet not over fuelling in a way that may dampen the beneficial adaptive responses, hence remaining within the proposed critical glycogen threshold/window. However, when oxidative adaptations, such as increasing lipid utilisation during exercise are the focus of training, low CHO nutritional strategies can be implemented to facilitate this outcome while also activating mitochondrial related signalling cascades in a work efficient manner by completing as much of the training as possible within the ranges of the critical glycogen threshold.

7.2.3. Effects of CHO availability on protein turnover

While the beneficial effects of training with low CHO availability on signalling regulating mitochondrial biogenesis are increasingly being understood, the energetic stress of this approach is not without potential limitations. Indeed, early studies noted increase in serum urea and amino acid efflux from skeletal muscle (Blomstrand and Saltin. 1999; Lemon and Mullin. 1980). These studies were followed by direct assessment of whole body protein balance and skeletal muscle protein turnover during 2 h of knee extensor exercise in conditions of high or low CHO availability (Howarth *et al.* 2010). Exercise with reduced

muscle glycogen availability produced a negative protein balance during exercise and recovery, an effect that was predominantly due to an increase in protein breakdown (Howarth *et al.* 2010).

To resolve these issues from a practical perspective, I examined the effect of leucine rich protein ingestion before during and after exercise as a potential method to compensate for the negative effects of low CHO training on protein balance. Indeed, I first confirmed that provision of leucine rich protein did not impair metabolic responses to exercise (i.e. circulating FFA availability and lipid oxidation) versus fasted conditions (Impey *et al.* 2015). Subsequently, I conducted a novel assessment of p70S6K1 activity following exhaustive endurance exercise in conditions of high or low CHO availability (Impey *et al.* 2016). To compensate for the negative effects of energy deficit on muscle protein turnover, the low CHO protocol was completed with leucine-rich protein provided before, during and after exercise. Despite the high leucine availability, I was unable to rescue p70S6K1 activity during recovery in the low CHO condition, thus demonstrating the negative effects of post-exercise CHO and energy restriction on MPS related signalling pathways (Impey *et al.* 2016). These findings indicated that whilst a level of glycogen depletion is required to augment mitochondrial related signalling (i.e. the potential glycogen threshold), keeping glycogen and energy at critically low levels may impair the regulation of post-exercise remodelling processes (Figure 7.1). Such data therefore suggested the requirement to consume post-exercise CHO in order to restore muscle glycogen to sufficient levels and/or provide the necessary energy required to facilitate remodelling processes.

I next tested the hypothesis that CHO and protein provision after a low CHO training session could restor p70S6K1 acitivty. The data in chapter 6 demonstrated that collagen and whey protein is indeed facilitative of reactivating post-exercise p70S6K activity when muscle glycogen remains in the suggested glycogen threshold (i.e. 150-350 mmol/kg dw) and CHO is consumed in the post-exercise period. However, an augmented activity of p70S6K1 was seen with whey protein. Given that this response was observed with comparable insulin and PKB activity, it suggests an effect of elevated leucine content within whey protein modulating the mTOR-p70S6K signalling axis. When taken together, such data suggest that recovery from train-low endurance training sessions should be supported with both carbohydrate and leucine rich protein to provide the necessary energy and substrate to support post-exercise remodelling processes. Indeed, while mitochondrial

adaptations and significant increases in lipid oxidation are beneficial components of a well-structured training program the ability to re-activate p70S6K1 (i.e. translation initiation) represents a vital component of the systems that remodel cellular structure as an adaptive response to training. While I acknowledge that the level of activity does not necessarily correlate with the rate of protein synthesis (myofibrillar or mitochondrial) the activity of p70S6K1 remains an essential component of the process (Figure 2.1) and thus ensuring the re-activation following exercise remains a pertinent goal for well-structured nutritional periodisation.

7.2.4. Effects of CHO availability on autophagy and mitophagy

Although a considerable bulk of research exists examining the signalling mechanisms that regulating mitochondrial biogenesis and MPS, a relative lack of data exists concerning the essential degradation and removal of malfunctioned cellular components, a process vital to sustain cellular functionality. The degradation of proteins by autophagy in response to exercise was initially characterised in the 1980's (Salminen and Virchows. 1984). Recent work elaborated on the contribution autophagy makes to adaptation from endurance exercise (He *et al.* 2012; Lira *et al.* 2013). While bulk autophagy clearly presents itself as an essential component of the adaptive response, in the context of low CHO training where a primary goal is mitochondrial biogenesis, mitochondrial specific autophagy (i.e. mitophagy), should also be considered. Recently, mitophagy has been shown to be upregulated by endurance exercise, an effect regulated in part by PGC-1α (Vainshtein *et al.* 2015; Vainshtein and Hood. 2016). Given these data, I chose to examine how exercise undertaken with reduced CHO availability would affect markers of bulk autophagy, and for the first time in humans, examine components of mitophagic signalling (Chapter 6). I demonstrate that training in a low CHO state appears to increase atuphagic flux independent of leucine availability, and observed significant transcriptional response of mitochondrial specific E3 ligase Parkin, therefore indicating that training with reduced CHO availability up-regulates markers of mitophagy. Nonetheless, I acknowledge that further research is required using a combination of methodological technique to fully characterise the effects of acute train-low paradigms on mitophagy. For example the incorporation of electron microscopy to directly observe number and structure of atuophagosomes as well as potentially identify the predominant cargo selected for degradation during low CHO training.

7.2.5. Practical application of the train-low paradigm

Experimental models of train-low thus far have typically studied twice per day models, fasted training, post-exercise CHO restriction and sleep-low (Table 2.1). Such models have consistently demonstrated that acute manipulation of CHO availability augments skeletal muscle cell signalling whilst short-term chronic CHO manipulation, can also improve exercise capacity and performance (Cochran *et al.* 2015; Marquet *et al.* 2016, 2016). Nonetheless, it is noteworthy that elite athletes rarely practice such models in isolation and indeed, CHO manipulation often presents itself as energy manipulation. As such, I deliberately studied an experimental model that represents an amalgamation of train-low paradigms. When examining the last decade of research in combination with the present experimental data, the picture that emerges therefore is that although high CHO availability unequivocally improves exercise performance, strategic periods of reduced CHO availability augments cell signalling related to mitochondrial biogenesis. From a practical perspective, it is becoming clear that CHO availability should be manipulated in a day-by-day and meal-by-meal manner depending on the upcoming workloads. A practical model for CHO and/or energy periodisation according to the principle of fuel for the work required within an endurance sport context is displayed in Table 7.1. In this model, high CHO availability is ingested before, during and after training sessions where the intensity is likely to be intermittent in nature and would be greater than lactate threshold and over a sustained duration (>60 min). In contrast, CHO availability is reduced for those sessions undertaken below lactate threshold and where intensity is not likely to be compromised. As with any training tool, each sport must develop a unique method of applying nutritional periodisation that elicits the required adaptation when considered in addition to the other components of a training program (i.e. sport specific skills, strength and conditioning etc.) in a manner that suits the specific nuances of the sport.

Essentially, I propose a critical glycogen threshold in which beneficial effects may arise if training sessions are commenced with reduced CHO availability yet, post-exercise glycogen and energy should not be restricted to a level that may impair the regulation of post-exercise remodelling processes. For this reason, post-training meals should contain appropriate CHO to facilitate recovery based on the training session just completed and considering the time available for recovery, intensity and duration of the next training session. Additionally, CHO may be restricted in the evening meal (to achieve potential

sleep low effects) depending on, the intensity and duration of the training session on the subsequent morning (Table 7.1). In relation to the level of glycogen required to facilitate beneficial cell signalling responses, a suggested glycogen threshold is shown in Figure 7.2. Here I describe a range of glycogen that provides a metabolic milieu that is conducive to augmented mitochondrial related signalling (also see Figure 7.1). From a performance perspective, it is not currently known whether a block of train-low or a periodised model is superior to facilitate performance improvements. Clearly, future studies are now required to test the efficacy of the periodised model outlined in Table 7.1. In conclusion, there is now a considerable body of molecular and physiological evidence of the beneficial potential for undertaking some parts of training with low CHO availability, the focus and goal of sports scientists and nutritionists alike is to refine and develop the specific contexts and program methodologies (amount and timing of CHO intake around training) that is both practical for athletes and underpinned by robust scientific framework, to elicit beneficial performance outcomes.

Table 7.1 – Suggested practical model of the “fuel for the work” required paradigm.

	Breakfast	On Bike	After Ride	Dinner
Day 1 (Efforts)	High (2 - 3 g/kg)	High (60 - 90 g/h)	High (1.5 - 3 g/kg)	Low (<50 g)
Day 2 (5h General ride)	Low (<50 g)	Low (< 30 g/h)	High (1.5 - 3 g/kg)	High (1.5 - 3 g/kg)
Day 3 (Efforts)	High (2 - 3 g/kg)	High (60 - 90 g/h)	High (1.5 - 3 g/kg)	Low (<50 g)
Day 4 (Rcovery)	Low (<50 g)	Low (0 g/h)	High (1.5 - 3 g/kg)	Dependent on next day

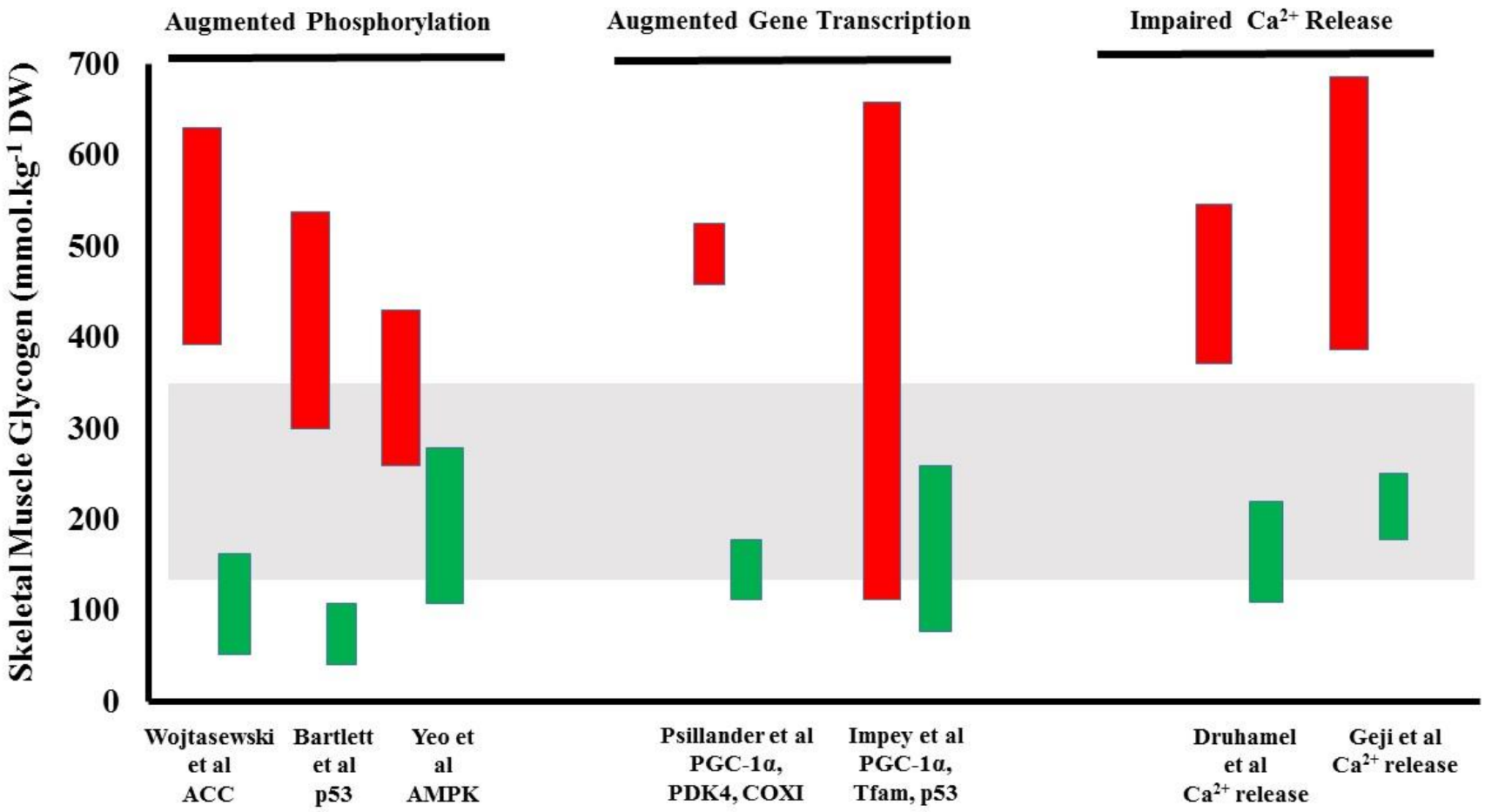

Figure 7.2 – The theoretical muscle glycogen threshold. Green bars represent enhanced signalling response. Red bars represent reduced signalling response. Length of bar represents reduction in muscle glycogen across exercise duration within each specific study.

7.3 Limitations

Chapter 4: While this chapter holds methodological rigor in standardising total work done, exercise duration, exercise intensity and energy availability, the use of indirect calorimetry *per se* (as opposed to tracer methodology) does not allow for measurement of protein oxidation. Additionally, the use of indirect calorimetry does not permit the assessment of lipid substrate from peripheral versus intramuscular stores, as has been examined previously (Hulston *et al.* 2010). However, I believe the methodologys employed facilitated a true representation of the physiological responses and thus present valid and reliable data from which to base conclusions.

Chapter 5: The different exercise capacity between the two conditions in this chapter resulted in different time points for post-exercise biopsy sampling, potentially limiting direct comparison of molecular responses. Had the low CHO trial been performed first in every subject thereby identifying the point of fatigue, then collecting a biopsy at the time point corresponding to low CHO fatigue in the high CHO trial would have allowed a more direct comparison of low vs high CHO training. However, this prevents the use of a randomised crossover counterbalanced design potentially influencing physiological and molecular responses as well as increasing the chances of statistically observing an order effect (Prescott, 1981). Additionally, a major limitation of this chapter was the inability to directly quantify muscle protein synthesis or breakdown owing to the lack of tracer methodology. Indeed, as it stands, I have provided a snapshot assessments of the regulation of relevant signalling pathways based on cell signalling assays conducted at set time-points. As such, I readily be acknowledge the requirement to provide dynamic assessments of both mitochondrial and muscle protein turnover. Of note, the use of a quantative measure of AMPK and p70S6K1 activity represent an attempt to present a more detailed understanding of the nature of intracellular signalling in response to nutrition-exercise interactions, rather than observing post-translational modifications which only act as proxy for activity.

Chapter 6: Here I present a study design that allows the distinction between collagen and whey protein on their ability to re-activate p70S6K activity following an acute train low endurance session. Additionally, I also examined how an acute train-low session may affect surrogate markers of autophagy and mitophagy. However, given the lack of a basal

or high CHO control I can identify that low CHO exercise induces activity of auto- and mitophagy markers but if this is different from exercise with high CHO can not be identified. Interpretation of autophagy activity from protein content and ratio of LC3bI and II in isolation can be problematic given the tissue specific expression and differences in basal rates of protein expression and lipidation (Kilonsky *et al.* 2011). By measuring p62 protein content and mRNA content in addition to LC3b, I can be more confident of the induction of autophagy given p62 acts as an autophagosome substrate (Kilonsky *et al* 2011). Despite the inclusion of additional measures to better quantify the activity of autophagy activity via western blotting and qRT-PCR, a potentially better method would be to use electron microscopy given its capacity to directly quantify changes in autophagosome formation and number as well as examining the constituents of the newly formed vacuoles to characterise the nature of specific vs bulk autophagy (Eskelinen *et al.* 2011). Like chapter 5, I wss also unable to directly quantify muscle protein synthesis or breakdown owing to the lack of tracer methodology.

7.4 Future studies

Recommendation 1: Following the formulation of the "fuel for the work required" strategy, the use of CHO periodisation in a well-structured real world representative training plan should be examined chronically. The work of Marquet *et al.* (2016) successfully demonstrated the potential for CHO manipulation to improve performance, however the use of a 'block' of low CHO training (as based on findings from this thesis) may not represent an optimal strategy to maximise the adaptive potential of low CHO training. Thus, incorporation of pre-determined selected train low sessions along with training sessions requiring considerable CHO availability should be utilised in a training study across several weeks. Furthermore, the effect of acute and chornic train-low interventions have yet to be tested in females and this also represents a considerable body of work still to be conducted.

Recommendation 2: This thesis presents the hypothesis of a critical glycogen threshold that must be achieved to induce the signalling required to elicit the augmented adaptive response. The use of step-wise reduction in muscle glycogen levels prior to exercise with muscle biopsy analysis, would allow better definition of the upper and lower limits of this

threshold. This data would considerably improve the understanding and ability to prescribe exercise-nutrition protocols to elicit the desired glycogen level appropriate to complete the pre-determined intensity and duration of training while still achieving the required threshold for augmented adaptive responses.

Recommendation 3: Glycogen depletion via exercise has a considerable effect on the transcription of several regulators of mitochondrial biogenesis and function (Cluberton *et al.* 2005; Pilegaard *et al.* 2002; Impey *et al.* 2016; Hammond *et al.* 2016), yet the full extent of low CHO training on transcriptional activity has not been well quantified. Screening all transcriptional responses and targeted analysis of the greatest responder genes holds significant potential to identify previously unreported regulators of low CHO training (Timmons *et al.* 2011). Indeed, use of 'omic' approaches to globally quantify protein, phosphorylation and metabolite (both intracellular and circulatory) changes holds considerable potential to better define the adaptive responses induced by low CHO training (Padro *et al.* 2009).

Recommendation 4: Much of the molecular targets measured here only provide a snapshot estimation of the regulation of key processes such as mitochondrial biogenesis, MPS and protein breakdown. The use of stable isotope methodology would therefore permit a more definitive assessment on the effects of reduced CHO availability on regulation of the above processes.

In summary, the novel data presented in this thesis allude to a potential muscle glycogen threshold (e.g. <350 but >150 mmol·kg^{-1} dw) hypothesis surmising that reduced pre-exercise muscle glycogen may enhance the activation of those pathways regulating mitochondrial biogenesis but also suggest that keeping glycogen (and energy intake) at critically low levels (i.e. <100 mmol·kg^{-1} dw) may impair the regulation of post-exercise remodelling processes. From a practical perspective, data lend support for a potential "fuel for the work required" train-low paradigm in that athletes could strategically reduce CHO availability prior to completing pre-determined training workloads that can be readily performed with reduced CHO availability, thereby inducing a "work-efficient" approach to training. Alternatively, when the goals of the training session are to complete the highest workload possible over more prolonged durations, then adequate CHO should be provided in the 24 h period prior to and during the specific training session. Future studies should

now examine the functional relevance of the signalling responses observed here, not only in terms of acute muscle protein synthesis but also the chronic skeletal muscle and performance adaptations induced by long-term use of this feeding strategy.

Chapter 8 – References

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Appendices

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Leucine enriched protein feeding does not impair exercise-induced lipid oxidation: implications for training in carbohydrate restricted states

Samuel G Impey[1], Daniel J Owens[1], Jon D Bartlett[1], Ken Smith[2], Graeme L Close[1] and James P Morton[1]

1: LJMU (Liverpool, UK), 2: University of Nottingham (Nottingham, UK),

Introduction

Training with reduced carbohydrate (CHO) availability augments skeletal muscle oxidative adaptations, an effect that may be mediated via free fatty acid (FFA) signalling (Hawley and Morton, 2014). However, a negative effect of CHO restriction during training is increased skeletal muscle protein breakdown (Howarth *et al.* 2010). A potential solution to overcome the latter is increased protein provision (especially leucine rich protein) so as to promote protein synthesis (Churchwood-Venne *et al.* 2013) though this strategy may be limited in that elevated insulin associated with protein feeding may actually attenuate lipolysis thereby negating FFA mediated signalling. The aim of the present study was therefore to test the hypothesis that leucine enriched protein feeding enhances circulating amino acid availability but does not impair lipid oxidation during exercise despite elevated insulin levels.

Method

Nine male cyclists performed 2 hours of cycling at 70% $\dot{V}O2_{peak}$ in fasted conditions (PLACEBO) or having consumed a whey protein isolate solution (WHEY) or a leucine enriched protein gel (GEL), administered as 20g 1 hour before, 10 g/h during and 20 g thirty minutes after exercise. Total leucine administration was 11.4 g and 4.6 in GEL and WHEY, respectively. Primary and secondary outcome variables were plasma FFA availability and rates of lipid oxidation during exercise, respectively.

Results

Mean plasma leucine concentrations during the 5 h collection period were elevated in GEL ($P= 0.001$) compared with WHEY and PLACEBO (375 ± 100, 272 ± 51, 146 ± 14 µmol.L^{-}

^{1} respectively). No differences (P= 0.153) in plasma FFA (WHEY 0.53 ± 0.30, GEL 0.45 ± 0.25, PLACEBO 0.65 ± 0.30, mmol.L^{-1}) were apparent between trials during exercise, despite elevated (P= 0.001) insulin in WHEY and GEL compared with PLACEBO (38 ± 16, 35 ± 16, 22 ± 11 pmol.L^{-1} respectively). Accordingly, whole body rates of lipid (WHEY 0.37 ± 0.26, GEL 0.36 ± 0.24, PLACEBO 0.34 ± 0.24 g/min) oxidation were not different (P= 0.955) between conditions.

Discussion

Leucine enriched protein feeding does not impair FFA availability or whole body rates of lipid oxidation during exercise. Data have practical applications for those athletes who deliberately train in CHO restricted states given that high leucine availability promotes protein synthesis and that high rates of lipid oxidation is one of the main aims of CHO restriction.

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Contact: s.g.impey@2007.ljmu.ac.uk

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Low muscle glycogen impairs post-exercise p70S6K activity despite high leucine availability: the critical glycogen hypothesis

Impey, SG.1, Hammond, K.1, Sheppard, S.1, Jeromson, S.2, Hamilton, DL.2, Close, GL.1, and Morton, JP.1

[1] LJMU, UK

[2] Stirling University, UK

Introduction

Reduced carbohydrate (CHO) availability during training augments mitochondrial biogenesis (Bartlett, *et al.* 2014) and ingestion of leucine rich protein elevates (Pasiakos *et al.* 2011) muscle protein synthesis (MPS). We tested the hypothesis that reduced CHO but simultaneous high leucine availability enhances cell signalling responses associated with regulation of mitochondrial biogenesis and MPS.

Method

In a repeated measures crossover design, eleven males completed an exercise-nutrition protocol to modulate muscle glycogen prior to completing 45 min cycling at 50% peak power output (PPO) followed by an exercise capacity test (1-min bouts at 80% PPO interspersed with 1-min bouts at 40% PPO) in conditions of high CHO and protein availability (HIGH) or low CHO but high protein (leucine enriched) availability (LOW). Vastus lateralis biopsies were obtained pre-, post- and 3 h post-exercise to assess muscle glycogen content and activity of p70S6K, AMPK and PKB using kinase assays. Two way RM ANOVA was used to compare differences between conditions and across time.

Results

Muscle glycogen was different ($P<0.05$) pre-exercise (HIGH: 583 ± 158, LOW: 271 ± 84 mmol/kg), similar post-exercise (HIGH: 114 ± 72, LOW: 85 ± 59 mmol/kg) and different ($P<0.05$) 3 h post-exercise (HIGH: 216 ± 132; LOW: 116 ± 60 mmol/kg). Despite differences ($P<0.05$) in exercise capacity (HIGH: 98 ± 29, LOW: 40 ± 17 min), exercise increased AMPK ($P<0.05$) at exhaustion in both groups (HIGH: 6 ± 4, LOW: 9 ± 5 mU/mg). In accordance with elevated leucine availability in LOW, p70S6K activity was

different (P<0.05) pre-exercise (HIGH: 45 ± 23, LOW: 65 ± 24 μU/mg) whilst exercise suppressed activity at exhaustion (HIGH: 22 ± 16, LOW: 22 ± 13 μU/mg). However, despite elevated leucine availability post-exercise in LOW, PKB activity was reduced (P<0.05) at 3h in LOW (HIGH 49 ± 24, LOW 29 ± 6 μU/mg) accordingly p70S6K activity was not restored at 3 h post-exercise (HIGH: 53 ± 38, LOW: 28 ± 14 μU/mg).

Discussion

We show for the first time that the post-exercise recovery of p70S6K activity is impaired when muscle glycogen remains low despite high leucine availability. Although reduced CHO availability augments mitochondrial adaptations, we also suggest that post-exercise glycogen stores should not remain depleted (< 150 mmol·kg) to restore activity of p70S6K; we suggest there is a 'critical glycogen threshold' below which remodelling is impaired following exercise. Athletes should minimise time spent below this threshold during periods of low CHO training to augment the adaptive response to training.

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Leucine availability regulates p70S6K activity when carbohydrate availability is sufficient in recovery from low carbohydrate training.

S G Impey[1], K M Hammond[1], R Naughton[1], C Langan-Evens[1], S O Shepherd[1], A P Sharples[1], J Cegielski[2], K Smith[2], S Jeromson[3], D L Hamilton[3], J P Morton[1]

1 Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, England

2 MRC-ARC Centre for Musculoskeletal Aging, University of Nottingham, Derby, England

3 Health and Exercise Sciences Research Group, University of Stirling, Stirling, Scotland

We assessed the ability of protein with augmented leucine content to reactivate p70S6K following low CHO training. In a repeated measures cross-over design eight males completed 75 minutes of cycling exercise comprised of HIT and steady state intensity in a low carbohydrate but high protein state. Protein was provided before, during and after (20g per hour) in the form of collagen gel (GEL) or conventional whey protein (WHEY). Following exercise carbohydrate was provided in addition to protein so as to facilitate muscle glycogen synthesis. Muscle glycogen was comparable before (GEL: 354 ± 44; WHEY: 339 ± 66 mmol·kg dw) exercise and decreased post exercise (GEL: 141 ± 25; WHEY: 158 ± 80 mmol·kg dw) ($P < 0.05$). Exercise induced comparable PGC-1α (8-fold), ATG12 (1.3-fold) and Parkin (1.3-fold) increases in gene expression at 90 minutes' post-exercise. Suppression of p70S6K activity was comparable between conditions post-exercise (~ 25 $fmol.min^{-1}.mg^{-1}$) ($P < 0.05$), while provision of carbohydrates and collagen protein was sufficient to re-activate p70S6K activity (GEL: 73 ± 42 $fmol.min^{-1}.mg^{-1}$), provision of leucine rich whey protein further augmented activity at 90 min post exercise (WHEY: 180 ± 105 $fmol.min^{-1}.mg^{-1}$) ($P < 0.05$). This augmented response occurred independent of insulin (WHEY AUC: 5296 ± 1478; GEL AUC: 3925 ± 602 AU) ($P > 0.05$) and PKB (WHEY: 47.7 ± 21.9; GEL: 40.4 ± 19.8 $fmol.min^{-1}.mg^{-1}$) ($P > 0.05$). These data support the notion of a critical glycogen threshold for augmented oxidative adaptations in addition to identifying optimal nutritional recommendations to augment signalling to facilitate recovery processes.