The Effects of Macronutrient and Energy Availability on Metabolic Responses to Exercise: Implications for Training Adaptation

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Abstract

Traditional nutritional guidelines for endurance athletes typically advise high carbohydrate (CHO) availability before, during, and after exercise in order to support high training intensities and volumes. However, accumulating data now suggest that restricting CHO and/or energy availability around training may augment the exercise-induced cell signalling responses associated with oxidative adaptations in human skeletal muscle. In addition to the manipulation of CHO availability, there is also a growing interest in the role of increased dietary fat intakes in augmenting components of training adaptation. The aim of this thesis was to therefore assess the effects of macronutrient and energy availability on the regulation of molecular signalling pathways associated with mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. A secondary aim was to also examine the acute effects of CHO and energy restriction on physiological markers associated with the syndrome of Relative Energy Deficiency in Sport (RED-S).

Study 1 (Chapter 4) examined the effects of reduced CHO but high post-exercise fat availability on the activation of cell signalling kinases and expression of genes with roles in the regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. In a repeated measures design, ten male participants completed a morning high intensity interval (HIT) running session (AM-HIT) followed by an afternoon steady state running session (PM-SS), under conditions of either high CHO (HCHO), or isocaloric low CHO but high fat (HFAT) availability in the post-exercise recovery period. Muscle glycogen was lower ($P<0.05$) at 3 (251 vs 301 $mmol.kg^{-1}dw$) and 15 h (182 vs 312 $mmol.kg^{-1}dw$) post afternoon exercise in HFAT compared to HCHO, however comparable increases (all $P<0.05$) in PGC-1α, p53, CS, Tfam, PPAR and ERRα mRNA were observed in HCHO and HFAT in response to exercise. AMPK-α2 activity was not increased post-exercise in either condition ($P=0.41$). HFAT induced greater increases in PDK4 ($P=0.003$), CD36 ($P=0.05$) and CPT1 ($P=0.03$) mRNA in the recovery period from afternoon exercise compared with HCHO. p70S6K activity was higher ($P=0.08$) at 3-h post-afternoon exercise in HCHO versus HFAT (72.7 ± 51.9 vs 44.7 ± 27 $fmol.min^{-1}$ mg^{-1}). Data demonstrate 1) that restricting CHO in the post-exercise recovery period has no further modulatory effect on the expression of genes associated with regulatory roles in mitochondrial biogenesis when overall energy availability is matched to a high CHO condition, and 2) high fat feeding may impair the regulation of muscle protein synthesis through reduced p70S6K signalling.

Having identified that high dietary fat intake does not augment mitochondrial biogenesis related signalling, the aim of Study 2 (Chapter 5) was to examine the effects of both post-exercise CHO and caloric restriction on the modulation of such pathways. In a repeated-measures design, eight male participants completed a twice per day exercise model whereby two bouts of HIT running were completed in the morning (AM-HIT) and afternoon (PM-HIT). These sessions were completed under three different dietary conditions consisting of either high CHO availability (HCHO) in the recovery period after both training sessions, reduced CHO but high fat availability (LCHF), or finally reduced CHO and reduced energy intake (LCAL). Muscle glycogen was reduced to comparable levels (~200-250 $mmol.kg^{-1}$ dw) in all trials immediately post PM-HIT and remained lower at 3-h (156, 182, and 345$mmol.kg^{-1}$ dw, $P< 0.001$) and 15-h post-exercise (171, 194, and 316$mmol.kg^{-1}$ dw, $P< 0.001$) in

LCHF and LCAL compared to HCHO. Phosphorylation of p38MAPK increased (P=0.037) immediately post-exercise, though no differences existed between dietary conditions (P = 0.755). Comparable increases (all P < 0.05) in PGC-1α, p53, CPT1 and CD36 mRNA were observed in HCHO, LCHF and LCAL. In contrast, PDK4 mRNA expression (P = 0.004) was greater in LCHF and LCAL in the recovery period from PM-HIT compared to HCHO, whilst SIRT1 mRNA expression was also greater in LCAL compared to HCHO and LCHF. Data demonstrate that under conditions where muscle glycogen is maintained within the range of 200-350 mmol.kg^{-1} dw, short-term periods of acute CHO and energy restriction (i.e. <24-h) does not potentiate skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism.

Whilst the acute manipulations of CHO and energy availability did not likely achieve absolute glycogen concentrations sufficient to constitute "true" train-low conditions, it is possible that such alterations in dietary intake may regulate other aspects of physiological function, many of which are associated with symptoms of RED-S. As such, Study 3 (Chapter 6) examined the effects of post-exercise CHO restriction and caloric restriction on markers of bone turnover, inflammation and appetite regulation. In an identical study design to Study 2 (Chapter 5), nine male participants exercised under three dietary conditions consisting of either high CHO availability (HCHO) in the recovery period after both training sessions, reduced CHO but high fat availability (LCHF), or finally reduced CHO and reduced energy intake (LCAL). Bone breakdown marker βCTX responses were significantly lower across all time points in the post-exercise period in HCHO (P=0.035) compared to LCHF and LCAL. Both AM-HIT (P=0.001) and PM-HIT (P=0.005) significantly increased bone formation marker P1NP responses but there was no difference between trials (P=0.633). IL-6 responses to exercise were higher in LCAL (P = 0.016) post PM-HIT compared to LCHF and HCHO. Circulating leptin levels were significantly lower (P = 0.04) in LCAL compared to HCHO in the post exercise sampling period. There was no difference in the short-term response of ghrelin to feeding (P= 0.408) with increases following both AM-HIT (P = 0.001) and PM-HIT (P = 0.025) in all three trials. Data demonstrate that consuming CHO before, during and after HIT running attenuates circulating β-CTX concentrations in the hours after exercise, effects that are independent of energy availability. In contrast, energy availability (but not CHO availability) modulates the regulation of post-exercise circulating leptin and IL-6 concentrations.

In summary, we provide novel data by demonstrating that in conditions where post-exercise muscle glycogen concentration is maintained within the range of 200-350 mmol.kg^{-1} dw, short-term periods of acute CHO and energy restriction (i.e. <24 hours) does not potentiate skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism. In addition, promotion of high CHO availability before, during and in recovery from exercise appears to be of greater importance for the acute regulation of bone turnover when compared with energy intake *per se*. In contrast, energy availability appears a more influential factor in regulating both IL-6 and leptin responses in recovery from exercise as opposed to CHO availability *per se*. Future studies should now examine the potential presence of a muscle glycogen threshold as an important regulator of skeletal muscle adaptations to endurance training. Additionally, the long-term implications (in relation to RED-S) of the acute within day fluctuations in both CHO and energy availability that occur when training twice per day should now be examined when performed as part of a periodised training programme.

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Contents

List of Abbreviations

AMP: Adenosine Monophosphate

AMPK: Adenosine Monophosphate activated Protein Kinase

ATP: Adenosine Triphosphate

AU: Arbitrary Units

β-CTX: COOH-terminal telopeptide region of collagen type 1

β-HAD – 3-hydroxyacyl-CoA dehydrogenase

Bp: base pairs

Ca^{2+} - Calcium

CaMKII – Calmodulin dependent protein kinase II

CD36: Cluster of differentiation 36

cDNA: complementary DNA

CHO: Carbohydrate

CPT1: Carnitine Palmitoyltransferase 1

COXIV – Cytochrome c oxidase IV

CS: Citrate Synthase

dw: Dry weight

ELISA: Solid Phase Enzyme-Linked Immunosorbent Assay

ERR-α: Estrogen-Related Receptor alpha

FFA: Free fatty acid

g: grams

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLUT4: Glucose Transporter Type 4

h: Hour

HIT: High-Intensity Interval Training

HCHO: High carbohydrate trial

HFAT: High fat trial

HR: Heart Rate

Kg: Kilogram

Km: Kilometer

KO: Knock-out

LCAL: Low calorie trial

LCHF: Low carbohydrate, high fat trial

LDH: Lactate Dehydrogenase

mmol: Milimole

mRNA: messenger RNA

mtDNA: Mitochondrial DNA

mTOR: Mechanistic target of rapamycin complex

NEFA: Non-Esterified Fatty Acids

NRF1/2: Nuclear Respiratory Factor 1/2

P1NP: NH_2-terminal propeptides of procollagen type 1

p38-MAPK p38: Mitogen-Activated Protein Kinase

P53: Tumour-suppressor protein 53

P70S6K: Ribosomal Protein S6 Kinase beta 1

PCR: Polymerase Chain Reaction

PDH: Pyruvate dehydrogenase

PDK4: Pyruvate Dehydrogenase Kinase 4

PGC-1α: Peroxisome proliferator-activated receptor γ coactivator-1α

PPARγ: Peroxisome Proliferator-Activated Receptor Gamma

RED-S: Relative Energy Deficiency in Sport

RPE: Rating of Perceived Exertion

rt-qRT PCR: Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction

SGLT-1: Sodium Glucose Linked Transporter

SIRT1: Sirtuin 1

Tfam: Mitochondrial Transcription Factor A

$\dot{V}O_{2max}$: Maximal Oxygen Uptake

$\dot{V}O_{2peak}$: Peak Oxygen Uptake

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Chapter 1
General Introduction

1.1 Background

The principle of promoting high carbohydrate (CHO) availability before, during, and after endurance exercise (both during training and competition situations) is the foundation on which traditional sports nutrition guidelines are based (Thomas *et al.*, 2016). Such recommendations have typically advised between 6-10 g/kg body mass of CHO, with the consensus being that athletes are able to train harder for longer periods and thus achieve a superior adaptive response (Coyle, 2000). Although it is well documented that high CHO availability is essential for promoting competition performance (Hawley *et al.*, 1997; Bergstrom *et al.* 1967; Burke, 2010), accumulating data from numerous independent laboratories now suggest that restricting CHO before, during, and in recovery from endurance based exercise training augments the cell signalling and gene expression responses associated with oxidative adaptations in human skeletal muscle (Impey *et al.*, 2018). As such, a reduction in CHO availability is now emerging as one of the most potent signals for activation of molecular signalling pathways associated with endurance adaptations (Philp *et al.*, 2012).

Indeed, both acute and training based studies have collectively observed that the reduction of both endogenous and/or exogenous CHO promotes 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 (Hulston *et al.*, 2010) lipid metabolism, and can improve both exercise capacity (Hansen et al. 2005) and performance (Marquet et al. 2016). The strategy that has emerged therefore suggests that athletes should deliberately complete specific training sessions with reduced CHO availability in order to augment muscle adaptive responses, but yet always ensure high CHO availability (as per traditional guidelines) during competition to promote optimal performance. This approach to CHO periodisation has been

termed '*train-low, compete-high'*, a model which simultaneously promotes both adaptive responses and competition performance through the manipulation of CHO availability (Burke *et al.,* 2010).

In addition to the manipulation of CHO availability to promote training adaptations, data also suggest a potential modulatory role of fat availability in augmenting the training response. Indeed, many studies have demonstrated shifts in substrate utilisation towards lipids during exercise following 'fat adaptation' protocols and when CHO availability is reduced (Rowlands and Hopkins *et al.,* 2002; Cameron-Smith *et al.,* 2003). It is therefore possible that the elevations in circulating free fatty acid (FFA) availability during exercise may regulate key cell signalling kinases and transcription factors, as well as modulating the expression of genes regulating both CHO and lipid metabolism (Zbinden-Foneca *et al.,* 2003; Cochran *et al.,* 2010). In this way, FFAs can act as signalling intermediates in conditions of CHO restriction in order to further up-regulate the muscle adaptive response. Indeed, studies have observed increases in resting intramuscular triglyceride stores, and increases in the protein content of those genes involved in the regulation of lipid metabolism in response to 5-days of high fat feeding (Cameron-Smith *et al.,* 2003). Such adaptations undoubtedly contribute to the enhanced rates of lipid oxidation observed during exercise following 'fat adaptation' protocols and would appear beneficial for endurance athletes.

Whilst it is well documented that restricting CHO around certain endurance-based sessions augments the adaptive responses to training, it is noteworthy that many of the studies investigating this concept have simultaneously adopted a CHO and calorie restriction approach to feeding around exercise (Bartlett *et al.,* 2013; Impey *et al.,* 2016). This raises the question as to whether the enhanced muscle adaptive responses observed when 'training-low' are due to CHO restriction *per se*, or whether it is in fact

a reduction in total energy intake post exercise, or indeed a combination of both. It is apparent that both CHO restriction and calorie restriction induce similar physiological alterations including increased FFA availability and lipid oxidation (Meynet and Ricci, 2014). In this way, it is possible to that metabolic adaptations may, in fact, be dependent on the energy state of the muscle rather than substrate selection. To date, no investigators have yet examined the effects of CHO restriction in comparison to total calorie restriction on the muscle adaptive responses to training in humans. This idea is especially relevant from an applied perspective given that many endurance athletes present daily with transient periods of both CHO and calorie restriction due to multiple training sessions per day, as well as longer term periods of sub-optimal energy availability in order to achieve desired body composition (Vogt *et al.* 2005; Fudge *et al.,* 2006). This highlights the requirement to study 'train-low' protocols that may be more reflective of real world athletic practice, i.e. both CHO and energy restriction.

Despite the rationale that training with reduced CHO, (or energy) and/or or high fat availability is beneficial from an adaptation perspective, such feeding strategies are not without potential limitations. Indeed, reduced CHO and energy availability have been shown to impair acute training intensity (Yeo *et al.,* 2008; Hulston *et al.,* 2010), immune function (Fischer 2006; Nieman *et al.,* 2007), and the ability to oxidise exogenous CHO during competition (Cox *et al.,* 2010). As such, training of this type could potentially lead to a detraining effect and impaired competition performance if performed long term. Additionally, low energy availability has also been shown to have negative effects on bone turnover (Sale *et al.,* 2015), the endocrine axis, and appetite regulation (De Souza *et al.,* 2008). In this regard, acute fluctuations in energy availability may lead to increased risk of athletes presenting with symptoms associated

with the Relative Energy Deficiency in Sport Syndrome, now referred to as RED-S (Mountjoy *et al.,* 2018). It is also noteworthy that high fat feeding may actually impair glycogen utilisation during exercise through reductions in pyruvate dehydrogenase (PDH) activity (Stellingwerff *et al.*, 2006), thus suggesting that rather than preparing elite athletes for competition, high fat feeding may actually negate the capacity to utilise CHO during high intensity exercise. It is therefore difficult to determine how best to periodise training-low or training with high fat in to an overall athletic training programme in order to minimise the aforementioned risks.

Although many endurance training-induced skeletal muscle adaptations are regulated at a transcriptional level, the turnover of myofibrillar (i.e. contractile) proteins are largely regulated through the translational machinery and the mechanistic target of rapamycin complex (mTOR) and ribosomal protein S6 kinase 1 (p70S6K) signalling axis (Moore *et al.,* 2014). In this regard, data collected from lipid and heparin infusion also suggests that high circulating FFA availability actually impairs muscle protein synthesis through negative effects on this pathway (Stephens *et al.,* 2015). In addition, it is also evident that this pathway is supressed post-exercise under conditions of low CHO and low energy availability (Impey *et al.*, 2016). When taken together these data suggest there is also a need to better understand the effects of both reduced CHO / energy and high fat availability on the signalling pathways regulating muscle protein synthesis (MPS).

1.2 Aims and objectives

The primary aim of this thesis is therefore to determine the effects of macronutrient and energy availability (with a specific emphasis on manipulation of CHO and fat) on the regulation of molecular signalling pathways associated with exercise-induced mitochondrial biogenesis, lipid metabolism, and muscle protein synthesis. A secondary aim is to examine the effects of such feeding strategies on markers of bone turnover, inflammation and appetite regulation.

These aims will be achieved by completion of the following objectives:

a) To examine the effects of post-exercise CHO restriction but high fat feeding on skeletal muscle cell signalling and gene expression associated with the regulation of mitochondrial biogenesis, lipid metabolism, and MPS (Study 1, Chapter 4).

b) To examine the effects of post-exercise CHO and caloric restriction on skeletal muscle cell signalling and gene expression associated with the regulation of mitochondrial biogenesis and lipid metabolism (Study 2a, Chapter 5).

c) To examine the effects of post-exercise CHO restriction and caloric restriction on markers of bone turnover, inflammation and appetite regulation (Study 2b, Chapter 6).

Objectives b and c will be achieved from data collected in one large study (Study 2) which will be split and presented as two separate chapters within this thesis. The original aims of study 2 from the outset of the PhD had to be changed following data collection due to an unforeseen issue with a collaborating institution were some analyses could not be performed resulting in a large delay. As such a separate data collection for a Study 3 could not take place and new analysis were performed on existing samples from Study 2 to form the third experimental chapter.

Chapter 2

Literature Review

2.1 Introduction

Being a highly malleable tissue, skeletal muscle has the ability to undergo major adaptations and alter its phenotype in response to exercise training. For endurance athletes, the key focus of training is to enhance their capacity to produce ATP and to delay the onset of fatigue. The most prominent adaptation observed in response to endurance exercise training is now widely recognised as the increase in mitochondrial mass (i.e. mitochondrial biogenesis), which ultimately permits higher intensities to be met for longer time periods (Holloszy, 1967). An increased understanding of the mechanisms by which regular exercise promotes mitochondrial biogenesis is therefore of paramount importance for those athletes with the goal of improving endurance performance. With the growing availability of modern laboratory techniques to investigate cellular signalling in response to exercise, it is now apparent that specific nutritional interventions can act to enhance the adaptive responses that exercise training alone can provide (Hansen *et al.*, 2005, Morton *et al.*, 2009, Yeo *et al.*, 2008, Bartlett, *et al.*, 2013).

The concept of manipulating CHO, fat, and/or overall energy availability around specific training sessions have now all been identified as a potential mediators to those training adaptations linked to improved performance in endurance athletes. However, further research is required to elucidate the precise molecular mechanisms underpinning the enhanced adaptations observed under these nutritional conditions. Furthermore, the optimal approach for which to practically apply these strategies within athletic populations in order to maximise adaptive responses while minimising the potential associated negative impact is still yet to be identified.

The present chapter provides an overview of some of the key signalling pathways currently thought to modulate exercise induced adaptations in human skeletal muscle, as well as highlighting some of the specific approaches to CHO and fat manipulation involved in further enhancing these adaptive responses. A brief discussion on some of the potential negative consequences of macronutrient and energy manipulation around training is also included. As such, this chapter aims to provide the reader with an extended introduction and rationale as to the nature of the studies undertaken in this thesis.

2.2 Training adaptations to endurance exercise

Being a highly malleable tissue, skeletal muscle has the ability to undergo major adaptations and alter its phenotype is response to exercise stimuli (Drake *et al.*, 2016). Endurance athletes typically focus their training to enhance those adaptations within the muscle which will subsequently increase exercise capacity and fatigue resistance. Such adaptations include increased mitochondrial content (Holloszy 1967), lipid oxidation (Costill *et al.*, 1979) and angiogenesis (Andersen and Henriksson, 1977), all of which are recognized functionally by increased whole-body oxygen uptake ($\dot{V}O_{2max}$) and rightward shift of the lactate threshold curve.

From an endurance perspective, perhaps the most important of these adaptive responses is the increase in mitochondrial content, termed *'mitochondrial biogenesis'*. In skeletal muscle, mitochondria are the key producers of ATP and thus play a critical role in overall energy balance (Hood, 2009). Mitochondrial biogenesis results in more efficient energy production through an increased abundance of proteins involved in ATP production, FFA transport and oxidation, glucose transport, and oxygen delivery to the muscle (Holloszy and Coyle, 1984). As such, increases in mitochondrial mass

ultimately allow endurance athletes to exercise at higher intensities for longer periods. Exercise training has long been linked to mitochondrial adaptation, with pioneering work from John Holloszy providing the first evidence of exercise induced mitochondrial biogenesis in skeletal muscle. This seminal study in rats demonstrated that three months of strenuous progressive endurance training led to significant increases in mitochondrial enzymatic activity and protein content in skeletal muscle (Holloszy, 1967). These adaptive responses were associated with increases in submaximal running time to exhaustion by up to 600% (from ~30 mins to ~90 mins). The results of this study have subsequently been replicated in human skeletal muscle in a number of investigations in well trained endurance athletes (Hoppeler *et al.,* 1973; Nielsen *et al.,* 2010), with both acute (Wilkinson et al 2008) and chronic (Scalzo et al 2014) exercise being associated with increases in mitochondrial proteins indicative of mitochondrial biogenesis. Indeed, data also demonstrates that mitochondrial content is reduced by prolonged periods of muscle disuse such as immobilization (Booth and Kelso, 1973). These enhanced enzymatic adaptations subsequently increase the muscles overall capacity for lipid oxidation and thus concomitantly reduces fatigue through attenuating CHO utilization (Holloszy and Coyle, 1984). When compared to the general untrained population, endurance trained individuals demonstrate a greater capacity to utilise triglycerides as a fuel source during exercise, while having less reliance on muscle glycogen (Hurley *et al.,* 1986). Indeed, investigators have observed increased levels of those mitochondrial enzymes involved in both fatty acid transport and oxidation (Costill *et al.,* 1979) - subsequently resulting in an enhanced capacity to utilise fat as a fuel source during exercise.

2.3 Molecular mechanisms regulating training adaptation

In response to each individual exercise bout, acute transcriptional changes take place within the muscle in the hours during recovery, and it is the accumulation of these acute responses over time that subsequently alters the muscle to a more oxidative phenotype through the expression of new proteins (Perry et al., 2010). Recent advances in molecular biology techniques such as polymerase chain reaction (PCR) and western blotting now allow exercise physiologists to examine the cellular and molecular responses to different exercise interventions in more detail, thus providing greater insight into the optimal training and/or nutritional interventions for elite athletes. A schematic overview of the proposed mechanisms underpinning skeletal muscle adaptive responses to training is displayed in Figure 2.1. And discussed in more detail below.

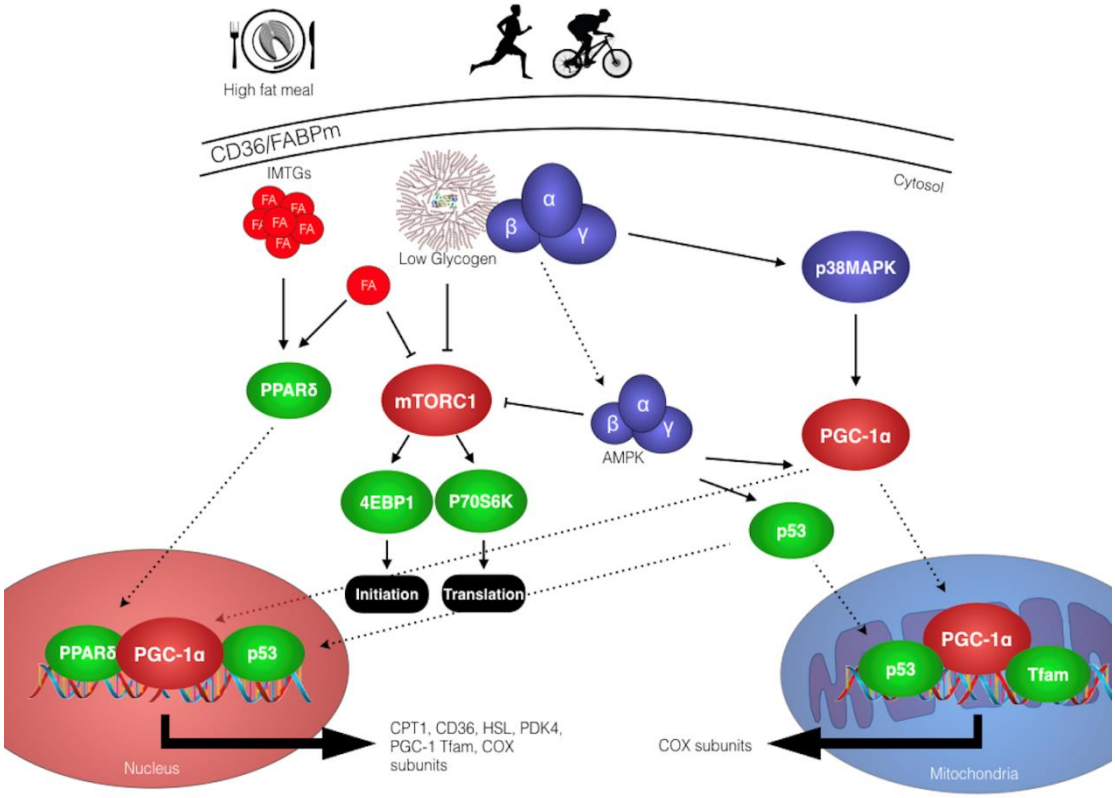

Figure 2.1. Overview of key molecular signalling pathways regulating endurance training adaptations

Upon the onset of muscle contraction, a number of metabolic perturbations within muscle cells (i.e. increased AMP/ATP ratio, Ca^{2+} flux, lactate, hypoxia and energy availability) occur which collectively trigger the activation of key regulatory protein kinases. The most extensively studied of these kinases are p38 mitogen-activated protein kinase (p38MAPK), AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1), and calmodulin-dependant protein kinase II (CaMKII). These kinases subsequently phosphorylate downstream targets such as transcription factors and transcriptional co-activators to induce the up-regulation of gene expression (Jager et al., 2007). The process of mitochondrial biogenesis requires fine-tuned co-ordination between both nuclear and mitochondrial (mtDNA) genomes (Scarpulla, 2002). The most well studied co-ordinator of these two processes, and thus regulator of

mitochondrial biogenesis is the transcriptional co-activator peroxisome proliferator activated receptor-γ co-activator-1α (PGC-1α).

Both AMPK and p38MAPK can directly phosphorylate PGC-1α during acute exercise resulting in its translocation to both the nucleus and the mitochondria. In the nucleus, it interacts with transcription factors such as NRF-1, NRF-2, oestrogen-related receptor (ERRα), peroxisome proliferator-activated receptor (PPARδ), and myocyte enhancer factor 2 (MEF2), to induce the up-regulation of proteins involved in glucose and fatty acid transport and oxidation. Upon localization to the mitochondria, PGC-1α forms a complex with mitochondrial transcription factor A (Tfam) at the D-loop region to co-ordinate up-regulation of muscle mitochondrial content and the capacity for substrate metabolism and oxidative phosphorylation (Safdar *et al.* 2011). In addition to PGC-1α, the tumour suppressor protein p53 has now emerged as a potential regulator of mitochondrial biogenesis. Indeed, acute exercise induces the post-translational modification of p53 (Bartlett *et al.,* 2012), and similarly to PGC-1α, this protein also translocates to the nucleus (Philp *et al*., 2011) and the mitochondria (Saleem & Hood 2013) to interact with Tfam.

2.3.1 PGC-1α

Since its discovery in 1998, where it was identified as the co-activator for PPARγ in brown adipose tissue (Puigserver *et al.,* 1998), PGC-1α has become widely termed as the *'master regulator'* of mitochondrial biogenesis, and as such, is commonly used as a marker of skeletal muscle adaptation to exercise training. The evidence of PGC-1α dependant mechanisms as mediators of mitochondrial biogenesis is now substantial. Indeed, initial rodent studies demonstrated that overexpression of PGC-1α increases oxidative enzyme activity (Lin *et al.,* 2002) and improves exercise capacity (Calvo *et*

al., 2008). It is also apparent in mice that genetic deletion of the PGC-1α gene blunts the mitochondrial biogenesis response to endurance exercise (Leick *et al.,* 2010). Although PGC-1α influences mitochondrial biogenesis through regulation of gene transcription, several other signalling proteins are also involved in the process. As a transcriptional co-activator, PGC-1α exerts its effects by direct interaction with downstream transcription factors or nuclear receptors (such as NRF-1/2, Tfam PPARs, and ERRα) which are important for the regulation of both nuclear and mitochondrial genomes in mitochondrial biogenesis (Hood *et al.,* 2016). In humans, there are now a wealth of studies investigating the PGC-1α response to both cycling (Gibala *et al.,* 2009) and running based (Bartlett *et al.,* 2012) exercise at varying intensities. Typically, elevated PGC-1α mRNA levels are observed following endurance exercise with the highest abundance present in the first 2-4 h of recovery (Pilegaard *et al.,* 2003; Gibala *et al.,* 2009; Bartlett *et al.,* 2012).

One of the first studies to examine the effects of both acute exercise and exercise training on PGC-1α mRNA expression in humans used a knee extensor exercise model (Pilegaard *et al.,* 2003). After 4-weeks of training one leg, subjects completed 3-h of two-legged knee extensor exercise with muscle biopsies obtained pre- and at various time points post-exercise in both legs. The single bout of exercise was shown to significantly increase PGC-1α mRNA in both the trained and untrained legs, peaking at 2-h post-exercise with concomitant increases in Tfam and PPAR mRNA in the untrained leg. Furthermore, the increase in PGC-1α was greater in the trained leg despite a lower relative workload.

The extent to which PGC-1α is increased in response to exercise also appears to be related to exercise intensity. Indeed, mRNA expression was significantly greater following exercise undertaken at 80% $\dot{V}O_{2max}$ compared to 40% $\dot{V}O_{2max}$ despite the

trials being matched for total work done (Egan *et al.*, 2010). Interestingly, there appears no further benefit when exercise is completed at workloads above 100% $\dot{V}O_{2max}$ (Edgett *et al.*, 2013).

A landmark study from Perry and colleagues (2010), documented the time course of increased PGC-1α mRNA and protein content in response to 2-weeks high intensity cycling training. Their results (displayed in Figure 2.2 below) demonstrated that each individual exercise session resulted in transient bursts of increased PGC-1α mRNA (at 4-h post cycling exercise), which returned back to baseline levels after 24-h. These repeat mRNA bursts following each individual training session subsequently lead to increases in PGC-1α protein content over time, thus indicative of a 'training adaptation' response. Interestingly, with each successive training session, the extent to which PGC-1α mRNA increased was attenuated over the 2-week training period thereby suggestive of becoming accustomed to the exercise stress. In this way it is possible that over time elite athletes will reach a plateau in their adaptive responses to training.

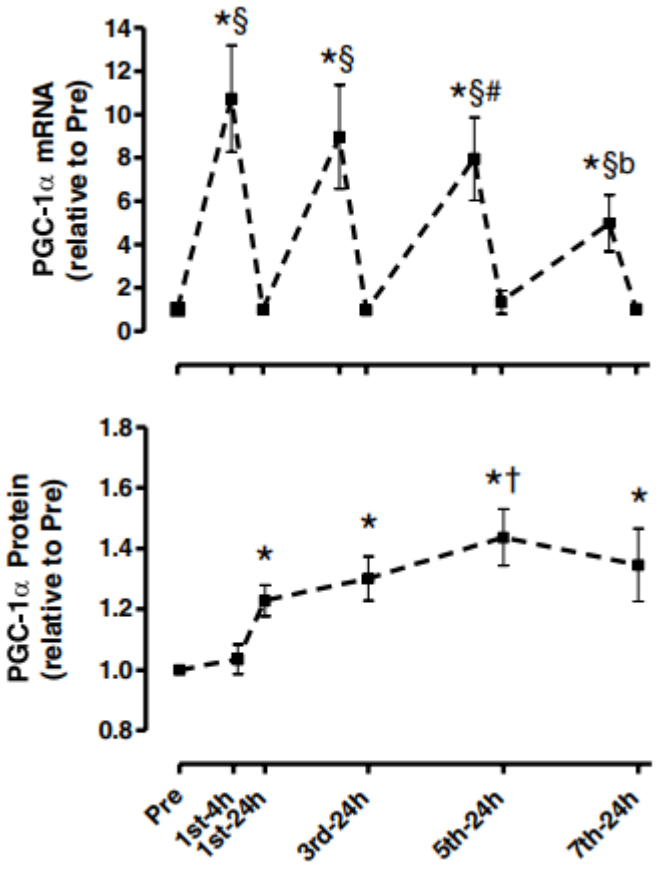

Figure 2.2 Time-course of human skeletal muscle PGC-1α mRNA and protein content following 2-weeks of high intensity interval training (Perry *et al.*, 2010). Values are means ± S.E.M. for 8–9 subjects. ∗Significantly different from Pre, §significantly different from all 24 h time points, #significantly different from 1st-4 h, b significantly different from all 4 h time points, †significantly different from 1st-24 h ($P < 0.05$).

Both the increases in activation and expression of PGC-1α in response to exercise are regulated by a number of upstream mechanisms. The metabolic sensors AMPK and p38 MAPK have been identified as two key activators of PGC-1α in skeletal muscle and will now be discussed in more detail.

2.3.2 AMP-dependant protein kinase (AMPK)

AMPK is a serine/threonine protein kinase that responds to the energy status in the cell (Kemp *et al.,* 1999). Being a heterotrimeric enzyme, AMPK is formed of one α catalytic subunit, and two regulatory β and γ subunits. The α-subunit contains the phosphorylation site threonine 172 which is activated by upstream kinases (Hawley *et al.,* 1997), the β-subunit contains a glycogen binding domain (McBride *et al.,* 2009), and the γ-subunit acts as a binding site for AMP and ATP. As such, AMPK is activated primarily by increases in cellular metabolic stress, as indicated by increases AMP, or increases in the rate of ATP hydrolysis such as during exercise (Drake *et al.,* 2016). Once activated, AMPK stimulates energy generating processes such as glucose uptake, GLUT4 translocation, and fat oxidation (Merrill *et al.,* 1997) whilst concomitantly decreasing energy consuming processes (e.g. protein synthesis) in order to restore energy levels (Richter and Ruderman 2009). In line with its role in the regulation of cellular metabolism therefore, AMPK is regularly referred to as an 'energy sensor'.

In human skeletal muscle, exercise is one of the most powerful activators of AMPK. While phosphorylation of AMPK has been demonstrated in response to multiple exercise modalities, it would appear that this activation takes place in an intensity-dependant manner (Egan *et al.,* 2010). Indeed, it appears that intense (~85% $\dot{V}O_{2peak}$) exercise is especially potent in activating AMPK in well-trained individuals (Clark *et al.,* 2004). In addition to exercise intensity, it appears that exercise duration may also play a role in the activation of AMPK. Indeed, during ~210-mins of low intensity cycling exercise at 45% $\dot{V}O_{2peak}$, AMPK became progressively more phosphorylated during the exercise bout (Wojtaszewski *et al.,* 2002). AMPK also appears to be activated in an isoform specific manner, for example both AMPK-α1 and AMPK-α2

are rapidly activated during 30-s maximal sprint exercise (Chen *et al.,* 2000). In contrast, when exercise intensity is increased from ~60% $\dot{V}O_{2peak}$ to ~80% $\dot{V}O_{2peak,}$ only AMPK-α2, but not AMPK-α1 activity was increased (Chen *et al.,* 2003), suggesting that AMPK-α2 should be measured at higher exercise intensities.

Interestingly, the exercised induced activation of AMPK appears to be reduced with endurance training status. For example, Nielsen et al. (2003) showed that AMPK-α2 phosphorylation was significantly blunted in well trained endurance athletes when compared to sedentary individuals. Similarly, AMPK activation in response to exercise was attenuated when untrained individuals had completed a short-term endurance training programme (McConnel *et al.*, 2005).

Once activated, it is apparent that AMPK can directly regulate the mitochondrial biogenesis pathway through PGC-1α. Indeed, muscle-specific knockout of both isoforms of AMPK results in dysfunctional mitochondria and impaired exercise capacity (Lantier *et al.,* 2014). Data have consistently demonstrated an increase in PGC-1α gene expression when AMPK is activated (Irrcher *et al.,* 2008; Zhang *et al.,* 2014). It is also apparent that the exercise-induced translocation of PGC-1α into the mitochondria and the nucleus is dependant on the activation of AMPK (Jager *et al.,* 2007).

Where AMPK possesses a glycogen binding domain within the β-isoform, data indicate that AMPK activity is actually inhibited by higher levels of muscle glycogen (McBride *et al.,* 2009). Indeed, Wojtaszewski et al. (2003) reported that at rest, AMPK-α1 and α2 activity were significantly higher when muscle glycogen concentration was ~160 mmol/kg. dry wt compared to a glycogen loaded state (~900

mmol/kg. dry wt). In addition, AMPK-α2 activity was shown to be higher when1-h of cycling exercise was commenced with low muscle glycogen availability.

2.3.3 p38 mitogen-activated protein kinase (P38 MAPK)

In addition to AMPK, p38 MAPK has also been identified as an upstream regulator of PGC-1α in the mitochondrial biogenesis pathway. p38 MAPK is a member of the mitogen-activated protein kinase family, and is often referred to as being 'stress inducible' due to its activation by a range of stimuli such as environmental stress, muscle contraction, insulin and pro-inflammatory cytokines. Following seminal work identifying exercise as a regulator of the p38 MAPK pathway in human skeletal muscle (Aronson *et al.,* 1997), the phosphorylation of p38 MAPK has been demonstrated following both running (Boppart *et al.,* 2000; Bartlett *et al.,* 2012) and cycling (Benziane *et al.,* 2008; Gibala *et al.,* 2009) based protocols in humans independent of exercise intensity (Egan *et al.,* 2010).

The role of p38MAPK in mediating mitochondrial biogenesis is now well established. From initial data in mice, it is apparent that where running based exercise increases the activation of p38 MAPK, there is also a corresponding increase in PGC-1α (Akimoto *et al.,* 2005). Using C2C12 cells, these authors demonstrated that PGC-1α promoter activity was enhanced following the activation of p38 MAPK, and blocked by specific p38 MAPK inhibitors (Akimoto *et al.,* 2005). They also found that overexpression of p38 MAPK in transgenic mouse models resulted in enhanced expression of both PGC-1α and COXIV in skeletal muscle. Subsequent *in vivo* studies have highlighted myocyte enhancing factor 2 (MEF2) and activating transcription factor 2 (ATF2) binding sites on the PGC-1α promoter as being critical for contraction induced promoter activity (Akimoto *et al.,* 2008). Indeed, activation of p38 MAPK in

response to endurance exercise has been shown to phosphorylate both ATF2 (Wright *et al.,* 2007) and MEF2 (Zhao *et al.,* 1999) and increase PGC-1α mRNA expression. It is likely that skeletal muscle contraction during exercise activates p38 MAPK through the increase in Ca^{2+} release (Wright *et al.,* 2007). When taken together, these data highlight an important role for p38 MAPK in regulating mitochondrial biogenesis through PGC-1α dependant mechanisms in response to an exercise stimulus.

2.3.4 Other factors involved in the regulation of endurance training adaptation

In addition to training status, exercise modality, intensity and duration, there are also several other factors which may augment the muscle adaptive responses to endurance-based exercise training.

Over the last decade, research has examined the potential of acute post-exercise cold exposure in modulating markers of training adaptation which are important for mitochondrial biogenesis such as increases in the expression of PGC-1α. In this regard, in human tissue both cold ambient temperatures (Slivka *et al.,* 2012; 2013) and post exercise cold-water immersion (Ihsan *et al*., 2014; 2015) have been shown to enhance the skeletal muscle PGC-1α gene transcriptional and protein translational response when compared with exercise alone. More recent data from our laboratory demonstrated that post-exercise cold-water immersion augmented the exercise induced expression of PGC-α mRNA in human skeletal muscle (~6 fold) following an acute bout of exercise when compared to the increase observed following exercise alone (~3-4 fold) (Joo *et al.,* 2016). The precise mechanisms underpinning this cold-induced regulation of the PGC-α transcriptional pathway are yet to be fully determined, however, data demonstrates that chronic cold-induced changes in PGC-

1α protein content correspond with an increased activity of the upstream signalling kinases AMPK and p38MAPK (Ihsan et al., 2015).

It is also apparent that exposing endurance athletes to hypoxic conditions during training may be beneficial from an adaptive perspective, and this has been shown to improve endurance performance when utilised frequently during a training programme (Levine, 2002). Intermittent exposure to hypoxic conditions while at rest, i.e., live-high train-low is currently though to be the most effective hypoxic method to increase endurance performance. In this regard, Katayama and colleagues (2004) examined the effects of exposing trained male endurance runners to 3-h per day in hypoxic conditions for 14 consecutive days in comparison with a control condition. Participants performed maximal, submaximal, and time trial running tests before and after the intervention, and findings demonstrated a tendency towards improvement in a 3000-m running time trial in the hypoxic group compared to the control group. There was also a significant relationship between the change in running time and the change in VO_2 during submaximal exercise following exposure to hypoxic conditions, suggesting that performance improvements shown could be due to an enhanced running economy when training in a hypoxic environment. Additionally, more recent data in runners has demonstrated an improvement in VO_{2max} following intermittent hypoxic training when compared to the same training undertaken in normoxic conditions (Nakamoto *et al.,* 2016). It is likely that these improvements in endurance exercise capacity are mediated by a higher oxygen carrying capacity of the blood following exercise training in a hypoxic environment (Katayama *et al.*, 2004).

Over the last decade there has been increased popularity in examining the role of nutrition in exercise performance and in modulating the muscle adaptive responses to endurance-based exercise training. – this will now be discussed in more detail through the remainder of this chapter.

2.4 The role of CHO for training and performance

Following seminal work in the 1920's, the importance of CHO as a fuel source during exercise has now long been recognised. Initial observations from Krogh and Lindhard (1920) revealed that fatigue occurred earlier during exercise when participants had consumed a high fat diet rather than a high CHO diet in the days prior to exercise. The study of CHO metabolism was then significantly advanced in the late 1960's with the incorporation of the percutaneous needle muscle biopsy technique. Primary work with this technique demonstrated that a high CHO diet (~80% of energy) was associated with elevated muscle glycogen content and augmented exercise performance when compared to a CHO restricted diet (Bergstrom et al. 1967). A developing body of research has since consistently demonstrated that muscle glycogen concentration displays a positive correlation with exercise time to exhaustion (Bergstrom et al., 1967, Tzintas et al., 1985), and that feeding CHO during exercise improves exercise capacity and performance (Coyle et al., 1986; Jeukendrup et al., 1997; Jeukendrup and Jentjens, 2000). Given the rate of glycogen utilization increases as exercise intensity increases (Gollnick et al., 1974) and that elite endurance athletes will typically train and compete at high exercise intensities (>60% of $\dot{V}O_2$ peak), the muscle will predominantly rely on CHO based fuels (muscle and liver glycogen, and blood glucose) to support the high energy demands. As such, muscle glycogen availability has now been identified as a major determinant of endurance exercise capacity, and promoting high CHO diets

prior to, during and after exercise (both in training sessions and competitive situations) is a common foundation on which many traditional nutrition practices are based.

2.4.1 Nutritional guidelines for CHO intake around training

Traditionally, typical nutritional guidelines for daily CHO intake followed a very generalised approach, giving a blanket recommendation aimed at all endurance-based athletes. For example, typical CHO intake recommendations were set at 65-70% of total daily energy from CHO (Americal Dietetic Association, 1993). However, discrepancies between longitudinal data from studies examining the beneficial effects of high CHO intakes highlighted the importance of individualising an athlete's nutrition plan to fit around their daily training sessions rather than adhering to a one size fits all approach. In this regard, the updated guidelines from 2000 then became more specific, but still advocated diets high in daily CHO (American College of Sports Medicine, 2000). These values were now suggested as ball park ranges, and it was noted that the CHO should be spread across the day to promote CHO availability during key training sessions or competition (Burke et al., 2007). Current recommendations for CHO intake to support endurance exercise training are outlined in reviews from Thomas et al. (2016), and Stellingwerff et al. (2018), suggesting a more individual and periodized approach to CHO intake. Since an athletes training load will vary day by day and week by week across specific training phases, it is important that CHO intake is also varied in order to support this (Stellingwerff *et al.*, 2018). In this way, athletes are able to manipulate their overall energy intake according to their individual goals (e.g. reductions in body fat/body mass and/or increases in lean mass) at certain strategic points in a training plan while minimising the effects this may have on training or competition performance.

2.4.2 CHO loading and Muscle Glycogen Super-compensation

CHO feeding before endurance based exercise becomes particularly important where the recovery time between training sessions or competitive situations is short, and as such it is important for athletes to have strategies in place whereby muscle glycogen storage is enhanced. Over the last 40 years of research, numerous studies have confirmed that high pre-exercise muscle glycogen stores can improve performance in those instances where exercise duration is > 60-90 mins (Hawley *et al.*, 1997). Typically, endurance trained individuals will have a normal resting muscle glycogen content of ~120 mmol/kg of muscle wet weight (w.w) (Bergstrom et al., 1972). In the 1980's, Sherman et al. (1981) observed that by undertaking exhaustive and thus glycogen depleting exercise followed by 24-48-hours of a high 'CHO loading' diet (>8g/kg BM), the muscle glycogen content of endurance athletes could be 'super-compensated' reaching up to ~200 mmol/kg w.w. As such, in terms of practical application, elite endurance athletes are now advised that dietary intakes of 8-12g/kg per day are required in the 24-36h prior to competition in order to augment muscle glycogen storage in both type I and type II fibres (Burke *et al.*, 2011). Indeed, Hawley et al. (1997) cited that CHO loading can increase exercise capacity by approximately 20%, and improve time trial performance by 2-3%. This enhanced performance effect observed with CHO loading is likely mediated initially by a delay in the time-point at which energy availability becomes limiting to the maintenance of the desired workload (Leckey *et al.*, 2016). It is now also apparent that as well as providing substrate availability for ATP production, muscle glycogen availability can directly modulate contractile function (Ortenblad *et al.*, 2011, 2013). Indeed, studies have collectively shown a preferential utilization of the intramyofibrillar storage pool during exercise in a manner correlating with impaired Ca^{2+} release from the

sarcoplasmic reticulum (Ortenblad *et al.,* 2011, 2013; Gejl *et al.,* 2014). This is likely of particular importance to endurance athletes during the stages of competition where high power outputs or sprint finishes are required.

2.4.3 Pre-Exercise CHO availability

The ingestion of CHO prior to and during endurance exercise has been studied extensively using both running and cycling modalities, resulting in both traditional and current nutritional recommendations advocating CHO consumption before and during both training and competition (Thomas *et al.,* 2016). Perhaps the first researchers to identify the need for CHO feeding prior to competitive performance were Christensen and Hansen in the 1930s. These authors demonstrated that performance improved when participants were fed a high CHO diet prior to exercise, whereas endurance capacity became impaired when CHO was restricted (Christensen and Hansen, 1939). Even without a CHO 'loading' phase in the lead up, performance has still been shown to improve when CHO is fed in the 1-4h prior to the onset of exercise (Neufer *et al.,* 1987; Sherman *et al.,* 1989; Wright *et al.,* 1991; Sherman *et al.,* 1991). Sherman and colleagues (1991) observed an improvement in time-trial performance following 90-mins of steady state exercise at 70% of $\dot{V}O_{2max}$ when 150-g of CHO was consumed pre-exercise in comparison to both 75-g of CHO or no meal. This improvement was associated with a maintenance in blood glucose concentrations in the later stages of exercise. These findings suggest that pre-exercise CHO feeding is beneficial not only for further increases in muscle glycogen content (Wee *et al.,* 2004), but also in the restoration of liver glycogen content which has been shown to be related to exercise capacity (Casey *et al.,* 2000). As such, the current guidelines for CHO ingestion pre-exercise advise an intake of 1-4g/kg body mass in the 3-4-h before training / competition (Thomas *et al.,* 2016). When considering total CHO

intake for a given day, current guidelines suggest a periodized approach to fuelling for training and competition, with recommendations to support total energy needs of a session, and specific training requirements on a day by day basis. As such CHO intake ranging from 3-12g/kg is recommended prior to training/competition depending on the specific session due to be undertaken (Thomas *et al.,* 2016). These guidelines also suggest manipulation of the timing of CHO ingestion through the day in order to promote CHO availability for a specific session or recovery from a previous session.

2.4.4 CHO Feeding During Exercise

In addition to the high endogenous pre-exercise muscle glycogen stores, it is now common practice for endurance athletes to consume CHO during exercise in order to maximise exogenous CHO and thus improve physical, cognitive and technical elements of performance (Stellingwerff and Cox, 2014). When taken together, numerous studies have demonstrated an ergogenic effect of CHO feeding during prolonged moderate to high intensity exercise (Bergstrom and Hultman, 1967; Coyle *et al.,* 1986; Sherman *et al.,* 1991). In a performance based study, Coyle et al. (1986) demonstrated that the ingestion of glucose (1.8g / min) increased exercise time to exhaustion from ~3h to ~4h during cycling based exercise undertaken at 71% of $\dot{V}O_{2max.}$ Similarly, when participants were required to perform 15-mins of maximal cycling immediately following the completion of 45-mins cycling at 77% of $\dot{V}O_{2max,}$ maximal performance was significantly improved following the consumption of 45g of CHO when compared to a placebo (Neufer et al., 1987). Data also demonstrate that when CHO is ingested in addition to a pre-exercise meal there is a further improvement in performance compared to consumption of a pre-exercise meal alone (Wright *et al.,* 1991).

Given the well documented observations that fatigue coincides with muscle glycogen depletion (Bergstrom *et al.,* 1967), exogenous CHO availability during exercise has been shown to attenuate the rate of glycogenolysis both in muscle and liver (Bjorkman *et al.,* 1984; Tsintzas *et al.,* 1998). As such it is currently thought that by feeding CHO during competition or training sessions, exogenous CHO levels will be increased and utilised, thus reducing fatigue and augmenting performance through a 'sparing' effect on both muscle (Stellingwerff *et al.,* 2007) and liver (Gonzalez *et al.,* 2015) glycogen stores. Indeed, early studies in this area focussed on increasing the oxidation of blood glucose through increased exogenous CHO availability from pre exercise feeding. In the 1980's Coyle and colleagues demonstrated that CHO oxidation rates were significantly reduced at the point of fatigue following high intensity cycling exercise under a control condition (Coyle *et al*., 1986) In contrast, when participants were fed with high CHO, the rate of CHO oxidation was maintained even when muscle glycogen levels were low, highlighting the importance of exogenous CHO as a fuel source and CHO feeding during exercise (Coyle *et al.,* 1986). The conventional approach to CHO fuelling during exercise is to consume 6-8% CHO beverages, however relying solely on this approach does not allow for flexibility in relation to individual variations (i.e. body mass or actual fluid requirements in various ambient conditions (Lee *et al.,* 2014). As such, many athletes rely on a CHO fuelling approach that is based on a combination of solids (e.g. bars), semi-solids (e.g. gels), and fluids (e.g. sports drinks) so as to collectively meet their personalised exogenous CHO targets – typically in the region of 30-90 g/h depending on the duration of exercise (Thomas *et al.,*2016). Current guidelines now advise different CHO targets (types and timings) during exercise based on the specific session undertaken (e.g. brief exercise, high intensity exercise, stop start exercise etc, Thomas *et al.,* 2016).

While the rate of CHO oxidation during exercise was initially thought to be maximal at ~1g/min due to the saturation of intestinal glucose (SGLT1) transporters (Leijssen *et al.,* 1995), it is now accepted exogenous rates of CHO oxidation can be increased up to ~1.8g/min through the addition of fructose or sucrose to the CHO blend (Jeukendrup, 2014). In this way, CHO can be delivered into the circulation via a different transporter (GLUT-5) increasing the overall total CHO delivery (Ferraris and Diamond, 1997). Indeed, many studies have now demonstrated an improvement in performance following the ingestion of multiple transportable CHOs, with CHO oxidation rates of up to ~1.75g/min (Jeukendrup, 2010). In this regard, it is now suggested that 30-60 g/h of CHO (glucose polymers e.g. maltodextrin) is consumed during events lasting <60-90-mins whereas in events >2-3-h, 60-90 g/h (glucose/fructose blends) is the recommended rate (Thomas et al., 2016).

2.4.5 CHO Feeding Post-Exercise

In addition to CHO feeding before and during endurance exercise, traditional nutritional guidelines also recommend CHO consumption during the post-exercise recovery period in order to refuel and replenish muscle glycogen stores. It was initially thought that the optimal amount of CHO required to facilitate maximal rates of glycogen re-synthesis post-exercise was ~0.7g/kg as a single bolus feed eliciting ~20mmol/kg dw/h rate re-synthesis over 4h (Ivy *et al.,* 1987). More recent work however now suggests that using smaller but more frequent CHO feeds is more beneficial. Indeed, data from several studies has consistently demonstrated that providing ~1g/kg CHO or greater at 15–60-min intervals in the first 0–5-h post exercise results in re-synthesis rates of 40–50 mmol/kg dw/h (Blom, 1989; Doyle *et al.* 1993; Hickner *et al.* 1997; Tarnopolsky *et al.* 1997; Jentjens *et al.* 2001; van Hall *et al.* 2000, van Loon *et al.,* 2000).

The type of CHO ingested and method of delivery may also play a role in muscle glycogen re-synthesis. CHO of high glycaemic index (GI) has been shown to produce 61% greater rate in glycogen re-synthesis than the provision of low-GI CHO sources (Burke, 1993). This is effect is likely mediated by increased gastric emptying and thus concentrations in the blood available for absorption by skeletal muscle. The provision of high-GI CHO in liquid versus solid form appears to be equivocal in relation to muscle glycogen synthesis (Reed *et al.* 1989). It is also possible that the use of multiple transportable CHO's may be a method to further augment glycogen re-synthesis. However, Wallis *et al.* (2008) found no benefit of addition of fructose to glucose for glycogen re-synthesis following exercise. It should be noted however, that glucose in the glucose-fructose trial was provided at 0.8 g/kg/h, well below the 1.2 g/kg/h typically recommended. Further work by Trommelen *et al.* (2016) examined glucose, glucose-fructose and glucose-sucrose combinations at 1.5 g/kg/h at 30 min intervals for 5-h post-exercise, this study also demonstrated no additive effect of fructose or sucrose over glucose alone at facilitating glycogen synthesis following exercise.

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.

2.5 Training and Nutrient interactions:

2.5.1 Low CHO training

As previously discussed, the principle of promoting high CHO availability before, during, and after exercise is the foundation on which traditional sports nutrition guidelines are based. Although this is essential for promoting competition performance and ensuring adequate recovery, accumulating data now suggest that restricting CHO before, during, and in recovery from endurance-based exercise augments the cell signalling and gene expression associated with oxidative adaptations in human skeletal muscle. Indeed, both acute and training-based studies have collectively observed that the reduction of both endogenous and/or exogenous CHO promotes mitochondrial enzyme activity and protein content, and increases both whole body (Yeo *et al.,* 2008) and intramuscular (Hulston *et al.,* 2010) lipid metabolism. This approach to CHO has now been termed '*train-low, compete-high'*- a model which promotes CHO restricted training for augmenting adaptation, but ensures high CHO availability during competition to promote maximal performance. This method is now becoming increasingly popular among elite athletes and an overview of the different approaches to 'train-low' are detailed below and summarised in Tables 2.1 and 2.2

2.5.1.1 Restricting CHO availability before training

The idea that CHO restriction augments markers of training adaptation first emerged when data demonstrated enhanced expression of genes involved in mitochondrial biogenesis and substrate oxidation following exercise undertaken with reduced muscle glycogen availability. For example, Pilegaard et al. (2002) demonstrated that the acute exercise induced increases in PDK4, UCP3 and CPT1 mRNA expression were all augmented to a greater extent when pre-exercise muscle glycogen levels were low

compared to normal levels. While the findings from this initial study provided mechanistic insight into the muscle adaptive responses associated with low glycogen training, the exercise training protocol employed consisted of one-legged cycling based exercise – a method of training which wouldn't mimic those practices used in an elite training environment, so it is difficult to determine whether these responses would be the same when using different exercise training methodologies. Using a similar idea, both Cluberton et al. (2005) and Civitarese et al. (2005) observed increases in those genes involved in metabolic regulation when commencing exercise following an overnight fast rather than the ingestion of a CHO rich breakfast. Data from the latter study also suggest that the expression of genes involved in the regulation of lipid metabolism was supressed when CHO was fed before, during and after 2-h cycling as opposed to the same exercise undertaken in the fasted state. Following on from these acute findings, in a 6-week training study design van Proeyen et al. (2011) attempted to elucidate whether these responses to a single bout of fasted exercise could be sustained over a longer period. The authors observed augmented citrate synthase (CS) and β-HAD activity when regular 2-h steady state cycling was performed in the fasted state compared to following the consumption of a breakfast. Despite this however, there were still similar performance improvements whether participants had trained in the fasted or the fed state in this particular study, with $\dot{V}O_{2max}$ improvements in both groups, likely due to the participants being untrained. Although the investigators provided some of the food to be consumed throughout the trial period, it is unclear whether all subjects were compliant to these diets. Additionally, they were only instructed on what to eat at the weekends so it is possible that participants own food choices may have effected the training protocol employed.

2.5.1.2 Restricting CHO during recovery

In addition to restricting CHO prior to endurance exercise training, data also demonstrate beneficial adaptive responses when restricting CHO during the post-exercise recovery period. Indeed, Pilegaard et al. (2005) explored this concept with participants completing 75-mins of cycling at 75% $\dot{V}O_{2max}$ followed by the consumption of an isocaloric diet either high (5g/kg body weight per 8 hour of the trial period) or low (0.5g/kg body weight per 8 hours of the trial period) in CHO for the next 24-h. Their data showed that although the mRNA expression of PDK4, LPL, UCP3, and CPT1 increased in response to exercise, activation was only sustained in the low CHO group following the 24-h. Furthermore, plasma FFA availability was significantly higher in the low CHO group compared to high during the 24-h recovery period. In addition to these findings, in a twice per day 6-week training study, it has also been observed that when glucose is consumed during recovery from the first session, the enhanced oxidative adaptations observed are actually blunted compared to when CHO is restricted despite reduced levels of muscle glycogen (Morton *et al.*, 2009). When taken together, responses from these studies suggest that reducing exogenous glucose availability also modulates the muscle adaptive process. Interestingly, data from Jensen et al. (2015) conflict these earlier findings, suggesting that when using trained athletes (average $\dot{V}O_{2max}$ ~66ml.kg.min), there is no beneficial effect of CHO restriction during recovery. Indeed, these authors observed that when participants cycled for 4-h at ~56% $\dot{V}O_{2max}$ there were no differences in the mRNA expression of key mitochondrial genes at 4-h or 24-h post-exercise whether CHO or water only was consumed during recovery. These findings suggest that more highly trained participants are required in order to translate the findings to elite athlete populations.

2.5.1.3 Twice per day training

On the basis of the molecular evidence derived from acute studies, Hansen and colleagues (2005) were the first to investigate the idea that repeated exercise (i.e. exercise training) with reduced CHO availability augments oxidative adaptations and subsequent endurance performance. In a 10-week long training study using single leg knee extensor exercise and training 5 days per week, participants either trained one limb every day with normal levels of muscle glycogen, or the contralateral limb twice every second day whereby the second session was undertaken with reduced muscle glycogen availability. Exercise during the twice per day sessions was interspersed with 2-h of recovery, during which time no CHO was consumed. In this way, both limbs performed identical work but one limb performed 50% of these sessions with low muscle glycogen availability. The authors observed greater increases in CS activity in the limb which had undertaken training with lower levels of muscle glycogen compared to normal. Additionally, greater improvements in exercise capacity were observed in the 'low' limb compared to normal, suggesting that repeatedly training in this way may lead to performance gains in the long term. Although these authors were the first to demonstrate the performance improvements of low CHO training over a longer period, the chosen exercise protocol of single leg knee extensor exercise does not translate to real-world endurance training practices. In addition, the participants recruited in this study were untrained, so it is unknown whether the same responses would be present in recreational or well-trained athlete populations.

Yeo and colleagues (2008) subsequently explored this idea using a more practical 'real world' design more applicable to elite athletes. Using well trained male cyclists in a 3-week training block, cyclists trained 6 times per week, again either once every day

with high muscle glycogen availability in one group, or twice every other day so the second session was undertaken with reduced levels of muscle glycogen in the other group. In the 'high' group cyclists alternated between steady-state and HIT exercise each day, whereas in the 'low' group, steady-state exercise was performed in the morning and HIT exercise performed after a 1-2-h recovery period during which time CHO was restricted. Before and after this training block, muscle biopsies were obtained to assess markers of adaptation, and a time-trial was completed to examine performance improvements in each group. Despite more significant increases in CS activity, β-HAD activity, COXIV and rates of fat oxidation in the 'low' group following training, time trial performance was still similar in both groups. Interestingly, the more significant adaptive responses still occurred in the 'low' group despite cyclists having to reduce exercise intensity during HIT training session. These findings suggest that even when overall training volume is reduced, low levels of CHO are still superior for inducing an adaptive response. In a similar study design, Hulston and colleagues (2010) also reported that despite greater increases in lipid oxidation and the expression of CD36 and β-HAD following training low, time-trial performance was still similar to the 'high' group.

2.5.1.4 Sleep-low / train-low

More recent train-low investigations have adopted a 'sleep-low/ train-low' approach, whereby participants perform an evening training session and then restrict CHO during the recovery period so they go to sleep with low levels of muscle glycogen. A morning training session is then subsequently performed the following day under levels of low muscle glycogen availability. This method was first examined using whole body exercise by Bartlett et al. (2013), whereby participants were required to perform an acute bout of HIT running under conditions of either high or low CHO

availability. In the low condition, participants had performed glycogen depleting exercise the evening prior to the trial, and CHO was restricted during and in recovery from exercise. The phosphorylation of p53 was significantly higher immediately post and 3-h post exercise in the low compared to the high trial. Additionally, the mRNA expression of PDK4, Tfam, COXIV, and PGC-1α were all significantly greater in the low trial at 3-h post exercise compared to when CHO was consumed before, during, and after exercise. Although this data suggests a beneficial adaptive response to training in this way, the feeding strategy employed simultaneously adopted a CHO and overall calorie restriction protocol rather than an isoenergetic match between the two conditions. Given the similarities in metabolic adaptation to both CHO and calorie restriction, such data raise the question as to whether the enhanced mitochondrial responses observed in the sleep-low train-low group were due to a restriction of CHO per se, or rather a reduction in total calories consumed compared to the high CHO trial. In a subsequent study, a sleep low strategy was employed whereby participants ingested isoenergetic diets containing 8g/kg CHO, but timing of ingestion was altered to elicit sleeping low. They consumed either 8g/kh CHO prior to evening HIT then slept low, or consumed 4g/kg CHO prior to the evening HIT then 4g/kg CHO before bed. The following morning they then completed a 2-h steady state cycling protocol. While fat oxidation and PDK4 mRNA expression were significantly greater following fasted morning exercise, those genes involved in the regulation of mitochondrial biogenesis showed similar exercise induced increases in both groups (Lane *et al.,* 2015). Since the participants in this study were highly trained, they still maintained high levels of muscle glycogen despite 'sleeping low', further highlighting that low muscle glycogen levels are required to elicit these increased responses particularly in more well trained populations. More recent work from Marquet *et al.*

(2016) focussed on incorporating the sleep-low strategy as part of a 3-week training block with elite triathletes. Using a similar CHO feeding approach to Lane and colleagues, these authors observed that when the sleep-low training strategy was employed, 10-km time trial performance was improved significantly compared to when normal levels of CHO were consumed across the training block. When taken together, these findings collectively suggest that the 'sleep low/train low' strategy is effective for not only further up-regulating the muscle adaptive responses to training, but also improving endurance performance.

While the mechanisms underpinning the aforementioned adaptive responses to both acute and chronic exercise are still not fully understood, they are likely mediated by upstream signalling from AMPK and p38MAPK. Indeed, AMPK has the capacity to be modulated by the glycogen status of the muscle through a glycogen binding domain on the β-subunit (McBride *et al*. 2009), with data suggesting that AMPK is more active when glycogen is depleted. Findings from Wojtaszewski *et al*. (2003) show that when pre-exercise muscle glycogen levels are low, AMPKα2 activity and ACC^{Ser221} phosphorylation are significantly elevated following steady state cycling compared to when muscle glycogen is high. In a subsequent study, Chan et al. (2004) also observed a significantly greater nuclear abundance of p38MAPK both pre and post exercise when muscle glycogen levels were low compared to high. In another twice per day train low study, Cochran et al. (2010) also reported significantly greater elevations in p38MAPK following the second exercise session when participants consumed no CHO during recovery. These data are highly suggestive of both AMPK and p38MAPK being nutrient sensitive, and thus likely regulating the downstream events leading to increases in mitochondrial biogenesis.

2.5.1.5 Muscle glycogen threshold

Although it is now accepted that muscle glycogen availability is a potent regulator of the adaptive responses of skeletal muscle to exercise training, the level of absolute glycogen required to augment the pathways regulating mitochondrial biogenesis is currently unknown. However, it appears that a 'glycogen threshold' may exist, whereby a critical absolute level of glycogen must be exceeded in order for significant activation of specific cell signalling pathways to occur (Impey *et al.*, 2018). The majority of studies that adopt a low glycogen model commence exercise with glycogen concentrations between 100-300 mmol/kg dw, where the activity of key cell signalling kinases, transcription factors and transcriptional co-activators and expression of various metabolic genes are augmented when compared with exercise commenced with high (350-900 mmol/kg dw) glycogen. As such, it would appear important that exercise is commenced with muscle glycogen concentrations below 350 mmol/kg dw when undertaking a 'train low' exercise session. Nonetheless, it also appears that significant activation of cell signalling pathways controlling mitochondrial biogenesis can still be achieved with high pre-exercise glycogen concentrations as long as a critical absolute amount of glycogen is exceeded during exercise. Indeed, Impey et al., (2016) recently demonstrated that exhaustive exercise induces significant activation of AMPK and expression of transcription factors (p53, Tfam) and co-activators (PGC-1α), even when commenced with high glycogen levels (600 mmol/kg dw). This is likely due to the fact that subjects surpassed a critical level of glycogen (~350 mmol/kg dw) during exercise and reached exhaustion at very low levels (~100 mmol/kg dw), therefore performing a significant proportion of exercise with low muscle glycogen. Although significant activation of cell signalling cascades appears possible with high pre-exercise glycogen levels, what is clear is that significantly more 'work' is required

to achieve the same signalling effects, whereby commencing exercise with low glycogen induces ‘work efficient’ cell signalling related to mitochondrial biogenesis. For instance, the aforementioned work demonstrates that training with low pre-exercise (~300 mmol/kg dw) glycogen induces a significant activation of AMPK in significantly less time (~60 min) than when training is commenced with high glycogen.

Table 2.1 Acute signalling and metabolic responses to exercise with reduced CHO availability.

Study	Participants	Train low strategy	Exercise protocol	Findings
Pilegaard *et al.* (2002)	6 active males	Fasted	a) Single leg depletion followed by double leg cycling the following day. b) 3-h of 2-legged knee extensor exercise with either normal or low muscle glycogen.	PDK4 increased pre exercise in the low glycogen leg only. PDK4 and UCP3 increased in response to exercise in the low glycogen leg only.
Wojtaszewski *et al.* (2003)	8 trained males	Pre-Exercise CHO restriction	60-min cycling at 70% $\dot{V}O_{2max}$ with either high (HIGH) or low (LOW) pre exercise glycogen levels.	AMPKα2 was activated in LOW only. ACC phosphorylation was greater in LOW compared to HIGH.
Cluberton *et al.* (2005)	6 active males	Fasted	60-min cycling at 75% $\dot{V}O_{2max}$ either CHO fed pre exercise (HIGH) or CHO restricted pre exercise (LOW).	PDK4, GLUT4, and UCP3 all increased in LOW, with no change in HIGH. PGC-1α increased in both conditions.
Civitarese *et al.* (2005)	7 untrained males	Fasted	2-h cycling at 50% PPO either CHO fed pre exercise (HIGH) or CHO restricted pre exercise (LOW).	GLUT4 and PDK4 increased in LOW, with no change in HIGH. HIGH resulted in suppression of AMPK, CD36, CPT1 and UCP3. ACC was unchanged in either condition.

Pilegaard *et al.* (2005)	9 active males	Post-Exercise CHO restriction	75-min cycling at 75% $\dot{V}O_{2max}$ followed by 24-h recovery consuming either LOW CHO or HIGH CHO.	PDK4, UCP3, LPL, and CPT1 all returned to baseline 8-h post-exercise in HIGH, whereas they remained elevated for 24-h in LOW.
Akerstrom *et al.* (2006)	9 active males	Fasted	2-h of one legged knee extensor exercise either CHO fed (HIGH), or fasted (LOW) before exercise.	AMPKα2 activity attenuated in HIGH No effect of CHO on AMPKα1
Steinberg *et al.* (2006)	7 active males	Pre-Exercise CHO restriction	60-min cycling at 70% $\dot{V}O_{2max}$ with either reduced (LOW) or normal (NORM) muscle glycogen availability.	Activation of AMPKα2 and ACC was enhanced in LOW.
Cochran *et al.* (2010)	10 active males	Twice per day	2 bouts of 5 x 4-mins cycling at 90-95% of heart rate reserve, separated by 3-h recovery during which either a high CHO drink (HIGH) or a placebo (LOW) was ingested.	Greater increase in p38MAPK in LOW in afternoon exercise compared to HIGH. PGC-1α and COXIV increased in both conditions post-exercise. AMPK increased post afternoon exercise in both conditions.
Yeo *et al.* (2010)	12 trained cyclists/triathletes	Twice per day	100-min cycling at 70% $\dot{V}O_{2max}$ followed either 2-h or 24-h later with 8 x 5-min cycling at max effort.	Greater increases in AMPK with low CHO. No change in p38MAPK between conditions.

Bartlett *et al.* (2013)	8 active males	Sleep low / train low	6 x 3-min bouts of running at 90% $\dot{V}O_{max}$ interspersed with 1-min at 50% with either high (HIGH) or low (LOW) CHO availability.	Phosphorylation of ACC^{Ser79} and $p53^{Ser15}$ higher in LOW immediately post-exercise and 3-h post-exercise. Pre and 3 h post-exercise PDK4, Tfam, COXIV & PGC-1α mRNA were greater in LOW. PGC-1α expression was increased by exercise independently of CHO.
Jensen *et al.* (2015)	15 triathletes	Post-Exercise CHO restriction	4-h cycling at 56% $\dot{V}O_{2max}$ followed by 4-h recovery consuming either CHO or water only.	mRNA expression of PGC-1a, TFAM, NRF-1, COX-IV, PPAR-a, and GLUT4 was not different between groups pre or post exercise.
Impey *et al.* (2015)	11 active males	Sleep low / train low	Cycling to exhaustion with either HIGH or LOW CHO availability.	Comparable increases in AMPKα2 activity, PGC-1α, p53, Tfam and SIRT1 in both trials despite significantly lower exercise capacity in LOW.
Lane *et al.* (2015)	7 trained male cyclists	Sleep low / train low	8 x 5-min cycling at 82.5% PPO in the evening followed by 2-h cycling at 65% max power the following morning after either previously consuming CHO (FED) or sleeping without eating (FAST).	PDK4 mRNA elevation greater in FAST. Resting phosphorylation of AMPK, p38MAPK and p-ACC was greater in FAST.

				Fat oxidation during 2-h cyle was higher in FAST. mRNA expression of CPT1, FABP and CD36 was higher in FAST.
Marquet *et al.* (2016)	11 trained male cyclists	Sleep low / train low	8 x 5-min cycling at 85% of max power in the evening followed by 1-h cycling at 65% max power the following morning after either sleeping with low (SL) or normal (CON) CHO availability. Repeated 3 times over a week.	No changes in substrate utilisation in either SL or CON. Mean 20km time trial performance (+3.2%) was improved in SL compared to CON.

Table 2.2 Signalling and metabolic responses to a period of exercise training with reduced CHO availability

Study	Subjects	Training duration	Train low strategy	Exercise protocol	Findings
Hansen *et al.* (2005)	7 untrained males	5 x per week for 10 weeks	Twice per day	Knee extensor exercise with one leg training in a low glycogen state (LOW) every other day and the other in a high glycogen state (HIGH) every day.	Greater increases in CS in LOW. β-HAD increased in LOW only.
De Bock *et al.* (2008)	20 active males	3 x per week for 6 weeks	Fasted	1-2-h cycling at 75% $\dot{V}O_{2max}$ either fasted (LOW) or consuming CHO before (HIGH).	SDH and GLUT4 increased to a similar level in both groups. FABP increased in LOW only.
Yeo *et al.* (2008)	14 trained cyclists/triathletes	4 x per week for 3 weeks	Twice per day	100-min cycling at 70% $\dot{V}O_{2max}$ followed either 2-h later or 24-h later with 8 x 5-min cycling at max power.	CS, β-HAD, and COXIV increased in LOW only. There was no change in PGC-1α in either group.
Morton *et al. (*2009)	30 active males	4 x per week for 6 weeks	Twice per day	5 x 3-min running at 90% $\dot{V}O_{2max}$ interspersed with 6 x 3-mins at 50% with either CHO before and during (Low+GLU),	PGC-1α, HSP70, and HSP60 increased in all conditions. Increases in SDH were greater in Low+placebo

				a placebo before and during, or water only.	compared to Low+GLU and norm.
Hulston *et al.* (2010)	14 trained cyclists	6 x per week for 3 weeks	Twice per day	In the HIGH condition participants exercised each day alternating between HIT (8 x 5-min efforts) and stead state (90-mins at 70% intensity). In LOW HIT was performed in the morning and steady state in the afternoon every other day.	β-HAD increased in the LOW trial only. Increases in GLUT4 were greater in HIGH than LOW.
Van Proeyen *et al.* (2011a)	20 active males	4 x per week for 6 weeks	Fasted	1-1.5-h cycling at 70% $\dot{V}O_{2max}$ either fasted (LOW) or CHO fed before (HIGH).	CS and β-HAD increased in LOW only.
Van Proeyen *et al.* (2011b)	20 active males	4 x per week for 6 weeks	Fasted	60-90-min cycling at 70-85% $\dot{V}O_{2max}$ either fasted (LOW) or CHO fed before and during exercise (HIGH).	GLUT4 and AMPK phosphorylation increased in LOW only. CD36 and CPT1 mRNA post training increased in LOW only. CS increased in both conditions.
Marquet *et al.* (2016)	21 trained male triathletes	3 x per week for 3 weeks	Sleep low	HIT in the evening (either 8 x 5-min cycling at 85% max power or 6 x 5-min running at 10km intensity) followed by low	Significant improvement in cycling efficiency in SL.

		intensity cycling (60-min at 65% max power) the following morning. Participants either slept low (SL) or had a control diet (CON).	Fat mass decreased in SL and 10-km TT performance was improved.

2.5.1.6 Potential negative effects of 'training low'

Despite the clear benefits of the 'train-low' paradigm, there are a number of potential limitations to this type of training which can make it difficult for exercise physiologists and nutritionists to best periodise this type of training in to an elite athletes training schedule. Indeed, reduced CHO availability has been shown to impair acute training intensity in several studies (Yeo *et al.,* 2008; Hulston *et al.,* 2010) which if performed consecutively over time may lead to a de-training effect. Additionally, given the role of CHO in preventing immunosuppression, it is possible that repeated high intensity training under conditions of low CHO may increase susceptibility to illness and infection (Gleeson *et al.,* 2004). Restriction of CHO availability has also been shown to increase muscle protein breakdown (Howarth *et al.,* 2010), so if performed chronically over time may lead to muscle mass loss. Finally, data also demonstrate a reduced ability to oxidise exogenous CHO following regular training with low CHO, which could lead to a negative effect on competition performance (Cox *et al.,* 2010). Taking the above limitations into account, it is apparent that training with low CHO availability should be carefully periodised in an athletes training programme.

2.6 Energy restriction

In addition to role of 'low CHO' training in augmenting markers of training adaptation, it is also possible that many of the muscle adaptive responses associated with training of this type are mediated by overall energy restriction rather than CHO restriction *per se*. Indeed, some of the aforementioned 'train low' studies demonstrating increases in muscle adaptive responses to exercise have adopted a simultaneous reduction in both CHO and overall energy prior to exercise. In this regard, Bartlett and colleagues (2012) adopted a 'sleep-low/ train-low' approach

whereby participants performed a glycogen depleting exercise protocol in the evening in the 'LOW' condition prior to performing an acute bout of HIT running the following morning, where CHO was also restricted before, during, and after exercise. In the 'HIGH' condition however, participants had consumed a diet high in CHO the day before, a high CHO diet on the morning of testing, and were fed with CHO during the HIT running bout. In addition to 3g/kg CHO in the low trial versus 8g/kg CHO in the high trial, the total number of calories consumed in HIGH was more than double that of LOW, suggesting that the increased p53 signalling observed in LOW may be due to the combined reduction in both CHO and energy rather than CHO restriction alone. More recent data in rodent models has examined the effects of alternate day food deprivation versus ad libitum feeding in combination with daily training over 1 month on endurance related adaptive responses and running performance (Marosi *et al.*, 2018). These authors demonstrated that when mice were intermittently deprived of food (i.e. in an energy restricted state) in combination with daily training, their run to exhaustion performance was superior to those mice who had fed ad libitum. Furthermore, cell signalling and gene expression associated with mitochondrial biogenesis was increased to a greater extent in the intermittent food deprivation group compared to ad libitum. These findings suggest that it is the restriction of total energy restriction rather than CHO restriction alone which augment the muscle adaptive responses to endurance exercise training albeit in a rodent model.

In the real-world environments of elite endurance training, athletes will likely train 20-30-h per week, often with multiple training sessions per day, whereby they likely present daily with transient periods of both CHO and calorie restriction, as well as long-term periods of sub-optimal energy availability. Many endurance athletes also practice longer-term periods of energy restriction in an attempt to reduce body mass

and fat mass in preparation for competition (Vogt *et al.*, 2005; Stellingwerff, 2012). Indeed, the performance improvements observed in the 'sleep-low study by Marquet et al, (2016) were also associated with a 1kg reduction in fat mass, thus suggestive of a combined overall CHO and energy restriction rather than just low CHO alone.

2.7 Relative Energy Deficiency in Sport (RED-S)

In addition to its potential effects on the muscle adaptive responses to endurance training, reduced energy availability also has widespread effects on physiological and biological function in elite athletes (Burke *et al.*, 2018), many of which are associated with Relative Energy Deficiency in Sport (RED-S) syndrome (Mountjoy *et al.*, 2014). The underlying problem of RED-S is a decreased energy availability which is inadequate to support the physiological functions involved in health and performance. Energy availability is calculated as energy intake minus the energy cost of exercise relative to fat-free mass (FFM), and in healthy adults a value of 45 kcal/kgFFM/day equates energy balance (Loucks, 2004). When energy availability decreases, physiological functions are compromised in order to reduce the energy they require, thus leading to a disruption in metabolic characteristics (Loucks, 2004). Those aspects that may be negatively affected by reduced energy availability include but are not limited to; bone health, immune function, endocrine function, and metabolic rate (Mountjoy *et al.*, 2014). Although the literature on low energy availability and the symptoms of RED-S mainly focusses on female athletes (Mountjoy *et al.*, 2014), research has shown that a number of male athletes also have severely restricted energy availabilities and low bone mineral density (Nichols *et al.*, 2003; Vogt *et al.*, 2005; Rector *et al.*, 2008) which is most common in endurance athletes.

Athletes participating in endurance sports such as distance running, triathlon, and road cycling often have sustained periods of reduced energy availability resulting from the extreme energy expenditures associated with high volume training and frequent racing (Jeukendrup *et al.,* 2000). Additionally, the culture within many of these sports often promotes extremely low body mass and body fat levels for their beneficial effect on factors such as running economy (Mooses & Hackney, 2017). Indeed, Fudge et al. (2006) demonstrated through the use of doubly labelled water (the gold standard measure of energy expenditure), that elite male Kenyan runners presented with energy availabilities as low as 6 kcal/kgFFM/day. Drenowatz et al. (2012), also demonstrated that endurance athletes ranging from 10 km runners to Ironman distance triathletes present with energy availabilities ranging from 24 kcal/kgFFM/day to 33 kcal/kgFFM/day. As such, where 'train-low' and/or energy restriction may be beneficial for certain aspects of training adaptation, this is not without limitations to other aspects of physiological function.

2.8 High-fat feeding

In addition to CHO manipulation around training, data also suggest a potential modulatory role of fat availability in augmenting the adaptive responses to endurance exercise. Since endogenous CHO stores are limited and can only fuel ~3-h of submaximal exercise, it would be of benefit to elite endurance athletes to utilize an alternative fuel source during exercise in order to 'spare' muscle glycogen (Burke and Hawley, 2002). As previously discussed, endurance trained athletes typically have a greater capacity to utilise fat as a fuel source during exercise (Coyle *et al.*, 1984), and as such, one possible strategy of sparing muscle glycogen is to maximise the contribution of fat as a substrate by following a low CHO, high-fat (LCHF) diet. Indeed, it is well established that both acute and chronic modifications in fat availability alter substrate utilisation both at rest and during exercise (Krogh and Lindhard, 1920; Burke and Hawley, 2002). It is also possible that acute elevations in circulating FFA availability during exercise may regulate key cell signalling kinases and transcription factors that modulate the expression of genes regulating both CHO and lipid metabolism (Zbinden-Foneca *et al*., 2003; Cochran., *et al.*, 2010).

Over the last few decades numerous research studies have examined the effects of *'fat adaptation'* protocols on the adaptive responses to endurance exercise training. Such strategies have included chronic exposure to either keotgenic high-fat (< 20g per day CHO), 80% energy from fat) diets, or restricted CHO, high-fat (15-20% energy from CHO, 60-65% energy from fat) diets, as well as short term fat adaptation. Despite this body of research however, there still remains equivocal evidence that feeding of this type has any actual beneficial effect on performance long term (Burke, 2015). Though it is apparent that LCHF feeding can both increase the rates of lipid oxidation during varying exercise intensities and improve endurance performance in some cases

(Muoio *et al.,* 1994; Lambert *et al.,* 1994), other investigators have observed a negative effect on performance following nutritional manipulation of this type (Havemann *et al.,* 2006; Burke *et al.,* 2017).

2.8.1 Adaptations to high-fat diets

The idea of using LCHF diets for enhancing endurance performance has re-emerged in recent years, however a lot of this newfound interest is based upon claims and testimonials from social media rather than any actual scientific evidence in athletic populations. A summary of this research is discussed below, with further detailed information available in an excellent review from Burke (2015).

Perhaps the first study to examine the effects of LCHF feeding on endurance performance and biochemical adaptation was performed by Dr Stephen Phinney and colleagues in the early 1980's (Phinney *et al.,* 1983a; Phinney *et al.,* 1983b). In this initial study, five well trained cyclists performed both $\dot{V}O_{2max}$ and exercise to exhaustion tests at 62%-64% maximal intensity on two separate occasions. Firstly, following the consumption of a CHO rich diet (two thirds of energy from CHO) for a week, and then again following 4-weeks of an isoenergetic CHO restricted (<20g/day) but high-fat (~85% of energy) diet which induced ketosis. The authors observed that time to exhaustion in the cycling capacity test was unchanged following 4-weeks of LCHF feeding, suggesting that a diet of this type did not compromise aerobic capacity. Furthermore, it was also observed that the contribution of blood glucose was significantly lower, and lipid oxidation significantly higher during exercise following the high-fat diet, suggesting enhanced utilisation of fat and sparing of CHO. Despite the apparent potential benefits of high-fat feeding displayed here however, it was evident that one particular subject was a high responder to LCHF feeding and as such

skewed the data with a large increase in endurance compared to others. In this way, it is possible that individuals respond differently to this type of feeding, and that certain trained athletes possess more metabolic flexibility to tolerate high-fat diets compared to untrained individuals.

Following this early work, researchers from a number of laboratories went on to examine the effects of LCHF diets on metabolic adaptations and exercise performance (summarised in Table 2.3). It is evident from these studies that fat adaptation significantly increases rates of whole-body fat oxidation in well trained subjects, likely due to alterations within the muscle such as substrate availability, transport, and breakdown. Indeed, muscle triglyceride stores and fat oxidation have both been shown to increase at rest and during exercise following 5-days high-fat feeding compared with a high CHO diet (Yeo *et al.,* 2008). The transporter protein FAT/CD36 is key for the movement of FFA's in to skeletal muscle for breakdown both at rest and during exercise. In this regard, 5-days of LCHF feeding in well-trained subjects has been shown to increase both the mRNA and overall protein content of FAT/CD36 (Cameron-Smith *et al.,* 2003). Once transported in to the muscle cells, FFA's then require transport in to the mitochondria via carnitine palmitoyltransferase 1 (CPT1) before they can be broken down for oxidation. In this regard, both 15-days (Goedecke *et al.,* 1999), and 28-days (Fisher *et al.,* 1983) of LCHF feeding have been shown to increase the activity of CPT1. Such adaptations undoubtedly contribute to the enhanced rates of lipid oxidation observed during exercise following 'fat adaptation' protocols and would appear beneficial for endurance athletes.

When examining the potential real-world effects of LCHF feeding on metabolism and endurance performance, a more recent study determined the effects of a ketogenic LCHF diet (< 50g CHO; 78% fat; and 2.1g/kg^{-1} protein per day) over 3-weeks of

intense training in elite race walkers (Burke *et al.*, 2017). This study demonstrated that despite improvements in whole body lipid oxidation rates and peak aerobic capacity after following the LCHF diet, exercise economy and overall race walking performance were negatively impacted by this type of feeding when compared with periodised high CHO availability. These findings suggest that in elite athletes, keotgenic LCHF diets may be detrimental to performance if followed for long periods, and the observed increases in fat oxidation and aerobic capacity do not necessarily translate into any performance gains despite what early research may suggest. In addition, as this study was performed on elite level participants, muscle biopsies were not taken across the 3-week training period, so it is unclear as to what adaptive responses took place at a muscle tissue level, or at what time course any adaptive responses took place.

2.8.2 Mitochondrial biogenesis related adaptations

In addition to the beneficial effects observed on the regulation of fat transport and oxidation, data also suggest that high-fat diets may regulate the upstream cell signalling associated with increases in mitochondrial biogenesis. Indeed, Yeo and colleagues (2008) investigated this idea, whereby trained participants completed 1-h of cycling at 70% $\dot{V}O_{2peak}$ after following either 5-days of LCHF feeding followed by 1-day high CHO feeding, or after 6-days high CHO feeding. Muscle biopsies obtained pre- and post- this exercise bout revealed significant increases in AMPK-α2 activity after the fat adaptation trial compared with the high CHO trial (Yeo *et al.*, 2008a). However, in this study no further measures relating to mitochondrial biogenesis related adaptations were taken, so it is unknown whether the increased AMPK-α2 activity corresponded to any further beneficial increases in downstream signalling in the mitochondrial biogenesis pathway. It is also possible that the elevated circulating

FFAs following high-fat feeding may act as signalling intermediates for p38MAPK when CHO is low. Indeed, Zbinden-Foneca et al. (2003) observed suppressions in p38MAPK during exercise following the pharmacological ablation of FFA availability when compared with control conditions. Additionally, the enhanced p38MAPK phosphorylation observed by Cochran et al. (2010) using a twice per day exercise model was associated with enhanced circulating FFA availability during the afternoon exercise. These findings however were in an active population and employed an acute exercise protocol, so it is unclear if these would translate to a well-trained athlete population over a longer term exercise training programme.

Table 2.3 Muscle adaptive responses and exercise performance responses to LCHF feeding protocols

Study	Participants	High-fat feeding strategy	Adaptive responses	Performance responses
Phinney *et al., (*1983)	5 well-trained male cyclists	1-week eucaloric balanced diet followed by 4-week ketogenic diet (HFAT).	Increased fat oxidation and reductions in CHO oxidation and glycogen utilisation in HFAT.	Cycling time trial time to exhaustion was not different between conditions.
Muoio *et al.,* (1994)	6 well-trained male cyclists	HFAT, HCHO or normal diet for 7-days.	Plasma FFA levels were higher during the HFAT diet.	Running time to exhaustion and $\dot{V}O_{2max}$ were greatest after HFAT compared to HCHO and normal.
Lambert *et al.,* (1994)	5 trained male cyclists	2-weeks of either HFAT or HCHO diet.	Resting muscle glycogen was lower after HFAT. RER and CHO oxidation were lower during exercise after HFAT.	Exercise time to exhaustion at 60% $\dot{V}O_{2max}$ was greater following HFAT. Exercise time to exhaustion at 90% $\dot{V}O_{2max}$ was not different between trials.
Goedecke *et al.,* (1999)	16 endurance-trained male cyclists	15-days of either HFAT or control (normal) diet.	No change in CS or β-HAD activity. Increased fat oxidation during exercise after 5 and 10 days. Reduced glycogen oxidation following HFAT.	No change in 40-km time trial performance following HFAT.
Burke *et al.,* (2000)	8 well-trained male cyclists	Either 6-days HCHO, or 5-days HFAT followed by 1 HCHO.	Increased fat oxidation following HFAT + 1-day HCHO. Muscle glycogen was lower after 5-days HFAT but restored after 1-day HCHO. Muscle glycogen utilization was lower following HFAT + 1-day HCHO.	No difference between trials in cycling time trial performance.

Lambert *et al.,* (2001)	5 endurance-trained male cyclists	10-days either HFAT or control followed by 3-days HCHO feeding.	Mean serum glycerol concentrations were higher in HFAT. Fat oxidation was higher and CHO oxidation lower during exercise following HFAT. Muscle glycogen utilization was lower following HFAT.	20-km time trial performance increased following HFAT.
Rowlands and Hopkins (2002)	7 well-trained male cyclists	14-days of either HFAT, or HCHO diet, or 11.5-days HFAT with 2.5-days CHO loading.	Lipolysis and peak fat oxidation were greater in HFAT.	Both HFAT and HFAT + CHO loading reduced the decline in power observed during 100-km time trial.
Burke *et al.,* (2002)	8 well-trained male cyclists	Either 6-days HCHO, or 5-days HFAT + 1-day HCHO.	Increased fat oxidation and decreased muscle glycogenolysis following HFAT + 1-day HCHO.	No difference between trials in cycling time trial performance.
Cameron-Smith *et al.,* (2003)	14 well-trained male cyclists	5-days either HFAT or HCHO diet.	Greater expression of CD36 and β-HAD following HFAT. Fat oxidation was greater following HFAT.	NA
Havemann *et al.,* (2006)	8 well-trained male cyclists	Either 6-days HCHO, or 5-days HFAT diet followed by 1 CHO loading.	Plasma FFA and fat oxidation were greater following HFAT.	No difference in 100-km time trial performance between trials. Reduced 1-km sprint power output following HFAT.
Stellingwerff *et al.,* (2006)	7 well-trained male cyclists	Either 6-days HCHO or 5-days HFAT + 1-day HCHO.	Fat oxidation was higher following HFAT + 1-day HCHO. Reductions in PDH activity and glycogenolysis following HFAT + 1-day HCHO.	NA
Yeo *et al.,* (2008)	8 trained males	Either 6-days HCHO, or 5-days HFAT + 1-day HCHO.	Whole body fat oxidation was higher and muscle glycogenolysis lower	NA

			following HFAT + 1-day HCHO. Resting AMPKα-1 and α-2 was higher following HFAT + 1-day HCHO. HFAT attenuated increases in AMPK during exercise compared to HCHO.	
Zajac *et al.,* (2014)	8 trained male cyclists	4-weeks following either HFAT diet or a mixed diet.	Resting plasma FFA levels and fat oxidation were higher following HFAT.	$\dot{V}O_{2max}$ and lactate threshold were higher following HFAT. Max workload and workload at lactate threshold were higher following the mixed diet.
Webster *et al.,* (2016)	7 well-trained male cyclists	At least 8-months following either a HFAT diet or a mixed diet.	Rates of endogenous glucose production and glycogenolysis were significantly lower at rest and during 2-h cycling at 72% $\dot{V}O_{2max}$ following HFAT. Resting muscle glycogen was lower following HFAT. Fat oxidation during exercise was higher following HFAT.	NA
Volek *et al.,* (2016)	20 elite ultra-marathon runners and iron man triathletes	Either HCHO or HFAT for an average of 20-months (range from 9-36 months).	Fat oxidation was higher in the HFAT group. No differences in resting muscle glycogen between groups.	NA
Burke *et al.,* (2017)	21 elite male race walkers	3-weeks either HCHO, periodised CHO (PCHO) or HFAT.	Whole body fat oxidation was higher following HFAT during exercise.	Peak aerobic capacity increased in all conditions due to training. Exercise economy was lower in HFAT .

2.8.3 Fat adaptation and CHO restoration

Despite elevating the oxidation of fat during exercise, it is important to note that LCHF feeding also results in a reduction in muscle glycogen content, which as previously discussed will ultimately limit high intensity exercise performance and contribute to fatigue (Bergstrom *et al.*, 1967). Since initial research demonstrated no clear benefits of LCHF diets on exercise performance, there has also been focus on a dietary periodization strategy whereby short term LCHF feeding (~5-12 days) is followed by CHO loading or '*CHO restoration'* for 1-3 days (Havemann *et al.*, 2006; Burke *et al.*, 2000; Carey *et al.*, 2001; Stellingwerff *et al.*, 2006). Such strategies aimed to promote the increase in fat as a fuel source during exercise, while also promoting glycogen resynthesis and exogenous CHO availability to fuel high intensity exercise performance. In this way the ideal scenario for maximising endurance capacity would be present, whereby the short CHO restoration phase would be sufficient to resynthesize muscle glycogen content, but the elevated fat oxidation responses to high fat feeding would still persist (Burke, *et al.*, 2000).

This particular strategy was investigated by Havemann and colleagues (2006), whereby well-trained cyclists completed a real-life time trial simulation protocol containing high intensity sprint efforts. These subjects consumed either a 6-day LCHF diet followed by one day of high CHO feeding (or 'CHO restoration') or alternatively 7-days of a high CHO diet according to traditional textbook guidelines, before completing the time trial test protocol. Results demonstrated that although there was no significant difference in overall time between trials (high CHO times were ~3-min 44-s faster), the cyclists high intensity sprint ability (at >90% peak power) was impaired following the LCHF feeding strategy in all subjects. This was also associated with increased muscle recruitment, heart rate, and perception of effort. Further

findings have collectively demonstrated that while fat oxidation still remains elevated following LCHF feeding and CHO restoration (Burke *et al.*, 2000; Burke *et al.,* 2002; Stellingwerff *et al.,* 2006), muscle glycogen utilisation and CHO oxidation are reduced during subsequent exercise despite having been restored to a high level (Burke *et al.,* 2000; Lambert *et al.,* 2001).

2.8.4 Potential negative effects of LCHF feeding

Despite some of the aforementioned beneficial responses to LCHF diets, it is also noteworthy that feeding of this type may come with limitations. It is evident that where initial research examining the effects of LCHF diets has focussed on the potential 'sparing' of muscle glycogen during endurance exercise, it may in-fact have the opposite effect and actually impair glycogen utilisation during exercise even following a CHO restoration phase. This would provide explanation for the impaired high intensity exercise performance observed in some of the previous research studies in response to LCHF feeding. As such, in an attempt to identify the potential mechanisms behind these findings, Stellingwerff et al. (2006) conducted a study whereby well-trained cyclists followed either 5-days of fat adaptation followed by 1-day CHO restoration or 6 days of a high CHO diet. These authors observed a reduction 1-km sprint power output, along with a reduction in glycogenolysis, and a significant reduction in pyruvate dehydrogenase (PDH) activity both at rest and during exercise following 5-days of LCHF feeding compared to the high CHO diet. It is likely that the down-regulation of the PDH enzyme subsequently inhibits the entry of CHO in to the Krebs cycle. Indeed, Peters et al. (2001) had previously observed increases in PDH kinase (the enzyme responsible for inhibiting the PDH complex) activity following 3-days LCHF feeding compared to a standardized high CHO diet. In addition, a more recent study in well-trained cyclists examined the effects of ~8-months LCHF feeding

on endogenous glucose production and hepatic glycogenolysis. These authors observed significantly lower rates of glucose production and glycogenolysis both at rest and during 2-h of cycling at 75% $\dot{V}O_{2max}$ (Webster *et al.*, 2016). When taken together, these findings suggest that rather than preparing elite athletes for competition, high fat feeding may actually impair the capacity to utilise CHO during high intensity exercise thus impairing performance. Moreover, when considering the turnover of myofibrillar proteins, it is apparent that high circulating FFA availability may actually impair MPS. In this regard, recent data display a reduction in mixed muscle fractional synthesis rate following 7-h of lipid and heparin infusion, despite the intake of high quality protein (Stephens *et al.*, 2015). Furthermore, data in rodents showed a significant reduction in the phosphorylation of p70S6K1 following 5-weeks of a high fat diet compared to control conditions (Kimball *et al.*, 2015). It is also noteworthy that both 6-weeks and 20-weeks of LCHF feeding in mice significantly increased markers of the unfolded protein response, likely decreasing mTORC1 activity (Deldicque *et al.*, 2010).

2.9 Summary

While high CHO intakes are still essential for competitive endurance performance, there is now a body of knowledge to suggest that strategically restricting CHO and/or energy availability around training sessions activates the acute cell signalling pathways that increase many of the hallmark muscle adaptations to endurance exercise training such as mitochondrial biogenesis and lipid oxidation. It is also apparent that the availability of fat may play a role in further upregulating some of these muscle adaptive responses to exercise when CHO is low. To date however, it is apparent that research investigating this type of feeding strategy (i.e. low CHO/energy but high fat availability) in well-trained participants (particularly in running populations) exercising at intensities corresponding to real-world training and competition demands still remains very limited. Accordingly, with the growing interest and popularity in high-fat diets for endurance exercise, there is still a need to better understand the adaptive responses to this type of feeding when under conditions of low CHO and/or low energy availability, from both a mitochondrial biogenesis and MPS perspective. The effects of repeated training sessions commenced with low CHO and/or energy availability (as is often common practice in elite endurance populations) on some of the symptoms associated with RED-S also warrants further investigation. As such, the chapters in this thesis attempt to expand on the mechanistic and practical understanding of macronutrient and energy availability for augmenting training adaptation.

Chapter 3

General Methodology

3.1 Location of testing and ethical approval

All exercise testing took place in the Physiology Research laboratories in the Research Institute for Sport and Exercise Sciences. Analysis of skeletal muscle cell signalling activity took place at the University of Sterling, and analysis of bone turnover markers took place at the University of East Anglia. All other biochemical analyses were conducted at the Research Institute for Sport and Exercise Science. All experimental procedures were approved by the Research Ethics committee at Liverpool John Moores University.

3.2 Subjects

All of the subjects involved were recreational male runners recruited from local running clubs and University sports clubs who participated in structured running based exercise at least twice per week. All subjects gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. None of the subjects had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment over the course of the testing period. Subjects were instructed to refrain from any strenuous physical activity, alcohol and caffeine consumption in the 48-h prior to each experimental trial. Subject characteristics are displayed in Table 3.1 below.

Table 3.1 Subject characteristics

	Study 1 (n = 10)	**Study 2a (n = 8)**	**Study 2b (n = 9)**
Age (years)	24 ± 1.5	21 ± 1.9	22 ± 2.1
Height (cm)	177.3 ± 7.2	177.6 ± 3.8	175.9 ± 6
Body mass (kg)	75.9 ± 6	71.4 ± 7	71 ± 6.7
$\dot{V}O_{2peak}$**(ml.kg^{-1}.min^{-1})**	60 ± 3.6	56 ± 5.1	57 ± 4.6

3.3 Anthropometry

Upon their first visit to the laboratories, subjects height was measured using a stadiometer (SECA, Birmingham UK). Nude body mass (SECA, Hamburg, Germany) was recorded upon each subjects first visit to the laboratory, then again during each visit thereafter.

3.4 Physiological measures

3.4.1 Heart rate

During all exercise testing, subjects were fitted with a short-range radio telemetry system (Polar, Kempele, Finland) to monitor their heart rate.

3.4.2 Assessment of respiratory gases

During exercise trials, expired oxygen and carbon dioxide was collected and analysed at regular intervals via breath-by-breath gas measurements obtained through a mouth-piece connected to a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US).

3.4.3 Assessment of substrate utilisation during exercise

Carbohydrate and lipid oxidation rates during exercise were calculated via indirect calorimetry according to the Jeukendrup and Wallis equations assuming negligible contribution of protein oxidation (Jeukendrup and Wallis 2005).

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

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

3.4.4 Assessment of peak oxygen uptake ($\dot{V}O_{2peak}$)

For assessment of $\dot{V}O_{2peak,}$ all subjects performed a maximal incremental running test to volitional fatigue on a motorised treadmill which attached to a harness (h/p/Cosmos, Nussdorf-Traunstein, Germany). Following a 10 minute warm up at a self-selected treadmill speed the maximal incremental test commenced, beginning with a 2-min stage at a treadmill speed of 10km/h. Running speed was then increased by 2km/h every 2-min until a speed of 16km/h was reached, after which the treadmill inclined by 2% every 2-min until volitional exhaustion. $\dot{V}O_{2peak}$ was defined as the highest $\dot{V}O_2$ value obtained during any 10-s period and was stated as being achieved by two of the following criteria: 1) heart rate was within 10 beats.min^{-1} (bpm) of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. In an attempt to accurately match subjects individual running speeds to the respective $\dot{V}O_2$ percentages in subsequent experimental trials, a further running economy test was subsequently completed. Following a warm up, this

test began with a 5-min stage at a treadmill speed of 8km/h with 1% incline and speed was then increased by 1km/h every 5-mins thereafter . The test was stopped when >90% of the previously determined $\dot{V}O_{2peak}$ was reached. These measurements were recorded via breath-by-breath gas measurements obtained continuously throughout both tests using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US), with heart rate (Polar, Kempele, Finland) also recorded continuously during exercise.

3.5 High-Intensity interval (HIT) protocol

The HIT protocol performed by participants in Chapter 4 and Chapter 5 (Study 2a and Study 2b) consisted of 8 x 5-min bouts running at a velocity corresponding to 85% $\dot{V}O_{2peak}$ interspersed with 1-min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm up and cool down at a velocity corresponding to 50% $\dot{V}O_{2peak.}$ This running protocol was developed based on previous work from our laboratory (Bartlett *et al.*, 2013), and to mimic the real-world training practices of elite endurance athletes based on observations from practitioners working in the English Institute of Sport.

3.6 Steady state (SS) protocol performed in Study 1 (Chapter 4)

After a 5-min warm up at a self-selected treadmill speed, participants subsequently commenced a 60-min steady state running (SS) protocol at a velocity corresponding to 70% $\dot{V}O_{2peak}$

3.7 Perceptual measures

3.7.1 Ratings of perceived exertion

All subjects reported their rating of perceived exertion (RPE) at regular intervals during exercise according to a 15-point scale (Borg 1970), as displayed in Table 3.2.

Table 3.2 Borg scale for ratings of perceived exertion during exercise.

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

3.8 Blood sampling and analysis

All blood samples were drawn from a superficial vein in the anticubital crease of the forearm using a standard venepuncture technique. Samples were collected into vacutainers (BD Biosciences, UK) containing serum, EDTA or lithium heparin and stored on ice or at room temperature for serum tubes until centrifugation at 1500g for 15-mins at 4ºC. Following centrifugation, aliquots of serum were stored in a freezer at -80ºC for subsequent analysis.

3.8.1 Analysis of circulating metabolites and hormones

Plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol, and β-hydroxybutyrate were analysed with a Randox Daytona spectrophotometer using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, Co. Antrim, UK) as per the manufacturers' instructions. The coefficient of variation for plasma glucose, lactate, NEFA and glycerol in our laboratory is ≤ 5 %. Serum samples were also analysed using commercially available solid phase enzyme-linked immunosorbent assays (ELISA) for insulin (DIAsource, Belguim), leptin (R&D systems, Minnesota, USA) total ghrelin (Invitrogen, Thermo Fisher Scientific, UK) and adiponectin (Abcam, Cambridge, UK) as per the manufacturers instructions.

3.8.2 Analysis of bone turnover markers

Plasma β-CTX and P1NP were measured by ECLIA on a fully automated COBAS c501 system (Roche Diagnostics, Mannheim, Germany). The inter-assay CV for β-CTX was ≤3% between 0.2 – 1.5 μg.L-1, with a sensitivity of 0.01 μg.L-1, and the

inter-assay CV for P1NP was ≤3% between 20 – 600 µg.L-1, with a sensitivity of 8 µg.L-1. The assays used a sandwich test principle involving two incubations.

3.8.3 Analysis of inflammatory cytokines using Flow Cytometry

Flow cytometric detection of phosphoproteins was performed using a dual-laser BD C6 accuri flow cytometer (BD biosciences, USA). Phospho-p38 concentrations were measured using a cytometric bead array (CBA, BD Biosciences, San Diego, USA) according to the manufacturers instructions. Bead populations with distinct fluorescence intensities coated with phycoerythrin (PE)-conjugated capture antibodies specific for phospho-p38 were used. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BDA CBA Analysis Software (Hungary Software Ltd., for BD Biosciences, San Jose, CA, USA) where median values were used. Briefly, muscle samples (~20 mg) were homogenised in 200µl of denaturation buffer (BD biosciences, UK) in beaded tubes (Roche, UK) using a Roche MagnaLyser instrument. Samples were homogenized for 45 seconds at 6000 rpm x 3 with 5-mins on ice between runs. Protein concentrations were determined using a BCA protein assay, and samples added to the assay diluent provided (100µg/sample). Standards were prepared by serial dilution of a stock protein in the BD CBA Cell Signalling Flex Set. All samples and standards were incubated with the capture beads conjugated to the p38MAPK $^{T180/Y182}$ antibody for 3-h then with the Phycoerythrin (PE) detection reagent for 1-h. Samples were then washed using the wash buffer provided and centrifuged at 300g for 5-mins before supernatant was removed and 300µl of wash buffer was added for re-suspension of the capture beads. All samples were analysed on the flow cytometer with 300 events captured per sample.

3.9 Muscle biopsies

Muscle biopsy samples (~50 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge x 10 cm length, (Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained from separate incision sites 2-3 cm apart under local anaesthesia (0.5% Marcaine), immediately frozen in liquid nitrogen and stored at -80ºC for later analysis. An example is displayed in Figure 3.1.

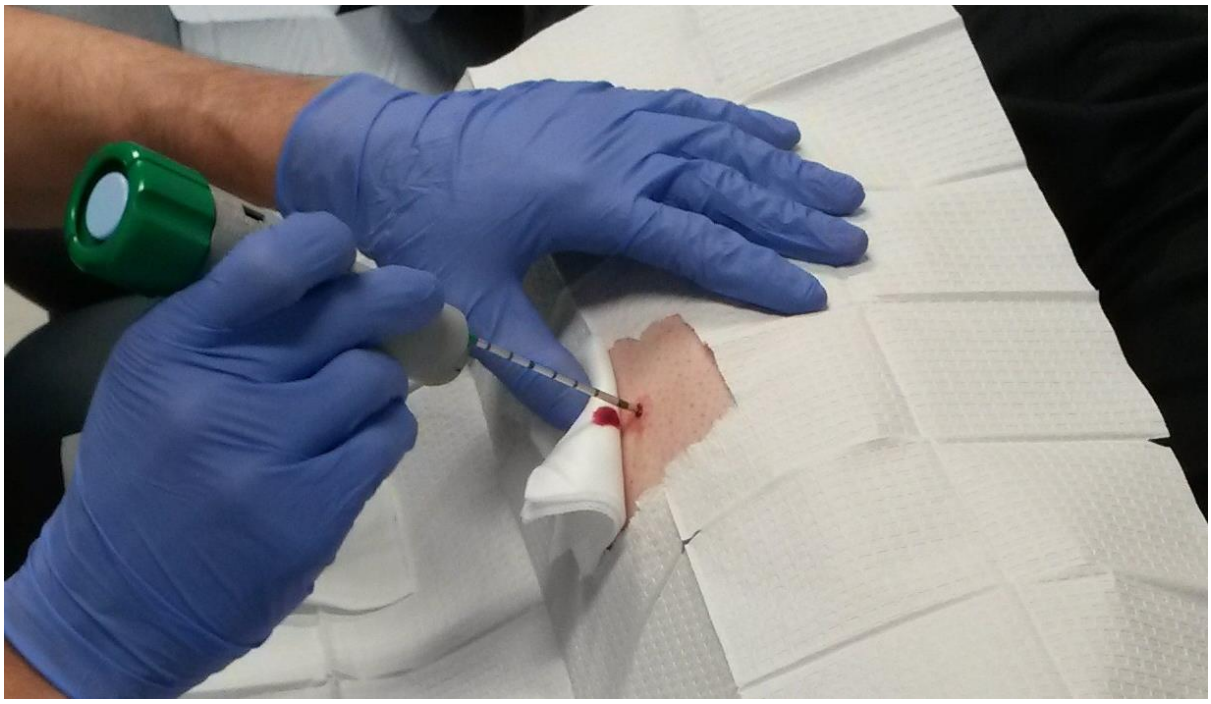

Figure 3.1 Photograph of a muscle biopsy being taken.

3.9.1 Analysis of muscle glycogen concentration

Muscle glycogen concentration was determined according to the methods described by van Loon et al (37). Approximately 3-5 mg of freeze dried muscle was powdered and all visible blood and connective tissue removed. The freeze dried sample was then hydrolysed by incubation in 500 µl of 1M HCl for 3 hours at 100˚C. After cooling to room temperature for ~20-min, samples were neutralized by the addition of 250µl 0.12 mol.L^{-1} Tris/2.1 mol.L^{-1} KOH saturated with KCl. Following centrifugation at 1500 RCF for 10-mins at 4ºC, 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 were <5%.

3.9.2 RNA isolation and analysis

Muscle biopsy samples (~20 mg) were homogenized in 1ml TRIzol reagent (Thermo Fisher Scientific, UK) and total RNA isolated according to manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Rosklide, Denmark). 70 ng RNA was then used for each PCR reaction. Samples were ran in duplicate.

3.9.3 Analysis of mRNA expression in skeletal muscle using Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR)

rt-qRT-PCR amplifications were performed using a QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene

software (Hercules, CA, USA). The following rt-qTR-PCR cycling parameters were used: hold 50°C for 10 min (reverse transcription/cDNA synthesis), initial denaturation and transcriptase inactivation at 95°C for 5 min, followed by PCR steps: 40 cycles of denaturation at 95°C for 10s, and annealing/extension at 60°C for 30s. Upon completion, dissociation/melting curve analysis were performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt analysis presented single reproducible peaks for each target gene suggesting amplification of a single product). Changes in mRNA content were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (Schmittgen and Livak, 2008) where relative gene expression was calculated as $2^{-\Delta\Delta ct}$ and where C_t represents the threshold cycle. GAPDH was used as a reference gene and did not change significantly between groups or time points. mRNA expression for all target genes was calculated relative to GAPDH for each subject, and the calibrator condition in the delta delta C_t equation was assigned to the pre-exercise condition.

Identification of primer sequences was enabled by Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and primers designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Specificity was ensured using sequence homology searches so the primers only matched the experimental gene with no unintended targets identified for primer sequences. In order to prevent amplification of gDNA, primers were ideally designed to yield products spanning exon-exon boundaries. 3 or more GC bases in the last 5 bases at the 3 end, and secondary structure interactions (hairpins, self-dimer and cross dimer) within the primers were avoided so there would be no non-specific amplification. All primers were between 16 and 25bp, and amplified a product between 141 – 244bp. All primers

were purchased from Sigma (Suffolk, UK) and sequences for each gene are shown in Table 3.3.

Table 3.3 Primer designs

Gene	Forward primer	Reverse primer
GAPDH	AAGACCTTGGGCTGGGACTG	TGGCTCGGCTGGCGAC
PGC-1α	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCCTCTCTGCT
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
CPT1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG
FAT/CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGGTTCTTATTCACA
PDK4	TGGTCCAAGATGCCTTTGAGT	GTTGCCCGCATTGCATTCTT
GLUT4	TCTCCAACTGGACGAGCAAC	CAGCAGGAGGACCGCAAATA
Tfam	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA
ERR-α	TGCCAATTCAGACTCTGTGC	CCAGCTTCACCCCATAGAAA
CS	CCTGCCTAATGACCCCATGTT	CATAATACTGGAGCAGCACCCC
PPAR	ATGGAGCAGCCACAGGAGGAAGCC	GCATGAGGCCCCGTCACAGC
SIRT-1	CGGAAACAATACCTCCACCT	CACATGAAACAGACACCCCA
Parkin	TCCCAGTGGAGGTCGATTCT	GGAACCCCCTGTCGCTTAG

3.9.4 [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.

3.9.5 Analysis of phosphorylated p38 MAPK $^{T180/Y182}$ phosphorylation using Flow Cytometry

Flow cytometric detection of phosphoproteins was performed using a dual-laser BD C6 accuri flow cytometer (BD biosciences, USA). Phospho-p38 concentrations were measured using a cytometric bead array (CBA, BD Biosciences, San Diego, USA) according to the manufacturers instructions. Bead populations with distinct fluorescence intensities coated with phycoerythrin (PE)-conjugated capture antibodies specific for phospho-p38 were used. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BDA CBA Analysis Software (Hungary Software Ltd., for BD Biosciences, San Jose, CA, USA) where median values were used. Briefly, muscle samples (~20 mg) were homogenised in 200µl of denaturation buffer (BD biosciences, UK) in

beaded tubes (Roche, UK) using a Roche MagnaLyser instrument. Samples were homogenized for 45 seconds at 6000 rpm x 3 with 5-mins on ice between runs. Protein concentrations were determined using a BCA protein assay, and samples added to the assay diluent provided (100µg/sample). Standards were prepared by serial dilution of a stock protein in the BD CBA Cell Signalling Flex Set. All samples and standards were incubated with the capture beads conjugated to the p38MAPK $^{T180/Y182}$ antibody for 3-h then with the Phycoerythrin (PE) detection reagent for 1-h. Samples were then washed using the wash buffer provided and centrifuged at 300g for 5-mins before supernatant was removed and 300µl of wash buffer was added for re-suspension of the capture beads. All samples were analysed on the flow cytometer with 300 events captured per sample.

3.10 Statistical analysis

All data were analysed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA). Metabolic responses (i.e. blood metabolites, muscle glycogen, kinase activity, mRNA data), physiological and perceptual responses (i.e. HR, RPE, and oxidation rates) were analysed using a two-way repeated-measures general linear model, where the within factors were time and condition. Post hoc LSD tests were used where significant main effects and interactions were observed in order to locate specific differences between time points and conditions. All data in text, figures and tables are presented as mean ± SD, with P values ≤ 0.05 indicating statistical significance.

Chapter 4

The effects of post-exercise CHO restriction but high fat feeding on skeletal muscle cell signalling and gene expression associated with the regulation of mitochondrial biogenesis, lipid metabolism, and MPS

This work was presented at ECSS Vienna 2016 and awarded the GSSI Nutrition Award for oral presentations. The full manuscript was published in Medicine & Science in Sport and Exercise in November 2016.

4.1 Abstract

Purpose: To examine the effects of reduced CHO but high post-exercise fat availability on cell signalling and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis (MPS). **Methods:** Ten males completed a twice per day exercise model (3.5 h between sessions) comprising morning high-intensity interval (HIT) (8 x 5-min at 85% $\dot{V}O_{2peak}$) and afternoon steady-state (SS) running (60 min at 70% $\dot{V}O_{2peak}$). In a repeated measures design, runners exercised under different isoenergetic dietary conditions consisting of high CHO (HCHO: 10 CHO, 2.5 Protein and 0.8 Fat $g.kg^{-1}$ per whole trial period) or reduced CHO but high fat availability in the post-exercise recovery periods (HFAT: 2.5 CHO, 2.5 Protein and 3.5 Fat $g.kg^{-1}$ per whole trial period). **Results:** Muscle glycogen was lower ($P<0.05$) at 3 (251 vs 301 $mmol.kg^{-1}dw$) and 15 h (182 vs 312 $mmol.kg^{-1}dw$) post-SS exercise in HFAT compared to HCHO. AMPK-α2 activity was not increased post-SS in either condition ($P=0.41$) though comparable increases (all $P<0.05$) in PGC-1α, p53, CS, Tfam, PPAR and ERRα mRNA were observed in HCHO and HFAT. In contrast, PDK4 ($P=0.003$), CD36 ($P=0.05$) and CPT1 ($P=0.03$) mRNA were greater in HFAT in the recovery period from SS exercise compared with HCHO. p70S6K activity was higher ($P=0.08$) at 3 h post-SS exercise in HCHO versus HFAT (72.7 ± 51.9 vs 44.7 ± 27 $fmol.min^{-1}\,mg^{-1}$). **Conclusion:** Post-exercise high fat feeding does not augment mRNA expression of genes associated with regulatory roles in mitochondrial biogenesis though it does increase lipid gene expression. However, post-exercise high fat feeding supresses p70S6K1 activity thus potentially impairing skeletal muscle remodelling processes.

Keywords: AMPK-α2, PGC-1α, p53, glycogen, mitochondrial biogenesis

4.2 Introduction

Traditional nutritional strategies for endurance athletes have largely focused on ensuring high CHO availability before, during and after each training session (ACSM, 2009). However, accumulating data from our laboratory (Morton, *et al.,* 2009, Bartlett *et al.,* 2013) and others (Hansen *et al.,* 2005, Yeo *et al.,* 2008, Hulston *et al.,* 2010, Cochran *et al.,* 2010, Lane *et al.,* 2015) have demonstrated a potent effect of CHO restriction (the so-called *"train-low"* paradigm) in augmenting the adaptive responses inherent to endurance training. Indeed, reduced CHO availability before (Psilander *et al.,* 2013) during (Akerstrom *et al.,* 2006) and after (Pilegaard *et al.,* 2005) training sessions augments the acute cell signalling pathways and downstream gene expression responses associated with regulating training adaptation. Accordingly, reduced CHO availability during short-term periods of endurance training augments markers of mitochondrial biogenesis (Hansen *et al.,* 2005, Yeo *et al.,* 2008, Morton *et al.,* 2009), increases both whole body (Yeo *et al.,* 2008) and intramuscular lipid metabolism (Hulston *et al.,* 2010) and also improves exercise capacity and performance (Hansen *et al.,* 2005, Cochran *et al.,* 2010, Marquet *et al.,* 2016). 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.

In addition to manipulation of CHO availability, many investigators have also demonstrated a modulatory role of high fat availability in augmenting components of training adaptation (Burke, 2015). For example, the acute elevation in circulating free fatty acid (FFA) availability during exercise regulates key cell signalling kinases and transcription factors and modulate the expression of genes regulating both lipid and CHO metabolism (Zbinden-Foncea *et al.,* 2003, Philp *et al.,* 2013). Additionally, 5-

15 days of high fat feeding increases resting intramuscular triglyceride stores (Yeo *et al.,* 2008a), hormone sensitive lipase (Yeo *et al.,* 2008b), carnitine palmitoyltransferase (CPT1) (Goedecke *et al.,* 1999), adenosine monophosphate activated protein kinase (AMPK)-α2 activity (Yeo *et al.,* 2008a) and protein content of fatty acid translocase (FAT/CD36) (Cameron-Smith *et al.,* 2003). Such adaptations undoubtedly contribute to the enhanced rates of lipid oxidation observed during exercise following "fat adaptation" protocols (Burke, 2015). Taken together, these data suggest carefully chosen periods of reduced CHO but concomitant high fat availability may therefore represent a strategic approach for which to maximise both training-induced skeletal muscle mitochondrial biogenesis and the enhanced capacity to utilise lipid sources as fuels during exercise.

However, such a feeding strategy is not without potential limitations especially if performed on consecutive days. Indeed, reduced CHO availability impairs acute training intensity (Yeo *et al.,* 2008b, Hulston *et al.,* 2010) and five days of high fat feeding reduces pyruvate dehydrogenase (PDH) activity (Stellingwerff *et al.,* 2006), thus potentially leading to a de-training effect, reduced capacity to oxidise CHO and ultimately, impaired competition performance (Yeo *et al.,* 2008b, Hulston *et al.,* 2010). Moreover, although many endurance training-induced skeletal muscle adaptations are regulated at a transcriptional level, the turnover of myofibrillar (i.e. contractile) proteins are largely regulated through the translational machinery and the mechanistic target of rapamycin complex (mTOR) and ribosomal protein S6 kinase 1 (p70S6K) signalling axis (Moore *et al.,* 2014). In this regard, recent data suggests high circulating FFA availability impairs muscle protein synthesis despite the intake of high quality protein, albeit examined via lipid and heparin fusion and euglycemic hyperinsulemic clamp conditions (van Loon *et al.,* 2000).

With this in mind, the aim of the present study was to examine the effects of reduced CHO but high post-exercise fat availability on the activation of key cell signalling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. In accordance with the original train-low investigations (Hansen *et al.*, 2005, Yeo *et al.*, 2008b, Morton *et al.*, 2009, Hulston *et al.*, 2010), we employed a twice per day exercise model whereby trained male runners completed a morning high-intensity interval training session followed by an afternoon training session consisting of steady-state running. Runners completed the exercise protocols under two different dietary conditions (both energy and protein matched) consisting of high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best practice nutrition) or alternatively, reduced CHO but high fat availability in the post-exercise recovery periods (HFAT). We specifically hypothesised that our high fat feeding protocol would enhance cell signalling and the expression of those genes with putative roles in the regulation of mitochondrial biogenesis and lipid metabolism but would also impair the activity of muscle protein synthesis related signalling.

4.3 Methods

4.3.1 Subjects: Ten male runners volunteered to participate in the study. All subject characteristics are described previously in Chapter 3, Section 3.2.

4.3.2 Design: In a repeated measures, randomised, cross-over design separated by 7 days, subjects completed a twice per day exercise model under two different dietary conditions (both energy and protein matched) consisting of high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best practice nutrition) or alternatively, reduced CHO but high fat availability in the post-exercise recovery periods (HFAT). The twice per day exercise model comprised a morning (9-10 am) high-intensity interval (HIT) training session (8 x 5-min at 85% $\dot{V}O_{2peak}$) followed by an afternoon (130-230 pm) training session consisting of steady-state (SS) running (60 min at 70% $\dot{V}O_{2peak}$). To promote training compliance during the HIT protocol in both the HCHO and HFAT trials, subjects adhered to a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery between the HIT and SS session and in the recovery period upon completion of the SS exercise protocol until the subsequent morning, subjects adhered to either a HCHO or HFAT feeding protocol. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre-HIT, immediately post-SS and at 3 h and 15 h post-SS as previously described. An overview of the experimental design and nutritional protocols are shown in Figure 4.1.

***4.3.3 Preliminary testing*:** At least 7-10 days prior to the first main experimental trial, subjects performed a maximal incremental running test to volitional fatigue on a motorised treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany) in order to determine peak oxygen uptake as previously described in Chapter 3, Section 3.4.4. On

their second visit to the laboratory (approx. 3 days later), subjects completed a running economy test in order to determine their individual running speeds for subsequent experimental trials as previously described in Chapter 3, Section 3.4.4.

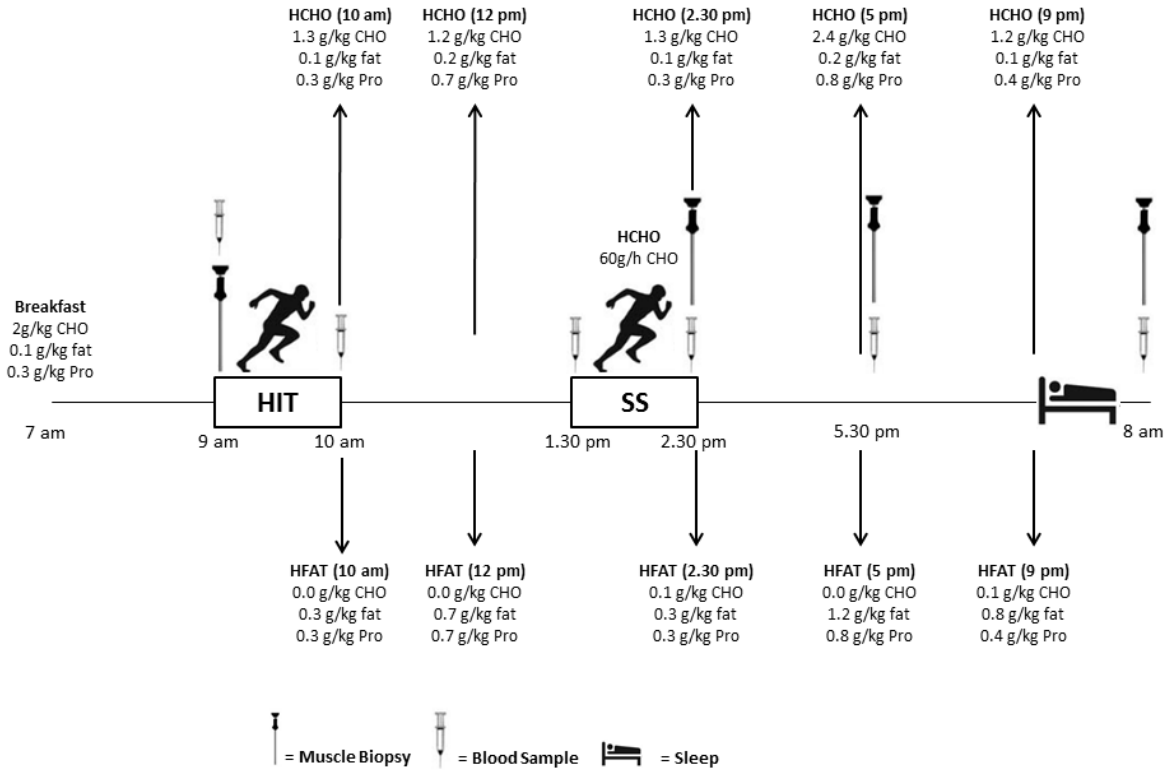

Figure 4.1 Overview of the experimental protocol employed in each trial. HIT = 8 x 5-mins running at a workload equal to 85% $\dot{V}O_{2peak}$ interspersed by 1-min recovery. SS = 1-hour steady state running at a workload equal to 70% $\dot{V}O_{2peak}$.

4.3.4 Experimental protocols:

***4.3.4.1 HIT protocol*:** In the 24-h preceding each main experimental trial, subjects consumed a standardised high CHO diet in accordance with typical nutritional recommendations (8 $g.kg^{-1}$ CHO, 2 $g.kg^{-1}$ protein, and 1 $g.kg^{-1}$ fat). On the morning of each experimental trial, subjects reported to the laboratory at ~7 am where they

were given a standardised high-CHO breakfast (2 $g.kg^{-1}$ CHO, 0.3 $g.kg^{-1}$ protein, and 0.1 $g.kg^{-1}$ fat). At 2-h post-prandial, a venous blood sample was then collected from an antecubital vein in the anterior crease of the forearm and a muscle biopsy sample taken from the vastus lateralis muscle. Subjects were then fitted with a heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the high intensity interval running (HIT) protocol which lasted ~1-h. The HIT protocol consisted of 8 x 5-min bouts running at a velocity corresponding to 85% $\dot{V}O_{2peak}$ interspersed with 1-min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm up and cool down at a velocity corresponding to 50% $\dot{V}O_{2peak,}$ and a further venous blood sample was obtained immediately upon completion of the protocol. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, Borg, 1973) were obtained upon completion of each HIT bout. In order to determine substrate utilisation during exercise (Jeukendrup and Wallis, 2005), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2-mins of each 5-min interval.

***4.3.4.2 SS protocol*:** During the 3.5 h recovery period between the HIT and SS protocols, subjects consumed either the HCHO (2.5 $g.kg^{-1}$ CHO, 1 $g.kg^{-1}$ Protein, 0.3 $g.kg^{-1}$ Fat) or HFAT (0 $g.kg^{-1}$ CHO, 1 $g.kg^{-1}$ Protein, 1 $g.kg^{-1}$ Fat) feeding protocols (the pattern and frequency of feeding is shown in Figure 1). Following the recovery period, another venous blood sample was obtained immediately prior to commencing the afternoon SS exercise protocol. After a 5-min warm up at a self-selected treadmill speed, subjects subsequently commenced the 60-min steady state running (SS)

protocol at a velocity corresponding to 70% $\dot{V}O_{2peak}$. During exercise, subjects also consumed 60 $g.h^{-1}$ of CHO (SiS GO Istonic Gels, Science in Sport, Blackburn, UK) in HCHO whereas no form of energy was consumed in the HFAT trial. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were also collected for 5-mins at 15-min intervals throughout the exercise trial (CPX Ultima, Medgraphics, Minnesota, US) and substrate utilisation again determined according to Jeukendrup and Wallis (2005). Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, 9) were obtained every 15 minutes during exercise. Upon completion of the SS protocol until sleep, subjects consumed either the HCHO (3.6 $g.kg^{-1}$ CHO, 1.5 $g.kg^{-1}$ Protein, 0.4 $g.kg^{-1}$ Fat) or HFAT (0.2 $g.kg^{-1}$ CHO, 1.5 $g.kg^{-1}$ Protein, 2.3 $g.kg^{-1}$ Fat) feeding protocols where the pattern and frequency of feeding is shown in Figure 1. Vastus lateralis muscle biopsies and venous blood samples were also collected immediately post- and at 3 h and 15 h post completion (i.e. ~8 am and in a fasted state) of the SS exercise protocol. The total energy intake across the whole trial period (i.e 7 am – 9 pm) in HCHO was: ~10 $g.kg^{-1}$ CHO, ~2.5 $g.kg^{-1}$ Protein and ~0.8 $g.kg^{-1}$ Fat, and in HFAT was: ~2.5 $g.kg^{-1}$ CHO, ~2.5 $g.kg^{-1}$ Protein and ~3.5 $g.kg^{-1}$ Fat, where both trials were matched for total energy intake. In order to monitor compliance to the diets administered, all food other than the evening snack was given to participants in the laboratory and consumed while supervised by the researcher. Participants were requested to take a photograph of any food left from the evening snack if they were unable to consume all of this.

4.3.5 Blood sampling and analysis: Samples were collected and later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol, and β-hydroxybutyrate as previously described in Chapter 3, Section 3.8.

***4.3.6 Muscle biopsies*:** Muscle biopsy samples (~50 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument as described in Chapter 3, Section 3.9.

4.3.7 Analysis of muscle glycogen: Muscle glycogen concentration was determined according to the methods described by van Loon et al (2000) as previously described in Chapter 3, Section 3.9.1.

4.3.8 RNA isolation and analysis: Muscle biopsy samples (~20 mg) were homogenized in 1ml TRIzol reagent (Thermo Fisher Scientific, UK) and total RNA isolated as detailed in Chapter 3, Section 3.9.2.

4.3.9 Primers: Identification of primer sequences was enabled by Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and primers designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). All primer sequences are listed in Chapter 3, Table 3.3.

4.3.10 Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): rt-qRT-PCR amplifications were performed using a QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA) as previously described in Chapter 3, Section 3.9.3.

4.3.11 [γ^{-32}P] ATP Kinase Assay: Approximately 10-20 mg of muscle tissue was used for the measurement of p70S6K1 and AMPKα2 activity as previously described in Chapter 3, Section 3.9.4.

4.3.12 Statistical analysis: All data were analysed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA) as described in Chapter 3, Section 3.10.

4.4 Results

4.4.1 Physiological responses and substrate utilisation during exercise.

Comparisons of subjects' heart rate, RPE and substrate oxidation during the HIT and SS protocols are displayed in Tables 4.1 and 4.2 respectively. Heart rate, RPE and lipid oxidation (all $P<0.01$) all displayed progressive increases during both HIT (see Table 4.1) and SS exercise (see Table 4.2) whereas CHO oxidation displayed a progressive decrease ($P<0.01$) during both exercise protocols. In accordance with identical pre-exercise feeding in HIT, no significant differences were apparent in any of the aforementioned variables between HCHO and HFAT ($P=0.06$, 0.19, 0.52 and 0.56, respectively). In contrast, however, during the SS exercise protocol CHO oxidation was significantly greater in HCHO compared to HFAT ($P<0.001$) whereas fat oxidation was significantly greater during HFAT compared to HCHO ($P < 0.001$).

Table 4.1 Heart rate, RPE and substrate oxidation responses during the HIT protocol in both the HCHO and HFAT trials. * denotes significant difference from HIT-1, P<0.05.

	HIT (Bout number)							
	1	2	3	4	5	6	7	8
Heart rate								
(b.min^{-1})								
HCHO	177 ± 9	181 ± 9	184 ± 8	185 ± 6*	186 ± 6*	185 ± 5	185 ± 5*	186 ± 7*
HFAT	173 ± 10	174 ± 8	180 ± 6	182 ± 7*	182 ± 7*	179 ± 6	182 ± 8*	184 ± 7*
RPE								
HCHO	14 ± 1.4	15 ± 1.5*	16 ± 2.0*	17 ± 1.6*	18 ± 0.9*	18 ± 1.0*	19 ± 0.6*	19 ± 0.7*
HFAT	14 ± 1.6	15 ± 1.8*	16 ± 1.9*	17 ± 1.1*	18 ± 1.2*	18 ± 1.0*	19 ± 0.9*	19 ± 1.0*
CHOoxidation								
(g.min^{-1})								
HCHO	5.55 ±2.61	5.10 ± 1.43	4.92 ± 1.43	4.40 ± 1.42	3.90 ± 2.17*	3.70 ± 1.91*	3.50 ± 2.36*	3.82 ± 2.77*
HFAT	5.52 ± 2.70	4.81 ± 1.80	4.50 ± 1.62	4.47 ± 1.50	4.11 ± 2.20*	3.63 ± 1.94*	3.60 ± 2.44*	3.71 ± 2.70*
Fat oxidation								
(g.min^{-1})								
HCHO	0.00 ± 0.29	0.00 ± 1.22	0.00 ± 1.40	0.06 ± 1.88	0.25 ± 2.26*	0.30 ± 2.54*	0.38 ± 3.10*	0.31 ± 3.55*
HFAT	0.00 ± 0.96	0.00 ± 1.15	0.03 ± 1.43	0.09 ± 1.87	0.19 ± 2.10*	0.34 ± 2.48*	0.37 ± 3.00*	0.34 ± 3.39*

Table 4.2 Heart rate, RPE and substrate oxidation responses during the SS protocol in both the HCHO and HFAT trials. * denotes significant difference from 15 min, P<0.05. # denotes significant difference between conditions, P<0.05.

	Time (mins)			
	15	30	45	60
Heart rate				
(b.min^{-1})				
HCHO	166 ± 12	169 ± 11	170 ± 12*	172 ± 12*
HFAT	161 ± 11	165 ± 12	166 ± 10*	168 ± 10*
RPE				
HCHO	13 ± 1.3	14 ± 1.5*	15 ± 1.8	15 ± 1.8*
HFAT	13 ± 1.4	14 ± 1.8*	15 ± 2.1	16 ± 1.4*
CHO oxidation (g.min^{-1})				
HCHO	3.42 ±0.81	3.13 ± 1.20*#	3.50 ± 0.89*#	3.33 ± 0.59*#
HFAT	2.88 ±0.52	2.33 ± 0.42*#	2.11 ± 0.54*#	2.00 ± 0.60*#
Fat oxidation				
(g.min^{-1})				
HCHO	0.00 ± 0.30	0.13 ± 0.30*#	0.26 ± 0.31*#	0.35 ± 0.31*#
HFAT	0.32 ± 0.30	0.55 ± 0.20*#	0.65 ± 0.31*#	0.71 ± 0.40*#

4.4.2 Plasma metabolite responses*:* Plasma glucose, lactate, NEFA, glycerol and β-hydroxybutyrate all displayed significant changes (all $P<0.01$) over the sampling period (see Table 4.3) in both experimental conditions. Plasma glucose was significantly higher than pre-exercise ($P<0.01$) values immediately post-HIT and immediately post-SS exercise (7.6 and 8.74mmol.L^{-1} respectively). Plasma lactate, NEFA and glycerol values were all significantly higher in the post exercise sampling period compared to baseline values. In accordance with the provision of post-exercise CHO feeding in the HCHO trial, plasma glucose was significantly higher compared with HFAT ($P<0.01$) whereas post-exercise high fat feeding in HFAT induced significantly greater plasma NEFA, glycerol and β-OHB (all $P<0.01$) in HFAT compared with the HCHO trial.

Table 4.3 Plasma glucose, lactate, NEFA, Glycerol and β-OHB before and after the HIT and SS exercise protocols. # denotes significant difference from Pre-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

		Pre HIT	Post HIT	Pre SS	Post SS	3-h Post SS	15-h Post SS
Glucose (mmol.L^{-1})							
	HCHO	5.22 ± 0.86	7.60 ± 0.74 #	4.72 ± 0.96*#	8.74 ± 1.00*#	5.90± 0.62 #	5.55 ± 0.33*
	HFAT	5.42 ± 0.84	7.59 ± 1.03 #	5.30 ± 0.34*#	5.62 ± 0.8*#	5.77 ± 0.6 #	5.10± 0.420*
Lactate (mmol.L^{-1})							
	HCHO	2.00 ± 0.42	7.84 ± 3.1 #	1.50 ± 0.33*#	1.62 ± 0.46 #	1.55 ± 0.16*#	1.10 ± 0.26 #
	HFAT	1.80 ± 0.41	7.01 ± 3.4 #	1.14 ± 0.22*#	1.33 ± 0.35 #	0.80 ± 0.21*#	0.90 ± 0.36 #
NEFA (mmol.L^{-1})							
	HCHO	0.01 ± 0.02	0.23 ± 0.27 #	0.09 ± 0.13 #	0.59 ± 0.56*#	0.09 ± 0.17*#	0.22 ± 0.22 #
	HFAT	0.05 ± 0.05	0.25 ± 0.28 #	0.32 ± 0.21 #	1.42 ± 0.74*#	0.48 ± 0.27*#	0.24 ± 0.14 #
Glycerol (mmol.L^{-1})							
	HCHO	14.84 ± 6.07	92.80 ± 27.11 #	22.92 ± 12.11*#	50.21 ± 37.41*#	13.20 ± 4.39*#	23.41 ± 20.12#
	HFAT	12.90 ± 4.82	79.35 ± 25.50 #	33.00 ± 7.30*#	122.90 ± 57.0*#	40.79 ± 12.0*#	30.85 ± 12.60#
β-OHB (mmol.L^{-1})							
	HCHO	0.07 ± 0.02	0.14 ± 0.04 #	0.07 ± 0.02*#	0.15 ± 0.08*#	0.08 ± 0.02*#	0.08 ± 0.03
	HFAT	0.07 ± 0.02	0.14 ± 0.04 #	0.11 ± 0.05*#	0.33 ± 0.21*#	0.29 ± 0.2*#	0.19 ± 0.017

4.4.3 Muscle glycogen and exercise induced cell signalling: Exercise induced significant decreases ($P<0.01$) in muscle glycogen immediately post-SS though no differences were apparent between HCHO and HFAT at this time-point (see Figure 4.2 A). However, in accordance with the provision of CHO after the SS exercise protocol in HCHO, muscle glycogen re-synthesis was observed such that significant differences in muscle glycogen between HCHO and HFAT ($P=0.01$) were observed at 3 h and 15 h post-SS exercise. Neither exercise ($P =0.407$) nor dietary condition ($P = 0.124$) affected AMPK-α2 activity at any time-point studied (see Figure 4.2 B). In contrast, p70S6K1 activity was significantly increased 3 h post-SS exercise (30-mins post-feeding) ($P<0.01$), although this increase was supressed ($P=0.08$) in HFAT (see Figure 4.2 C). Furthermore, p70S6K1 activity was significantly reduced at 15 h post-SS exercise when participants were fasted compared with pre-HIT when they were high CHO and protein fed ($P<0.01$).

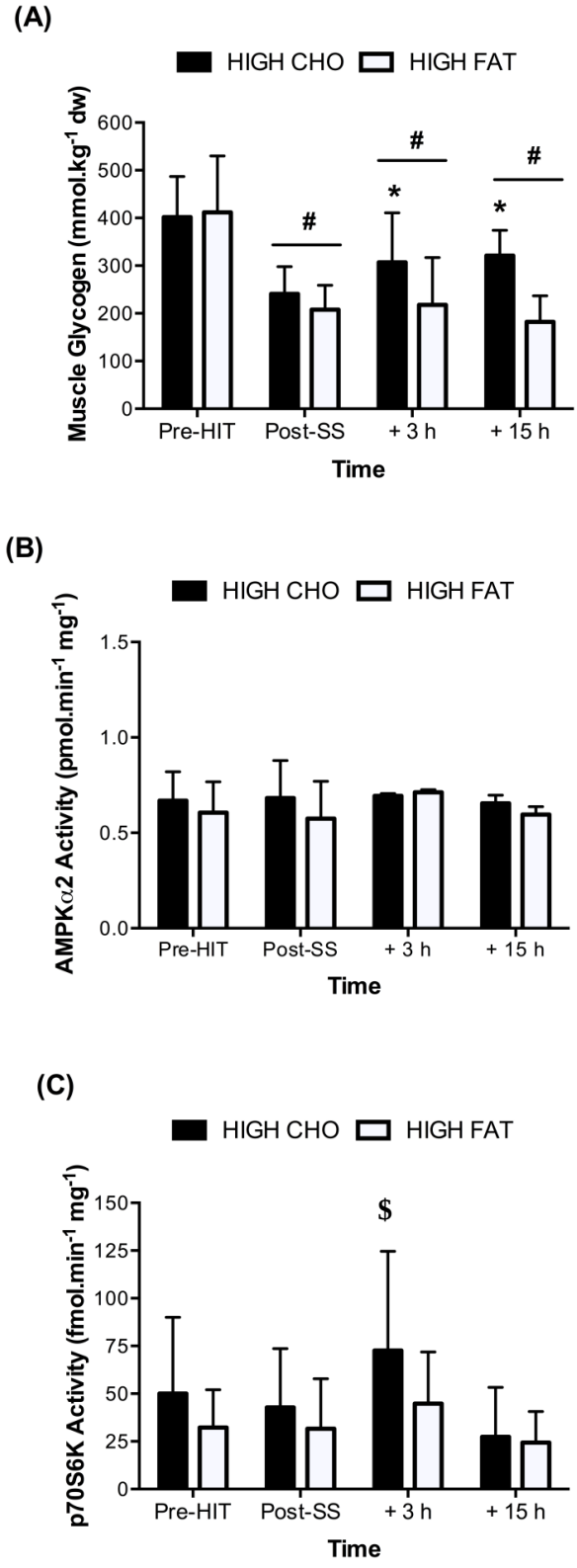

Figure 4.2 (A) Skeletal muscle glycogen content, (B) AMPK-α2 and (C) p70S6K1 activity before HIT exercise and after the SS exercise protocol. # denotes significant difference from Pre-HIT, $P<0.05$. * denotes significant difference between conditions, $P<0.05$. $ denotes difference between conditions, $P=0.08$.

4.4.4 Gene expression*:* Exercise increased the expression of PGC-1α ($P<0.001$), p53 ($P=0.032$), CS ($P=0.05$), Tfam ($P=0.05$), PPAR ($P<0.01$) and ERRα ($P=0.01$) however, there were no differences (all $P>0.05$) between HFAT and HCHO trials (see Figure 4.3 A-F). In contrast, the exercise-induced increase ($P=0.001$) in PDK4 mRNA was greater in HFAT versus HCHO ($P=0.003$). Similarly, mRNA expression of CD36 ($P=0.05$) and CPT1 ($P=0.02$) was significantly greater in HFAT in recovery from the SS exercise protocol (see Figure 4.3 I). In contrast, neither exercise ($P=0.12$) nor diet ($P=0.31$) significantly affected GLUT expression (see Figure 4.3 H).

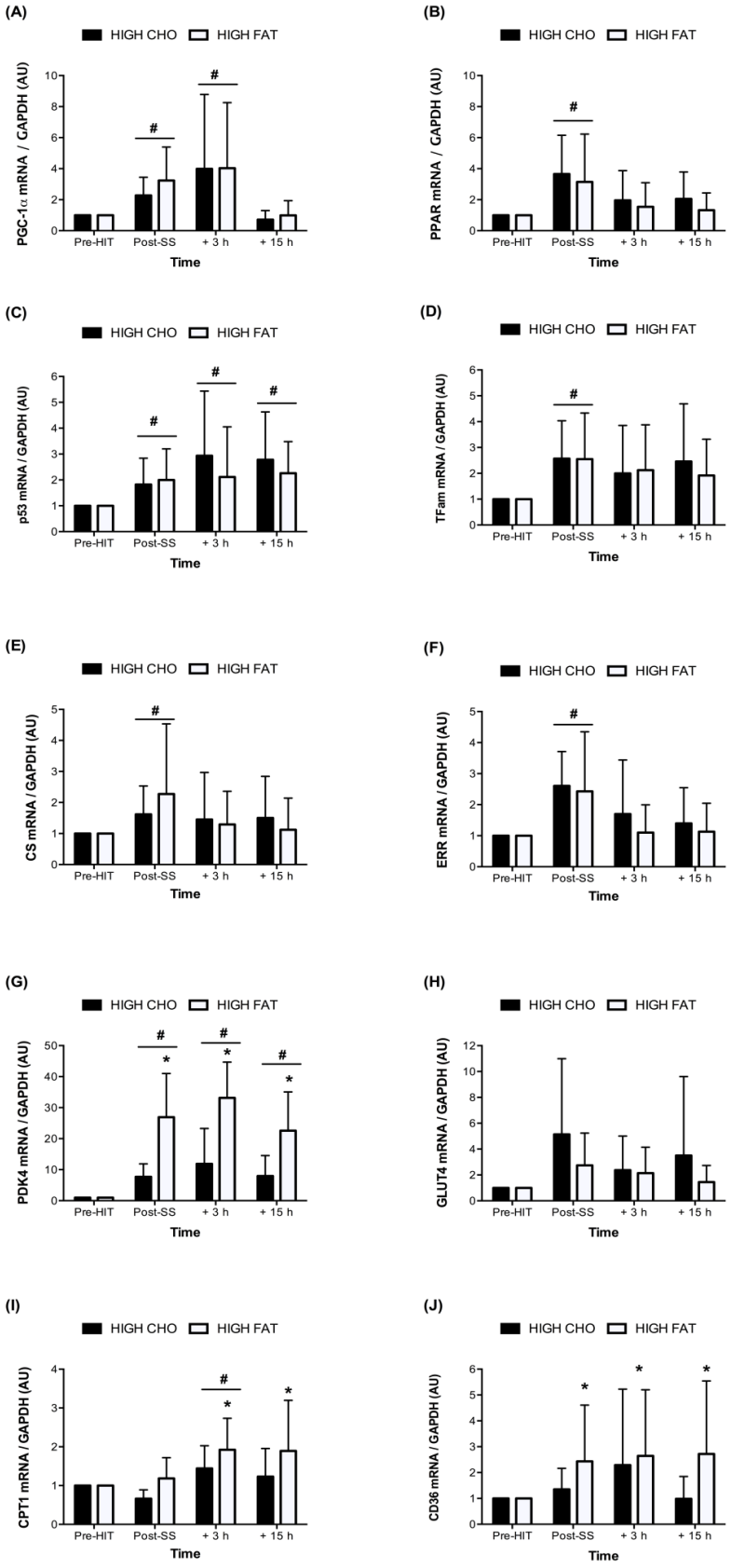

Figure 4.3 (A) PGC-1 α, (B) PPAR, (C) p53, (D) Tfam, (E) CS, (F) ERRα, (G) PDK4, (H) GLUT4, (I) CPT1 and (J) CD36 mRNA before HIT exercise and after the SS exercise protocol. # denotes significant difference from Pre-HIT, $P<0.05$. * denotes significant difference between conditions, $P<0.05$.

4.5 Discussion

The aim of the present chapter was to examine the effects of reduced CHO but high post-exercise fat availability on the activation of key cell signalling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. When compared with high CHO availability, we observed that post-exercise high fat feeding had no modulatory affect on AMPK-α2 activity or the expression of those regulatory genes associated with mitochondrial biogenesis. Furthermore, although post-exercise high fat feeding augmented the expression of genes involved in lipid transport (i.e. FAT/CD36) and oxidation (i.e. CPT1), we also observed suppression of p70S6K1 activity despite sufficient post-exercise protein intake. This latter finding suggests that post-exercise high fat feeding may impair the regulation of muscle protein synthesis and skeletal muscle remodelling processes, thereby potentially causing maladaptive responses for training adaptation if performed long-term.

In accordance with the original train-low investigations examining cycling or knee extensor exercise (Hansen *et al.*, 2005, Yeo *et al.*, 2008b, Morton *et al.*, 2009, Hulston *et al.*, 2010), we also employed a twice per day protocol, albeit consisting of morning HIT and afternoon SS running exercise protocol. This model is practically relevant given that many elite endurance athletes (including runners) train multiple times per day with limited recovery time between training sessions (Fudge *et al.*, 2006). Given that reduced CHO availability impairs high-intensity training capacity (Yeo *et al.*, 2008b, Hulston *et al.*, 2010), we also chose to schedule the HIT session in the morning period after a standardised high CHO breakfast. As expected, no differences in cardiovascular strain, ratings of perceived exertion, substrate utilisation and plasma metabolite responses were observed between the HCHO and HFAT trials during the

HIT session (see Tables). Following completion of the HIT protocol, subjects then adhered to a HCHO or HFAT feeding protocol in the 3.5 h prior to commencing the afternoon SS exercise. Given that exogenous CHO feeding during exercise reduces oxidative adaptations even in the presence of reduced pre-exercise muscle glycogen (Morton *et al.,* 2009), we also chose to feed exogenous CHO (at a rate of 60 g/h) during the afternoon SS protocol during the HCHO trial. Although we did not directly quantify muscle glycogen immediately prior to SS exercise, plasma metabolite and substrate utilisation during SS exercise were clearly suggestive of differences in both endogenous and exogenous CHO availability between the HCHO and HFAT trials. Indeed, plasma NEFA, glycerol, β-OHB and whole body lipid oxidation were all greater during SS exercise undertaken in the HFAT trial compared with the HCHO trial (see Tables). On the basis of comparable muscle glycogen data post-SS exercise (Figure..A) and greater whole body CHO oxidation during the HCHO trial (see Table), we also suggest that exercise-induced muscle glycogen utilisation was greater during the SS exercise protocol when completed in the HCHO conditions (Bartlett *et al.,* 2013).

Perhaps surprisingly, we observed that our SS exercise protocol did not increase AMPK-α2 activity in either the HCHO or HFAT trial. However, there are likely a number of physiologically valid reasons to explain the apparent lack of AMPK mediated signalling. Indeed, exercise-induced AMPK activation is known to be intensity dependent where >70% $\dot{V}O_{2max}$ is likely required to induce metabolic perturbations sufficient to mediate a signalling response (Egan *et al.,* 2010). Furthermore, the AMPK response to exercise is attenuated with exercise training (Benziane *et al.,* 2008), an effect that is especially relevant for the present investigation given the trained status of our chosen population and the low plasma lactate observed

(approximately 2 mmol.L^{-1}) during SS exercise. Reduced absolute muscle fibre recruitment from the vastus lateralis, when compared with other lower extremity muscles recruited during walking and running (Jensen *et al.,* 2012), or when exercising at similar relative intensities during cycling (Arkinstall *et al.,* 2004) and where AMPK activation is typically reported (Kristensen *et al.,* 2015) could also contribute, in part, to the lack of AMPK signalling observed here. Finally, although exercise-induced AMPK activity is also thought to be regulated, in part, via a glycogen binding domain on β-subunit of the AMPK heterotrimer (McBride *et al.,* 2009), it is possible that our runners did not exceed a potential "muscle glycogen threshold" that is required to fully activate the AMPK complex during prolonged endurance exercise (Philp *et al.,* 2012). Indeed, previous data from our laboratory also using running exercise protocols (Bartlett *et al.,* 2012, Bartlett *et al.,* 2013) have typically only observed AMPK related signalling when post-exercise whole muscle homogenate glycogen is <200 mmol.kg^{-1} dw. Despite previous suggestions that train-low training sessions should be targeted to SS exercise protocols so as to not compromise training intensity (Bartlett *et al.,* 2015), our data therefore suggest (at least for AMPK mediated signalling) that perhaps it is the actual completion of a high-intensity stimulus per se (especially in trained athletes) that is really required to create a metabolic milieu that is conducive to augmentation of necessary signalling networks.

In contrast to Yeo et al. (2008b), we also observed no modulatory effect of post-exercise high fat availability on resting AMPK-α2 activity. Indeed, these authors observed that 5 days of a fat loading protocol increased resting AMPK-α2 activity as well as the exercise-induced phosphorylation of ACC^{Ser221}. Such discrepancies between studies are likely due to the differences in duration of high fat feeding in that we adopted an acute high fat feeding protocol (<24 h) whereas the latter authors

adopted a 5 day “fat adaptation” protocol that also increased resting intramuscular triglyceride (IMTG) stores. In this regard, it is noteworthy that the magnitude of change in resting AMPK-α2 activity was positively correlated with the elevations in IMTG storage (Yeo *et al.*, 2008a).

In contrast to our hypothesis, we also observed comparable 2-3 fold changes between trials in mRNA expression of those genes with key regulatory roles associated with mitochondrial biogenesis. For example, the expression of PGC-1α, p53, Tfam, PPAR and ERRα mRNA were all elevated with similar magnitude and time-course in recovery from the SS protocol in both the HCHO and HFAT trials. Such data conflict with previous observations from our laboratory (Bartlett *et al.*, 2013) and others (Pilegaard *et al.*, 2005) where post-exercise CHO restriction (i.e. keeping muscle glycogen low) augments the expression of many of the aforementioned genes. However, in the previous report we simultaneously adopted a CHO but calorie restriction feeding protocol whereas the design implemented in the present chapter incorporated a reduced CHO but isocaloric and protein matched feeding protocol in our HFAT trial. Given the similarities in metabolic adaptation to both CHO and calorie restriction, such data raise the question as to whether the enhanced mitochondrial responses observed when “training low” are due to transient periods of CHO restriction, calorie restriction or indeed, a combination of both. This point is especially relevant from an applied perspective given that many endurance athletes present daily with transient periods of both CHO and calorie restriction due to multiple training sessions per day as well as longer term periods of sub-optimal energy availability (Fudge *et al.*, 2006).

In agreement with multiple studies demonstrating a role of both acute elevations in FFA availability (Lane *et al.*, 2015, Bartlett *et al.*, 2013) as well as high fat feeding

protocols (Cameron-Smith *et al.,* 2003), we also observed that post-exercise expression of PDK4, FAT/CD36 and CPT1 mRNA expression were elevated in the HFAT trial versus the HCHO trial. However, unlike Arkinstall et al. (2004), we did not detect any suppressive effects of high fat availability on GLUT4 mRNA expression though a longer and more severe period of CHO restriction utilised by these investigators (i.e. 48 h of absolute CHO intake <1 g/kg body mass resulting in muscle glycogen levels <150 mmol.kg^{-1} dw) may explain the discrepancy between studies. Nonetheless, the dietary protocol studied here clearly alters the expression of genes with potent regulatory roles in substrate utilisation and if performed long term, may increase the capacity to use lipids as a fuel but induce suppressive effects on CHO metabolism (through suppression of the PDH complex) thus potentially limiting high-intensity performance (Stellingwerff *et al.*, 2006). Whilst we did not directly quantify the signalling mechanisms underpinning these responses (owing to a lack of a muscle tissue), we suggest both p38MAPK and PPAR mediated signalling are likely involved. Indeed, using a twice per day exercise model, Cochran et al. (2010) also observed enhanced p38MAPK phosphorylation during the afternoon exercise protocol (despite similar pre-exercise muscle glycogen availability) that was associated with the enhanced circulating FFA availability during the afternoon exercise. Furthermore, pharmacological ablation of circulating FFA availability during exercise suppresses p38MAPK compared with control conditions (Zbinden-Foncea *et al.*, 2003). Additionally, FFA mediated signalling can also directly mediate PPAR binding to the CPT1 promoter thereby modulating CPT1 expression (Philp *et al.*, 2013).

We also examined the effects of post-exercise fat feeding on the regulation of p70S6K activity, a key signalling kinase associated with regulating MPS. In relation to the effects of endurance exercise *per se*, the majority of studies are typically limited to

measures of phosphorylation status with some studies reporting increases (Mascher *et al.*, 2007) and others, no change. When examined quantitatively using the [γ^{-32}P] ATP kinase assay, our data agree with previous observations from Apro et al. (2015) who also reported no change but yet, conflict with recent data from our group where we observed an exercise-induced suppression of p70S6K activity (Impey *et al.*, 2016). Nonetheless, the exhaustive (a fatiguing cycling HIT protocol) and muscle glycogen depleting (<100 mmol.kg^{-1} dw) nature of the latter exercise protocol versus the moderate-intensity nature of the afternoon SS running protocol studied here, likely explains the discrepancy between studies.

In relation to the effects of post-exercise feeding, we also provide novel data by demonstrating that post-exercise high fat feeding suppressed p70S6K activity (albeit P=0.08) at 3 h post-completion of the SS exercise protocol when compared with the elevated response observed in HCHO (when using both mean difference and standard deviation of differences of 50 fmol.min^{-1}.mg^{-1}, we estimate a sample size of 12-13 would be required to achieve statistical significance with 90% power, as calculated using Minitab statistical software, version 17). Although we did not measure circulating insulin levels in this study, it is of course possible that the suppressed p70S6K response observed here may be due to reduced upstream insulin mediated of protein kinase B (PKB) activity. Indeed, we recently observed post-exercise p70S6K activity to be suppressed in conditions of simultaneous carbohydrate and calorie restriction in a manner associated with reduced insulin and upstream signalling of PKB (Impey *et al.*, 2016). Alternatively, the suppression of p70S6K observed here may be mediated through direct effects of post-exercise high fat feeding that are independent of CHO availability, energy availability and insulin. Indeed, Stephens et al. (2015) observed infusion of Intralipid and heparin to elevate circulating FFA concentrations

attenuates MPS in human skeletal muscle in response to ingesting 21g amino acids under euglycemic hyperinsulemic clamp conditions. Furthermore, Kimball et al. (2015) also reported that high fat feeding impairs MPS in rat liver in a manner associated with reduced p70S6K phosphorylation, an effect that may be induced through sestrin 2 and sestrin 3 mediated impairment of mTORC signalling. Clearly, further research is required to examine the effects of high fat feeding on direct measures (and associated regulatory sites) of MPS within the physiological context of the exercising human.

In summary, we provide novel data by concluding that post-exercise high fat feeding has no modulatory affect on AMPK-α2 activity or the expression of those genes associated with regulatory roles in mitochondrial biogenesis. Furthermore, although post-exercise high fat feeding augmented the expression of genes involved in lipid transport and oxidation, we also observed a suppression of p70S6K1 activity despite sufficient post-exercise protein intake. This latter finding suggests that post-exercise high fat feeding may impair the regulation of muscle protein synthesis and post-exercise muscle remodelling, thereby potentially causing maladaptive responses for training adaptation if performed long-term. Future studies should now also examine the effects of post-exercise CHO versus calorie restriction in modulating the key cell signalling pathways associated with regulation of mitochondrial biogenesis.

Chapter 5

The effects of post-exercise CHO and caloric restriction on skeletal muscle cell signalling and gene expression associated with the regulation of mitochondrial biogenesis and lipid metabolism

Due to unfortunate issues with muscle sample analysis with a collaborating University, the original aims of Study 2 were changed during the PhD, and as such a separate data collection for a third study could not be performed due to a large delay. Subsequently, further analysis of muscle and blood samples collected in the second study was performed. The work from this one large study has now been split and presented as two separate chapters - Chapter 5 Study 2a (muscle cell signalling data and metabolic data), and Chapter 6, Study 2b (bone turnover, appetite regulation and inflammatory cytokine data).

5.1 Abstract

Purpose: To examine the effects of post-exercise CHO restriction, and caloric restriction on the modulation of skeletal muscle cell signalling pathways associated with mitochondrial biogenesis and lipid metabolism. **Methods:** Nine males completed a twice per day exercise model (3.5 h between sessions) comprising a morning and afternoon high-intensity interval (HIT) (8 x 5-min at 85% $\dot{V}O_{2peak}$) running protocol. In a repeated measures crossover design, runners exercised under three different dietary conditions consisting of 1) high CHO availability (HCHO: ~ 5030 kcals, CHO ~12.6, protein ~ 2.3, and fat ~0.6 $g.kg^{-1}$ per whole trial period), 2) reduced CHO but high fat availability (LCHF: ~ 5021 kcals, CHO ~3, protein ~ 2.3, and fat ~3 $g.kg^{-1}$ per whole trial period), or 3) reduced CHO and reduced energy availability (LCAL:~ 2714 kcals, CHO ~3, protein ~ 2.3, and fat ~0.4 $g.kg^{-1}$ per whole trial period). **Results:** Muscle glycogen was reduced to comparable levels (~200 $mmol.kg^{-1}dw$) in all trials immediately post PM-HIT ($P < 0.01$), and remained lower at 3-h (156, 182 and 345 $mmol.kg^{-1}dw$, $P < 0.001$) and 15-h (171, 194 and 316 $mmol.kg^{-1}dw$, $P < 0.001$) post PM-HIT in LCHF and LCAL compared to HCHO. Phosphorylation of p38MAPK increased ($P = 0.037$) immediately-post PM-HIT, though no differences existed between trials ($P = 0.755$). Tfam and Parkin mRNA were not increased post PM-HIT or during recovery from exercise in any of the three conditions, though comparable increases (all $P < 0.05$) in PGC-1α, p53, CPT1 and CD36 mRNA were observed in HCHO, LCHF and LCAL. In contrast, PDK4 mRNA expression ($P = 0.004$) was greater in LCHF and LCAL in the recovery period from PM-HIT compared to HCHO, whilst SIRT1 mRNA expression was also greater in LCAL compared to HCHO and LCHF. **Conclusion:** In recovery conditions where post-exercise muscle glycogen concentration is maintained within the range of 200-350 $mmol.kg^{-1}$ dw, our data therefore suggest that short-term periods of acute CHO and energy restriction (i.e. <24 hours) does not potentiate the metabolic signalling pathways that regulate the hallmark adaptations associated with endurance training.

Keywords: PGC-1α, PDK4, glycogen, mitochondrial biogenesis

5.2 Introduction

The role of nutritional manipulation strategies to support the adaptive responses to exercise training is now becoming increasingly well-established (Stellingwerff *et al.*, 2018). Accordingly, the concept of deliberately promoting low CHO availability in relation to a training session (i.e. the 'train-low' paradigm) has received much attention and is now becoming a more popular strategy amongst athletic populations (Marquet *et al.,* 2016). Indeed, over the last decade, a multitude of research designs examining the 'train-low' approach have consistently demonstrated that commencing and/or recovering from a training session with reduced CHO availability activates the key regulatory cell signalling pathways that increase many of the muscle adaptive responses to endurance training (Yeo *et al.,* 2008; Bartlett *et al.,* 2013; Psilander *et al.,* 2013). Most notably, exercise commenced with both reduced endogenous and exogenous CHO availability augments the AMPK-PGC-1α signalling axis (Yeo *et al.,* 2010; Bartlett *et al.,* 2013; Psilander *et al.,* 2013), and the expression of those genes involved in the regulation of mitochondrial biogenesis (Pilegaard *et al.,* 2002; Hansen *et al.,* 2005; Morton *et al.,* 2009) and substrate utilization (Hulston *et al.,* 2010). It is also apparent that the 'train-low' approach enhances performance in trained endurance athletes (Marquet *et al.,* 2016) when implemented more chronically in to a training programme.

Data presented in the previous chapter demonstrated that the post-exercise expression of those genes with putative roles in mitochondrial biogenesis and substrate metabolism were elevated to a similar magnitude following the consumption of a high CHO diet, and an isoenergetic reduced CHO but high-fat diet. Such data conflict with previous observations from our laboratory (Bartlett *et al.,* 2013) and others (Pilegaard *et al.,* 2005) where post-exercise CHO restriction (hence keeping muscle glycogen

low) augments the expression of many of these genes. However, where we have previously adopted post-exercise CHO restriction approach (Bartlett *et al.,* 2013; Impey *et al.,* 2016), there has also been a simultaneous restriction in total energy intake in contrast to an isoenergetic feeding protocol. Given the similarities in metabolic adaptation to both CHO and calorie restriction (Meynet and Ricci, 2014), such data raise the question as to whether the enhanced mitochondrial responses observed when "training low" are due to CHO restriction, calorie restriction or a combination of both. In this regard, recent data suggests that intermittent food deprivation in combination with endurance training (albeit in rodents) increases the activation of those cell signalling pathways involved in mitochondrial biogenesis and improves endurance performance to a greater extent than ad libitum feeding with exercise training (Marosi *et al.,* 2018).

When taking in to consideration the real-world training environments of elite endurance athletes, it is common practice to undertake 20-30 -h of training per week, (often with multiple training sessions per day) and as such it is likely that many endurance athletes present daily with transient periods of both CHO and calorie restriction through default. In other scenarios, day-to-day or longer-term energy periodization (as opposed to CHO *per se*) may also be deliberately implemented into a training programme in an attempt to reduce body (and fat) mass in preparation for competition (Vogt *et al.,* 2005; Stellingwerff 2012). Indeed, the performance improvements observed by Marquet and colleagues (2016) from elite triathletes following a 'sleep-low' approach to training-low were also associated with a 1-kg reduction in fat mass. It is therefore possible that many of the skeletal muscle adaptive responses observed using 'train-low' models are mediated by overall energy restriction as opposed to CHO restriction *per se*.

To further explore this concept, the aim of the present chapter was to examine the effects of post-exercise CHO and caloric restriction on the modulation of skeletal muscle cell signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism. We employed a repeated measures crossover design whereby trained male runners completed a twice per day exercise model whereby two bouts of a high-intensity interval training session were completed in the morning and afternoon. Runners completed the training sessions under three different dietary conditions consisting of high CHO availability (HCHO) in the recovery period after both training sessions (usually considered best practice recovery nutrition), reduced CHO but high fat availability (LCHF), or finally reduced CHO and reduced energy intake (LCAL). In this way, our experimental design allowed us to evaluate the effects of post-exercise CHO restriction in modulating skeletal muscle adaptive responses in conditions of both sufficient or reduced energy availability. We hypothesised that post-exercise CHO restriction would only augment cell signalling and gene expression when energy intake is simultaneously reduced.

5.3 Methods

5.3.1 Subjects: Eight male runners volunteered to participate in the study. All subject information is described in Chapter 3, Sections 3.1 and 3.2.

5.3.2 *Design*: In a repeated measures, randomised, cross-over design separated by 7 days, subjects completed a twice per day exercise model under three different dietary conditions, consisting of either high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best practice nutrition), reduced CHO but high fat availability (LCHF), or reduced CHO but reduced energy availability (LCAL). The twice per day exercise model comprised a morning (9-10 am) high-intensity interval (AM-HIT) training session (8 x 5-min at 85% $\dot{V}O_{2peak}$) followed by an afternoon (130-230 pm) training session consisting of the same high-intensity interval exercise bout (PM-HIT). To promote training compliance during the AM-HIT protocol in all three trials, subjects adhered to a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery between the HIT sessions and in the recovery period upon completion of the PM-HIT exercise protocol until the subsequent morning, subjects adhered to either the HCHO, LCHF, or LCAL feeding protocol. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre AM-HIT, immediately post PM-HIT and at 3 h and 15 h post PM-HIT. An overview of the experimental design and nutritional protocols are shown in Figure 5.1.

***5.3.3 Preliminary testing*:** At least 7-10 days prior to the first main experimental trial, subjects performed a combined running economy and maximal incremental running test to volitional fatigue on a motorised treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany). As such, both peak oxygen uptake and individual running speeds for the

subsequent experimental trials could be determined as previously described in Chapter 3.

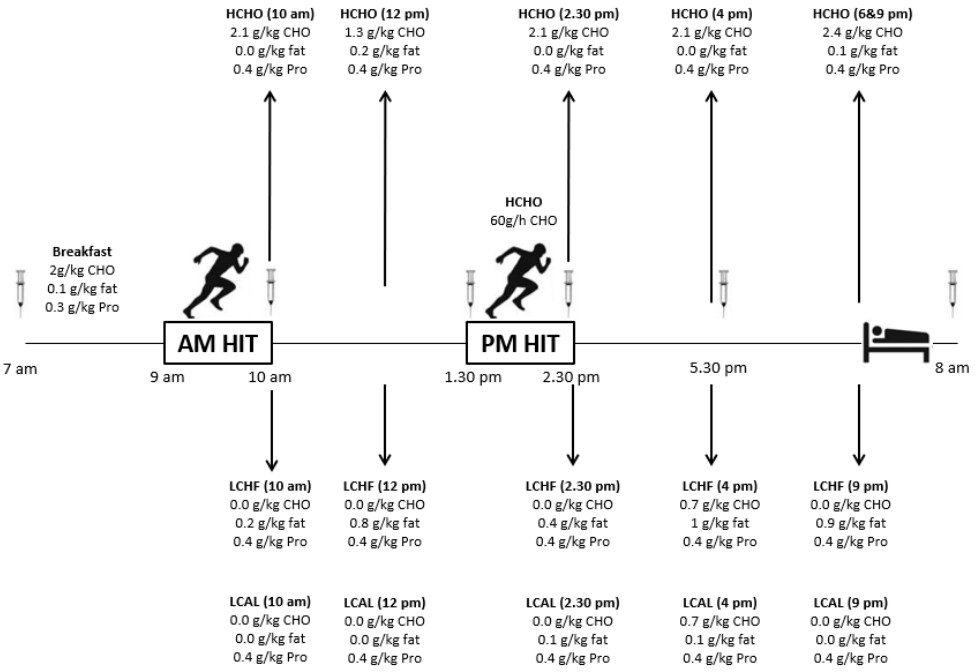

Figure 5.1 Overview of the experimental protocol employed in each trial. HIT = 8 x 5-mins running at a workload equal to 85% $\dot{V}O_{2peak}$ interspersed by 1-min recovery.

5.3.4 Experimental protocols:

***5.3.4.1 AM HIT protocol*:** In the 24-h preceding each main experimental trial, subjects consumed a standardised high CHO diet in accordance with typical nutritional recommendations (8 g.kg^{-1} CHO, 2 g.kg^{-1} protein, and 1 g.kg^{-1} fat). On the morning of each experimental trial, subjects reported to the laboratory at ~7 am where a venous blood sample was collected from an antecubital vein in the anterior crease of the forearm. Subjects were then given a standardised high-CHO breakfast (2 g.kg^{-1} CHO, 0.3 g.kg^{-1} protein, and 0.1 g.kg^{-1} fat), and at 2-h post-prandial, a muscle biopsy sample

was taken from the vastus lateralis muscle. Subjects were then fitted with a heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the first high intensity interval running (AM-HIT) protocol which lasted ~1-h. The HIT protocol consisted of 8 x 5-min bouts running at a velocity corresponding to 85% $\dot{V}O_{2peak}$ interspersed with 1-min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm up and cool down at a velocity corresponding to 50% $\dot{V}O_{2peak,}$ and a further venous blood sample was obtained immediately upon completion of the protocol. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, Borg, 1973) were obtained upon completion of each HIT bout. In order to determine substrate utilisation during exercise (Jeukendrup and Wallis, 2005), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2-mins of each 5-min interval.

***5.3.4.2 PM HIT protocol*:** During the 3.5 h recovery period between the AM HIT and PM HIT protocols, subjects consumed either the HCHO (3.5 $g.kg^{-1}$ CHO, 0.8 $g.kg^{-1}$ Protein, 0.2 $g.kg^{-1}$ Fat), LCHF (0 $g.kg^{-1}$ CHO, 0.8 $g.kg^{-1}$ Protein, 1.2 $g.kg^{-1}$ Fat), or LCAL (0 $g.kg^{-1}$ CHO, 0.8 $g.kg^{-1}$ Protein, 0.1 $g.kg^{-1}$ Fat) feeding protocols (the pattern and frequency of feeding is shown in Figure 1). Following the recovery period, another venous blood sample was obtained immediately prior to commencing the afternoon HIT exercise protocol. After a 10-min warm up at a velocity corresponding to 50% $\dot{V}O_{2peak}$, subjects subsequently commenced the PM HIT protocol. During exercise, subjects also consumed 60 $g.h^{-1}$ of CHO (SiS GO Istonic Gels, Science in Sport, Blackburn, UK) in HCHO whereas no form of energy was consumed in the

LCHF or LCAL trials. Again, water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were also collected for the last 2-mins of each 5-min interval throughout the exercise trial (CPX Ultima, Medgraphics, Minnesota, US) and substrate utilisation again determined according to Jeukendrup and Wallis (2005). Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, 9) were obtained at the end of each HIT bout. Upon completion of the PM HIT protocol until sleep, subjects consumed either the HCHO (6.4 $g.kg^{-1}$ CHO, 1.3 $g.kg^{-1}$ Protein, 0.3 $g.kg^{-1}$ Fat), LCHF (0.8 $g.kg^{-1}$ CHO, 1.3 $g.kg^{-1}$ Protein, 2.3 $g.kg^{-1}$ Fat), or LCAL (0.8 $g.kg^{-1}$ CHO, 1.3 $g.kg^{-1}$ Protein, 0.1 $g.kg^{-1}$ Fat) feeding protocols where the pattern and frequency of feeding is shown in Figure 1. Vastus lateralis muscle biopsies and venous blood samples were also collected immediately post- and at 3 h and 15 h post completion (i.e. ~8 am and in a fasted state) of the PM HIT exercise protocol. The total energy intake across the whole trial period (i.e 7 am – 9 pm) in HCHO was: ~12.6 $g.kg^{-1}$ CHO, ~2.3 $g.kg^{-1}$ Protein and ~0.6 $g.kg^{-1}$ Fat, in LCHF was: ~3 $g.kg^{-1}$ CHO, ~2.3 $g.kg^{-1}$ Protein and ~3 $g.kg^{-1}$ Fat (where both trials were matched for total energy intake), and in LCAL was ~3 $g.kg^{-1}$ CHO, ~2.3 $g.kg^{-1}$ Protein and ~0.4 $g.kg^{-1}$ Fat (where CHO intake was matched with HFAT).

5.3.5 Blood sampling and analysis: Samples were collected and later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol, and β-hydroxybutyrate as previously described in Chapter 3, Section 3.8.

***5.3.6 Muscle biopsies*:** Muscle biopsy samples (~50 mg) were obtained from the lateral portion of the vastus lateralis muscle as previously described in Chapter 3, Section 3.9.

5.3.7 Analysis of muscle glycogen: Muscle glycogen concentration was determined according to the methods described by van Loon et al (2000) as described in Chapter 3, Section 3.9.1.

5.3.8 Analysis of p38 MAPK phosphorylation: p38MAPK was measured using Flow Cytometry as previously described in Chapter 3, Section 3.9.5.

5.3.9 RNA isolation and analysis: Muscle biopsy samples (~20mg) were homogenised in 1mL TRIzol reagent (Thermo Fisher Scientific) and total RNA isolated as destailed in Chapter 3, Section 3.9.2.

5.3.10 Primers: Identification of primer sequences was enabled by Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and primers designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). All primer sequences are listed in Chapter 3, Table 3.3.

5.3.11 Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): rt-qRT-PCR amplifications were performed using a QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA) as previously described in Chapter 3 section 3.9.3.

5.3.12 Statistical analysis: All data were analysed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA).

5.4 Results

5.4.1 Physiological responses and substrate utilisation during exercise.

Comparisons of subjects' heart rate, RPE and substrate oxidation during both the AM HIT and PM HIT protocols are displayed in Figure 5.2. Heart rate, RPE and lipid oxidation (all $P<0.001$) all displayed progressive increases during both AM HIT and PM HIT exercise whereas CHO oxidation displayed a progressive decrease ($P<0.001$) during both exercise protocols (see Figure 5.2). In accordance with identical pre-exercise feeding in all trials prior to AM HIT, no significant differences were apparent in any of the aforementioned variables between HCHO, LCHF, and LCAL ($P=0.82$, 0.19, 0.30 and 0.72, respectively). In contrast, however, during the PM HIT exercise protocol, CHO oxidation was significantly greater in HCHO compared to LCHF and LCAL ($P=0.004$) whereas lipid oxidation was significantly greater during LCHF and LCAL compared to HCHO ($P=0.04$). No differences were observed in either CHO ($P = 0.595$) or lipid ($P = 0.403$) oxidation between LCAL and LCHF during PM HIT.

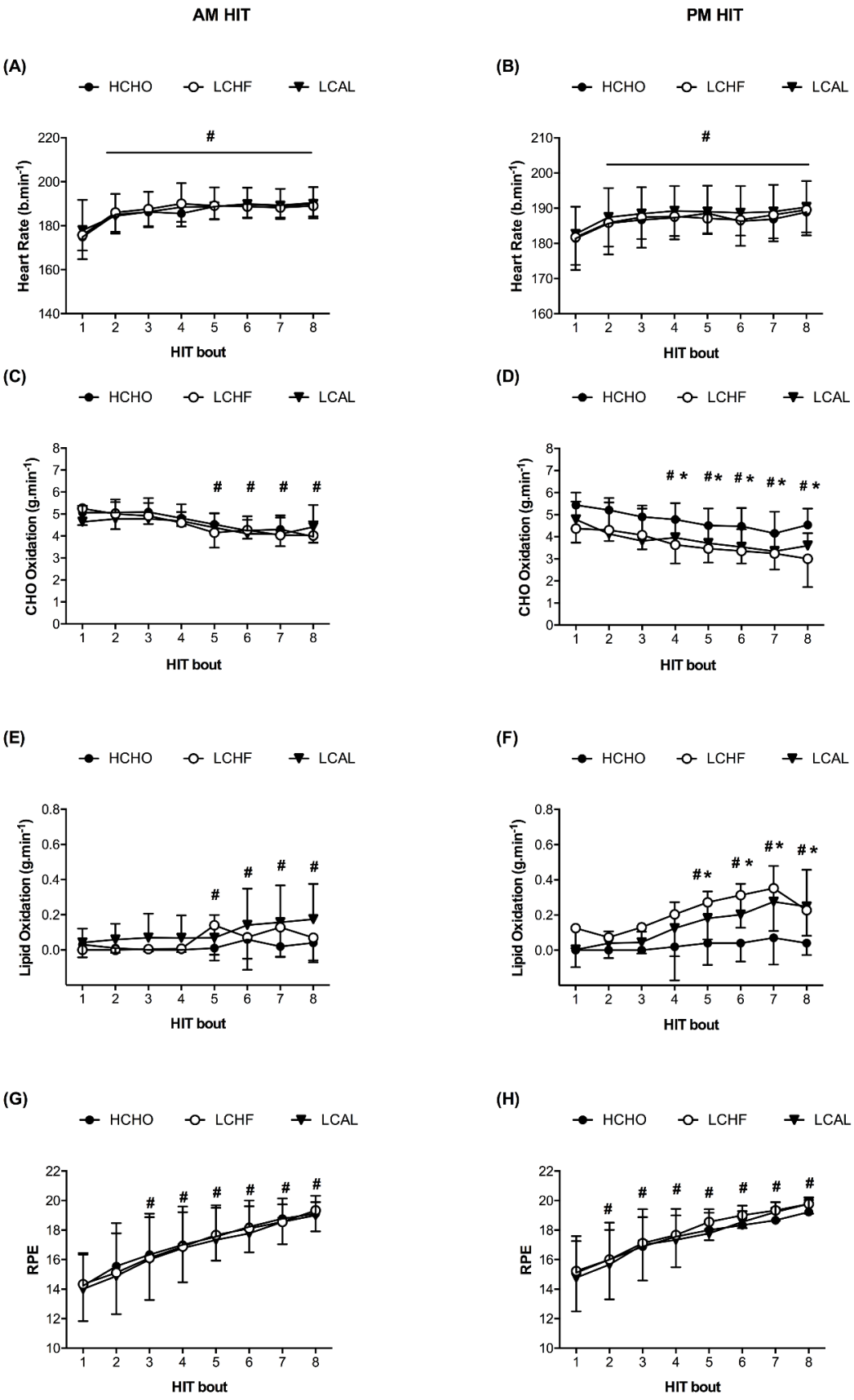

Figure 5.2 Heart rate (A,B), CHO oxidation (C, D), lipid oxidation (E, F), and RPE (G, H) responses during each HIT bout in the HCHO, HFAT, and LCAL trials. # denotes significant difference from HIT bout 1 ($P<0.05$). * denotes significant difference between conditions, ($P<0.05$).

5.4.2 Plasma metabolite responses: Plasma glucose, lactate, NEFA, glycerol and β-hydroxybutyrate all displayed significant changes (all $P<0.001$) over the sampling period (Figure 5.3). However, in accordance with the provision of post-exercise CHO feeding in the HCHO trial, plasma glucose was significantly higher compared with LCHF and LCAL ($P<0.05$) whereas restricting CHO and energy intake post-exercise induced significantly greater plasma NEFA and β-OHB (both $P<0.01$) in LCHF and LCAL compared with the HCHO trial. There was no difference between LCHF and LCAL on NEFA responses ($P = 0.06$), however β-OHB was higher ($P = 0.024$) in LCHF across all time points post- PM HIT. Plasma glycerol was significantly elevated in response to both AM HIT ($P=0.001$) and PM HIT ($P<0.001$), however this response was not different between the 3 trials ($P=0.362$). Similarly, plasma lactate concentrations were significantly elevated in response to AM HIT ($P<0.001$) and PM HIT ($P=0.004$), with no differences between feeding conditions ($P=0.383$).

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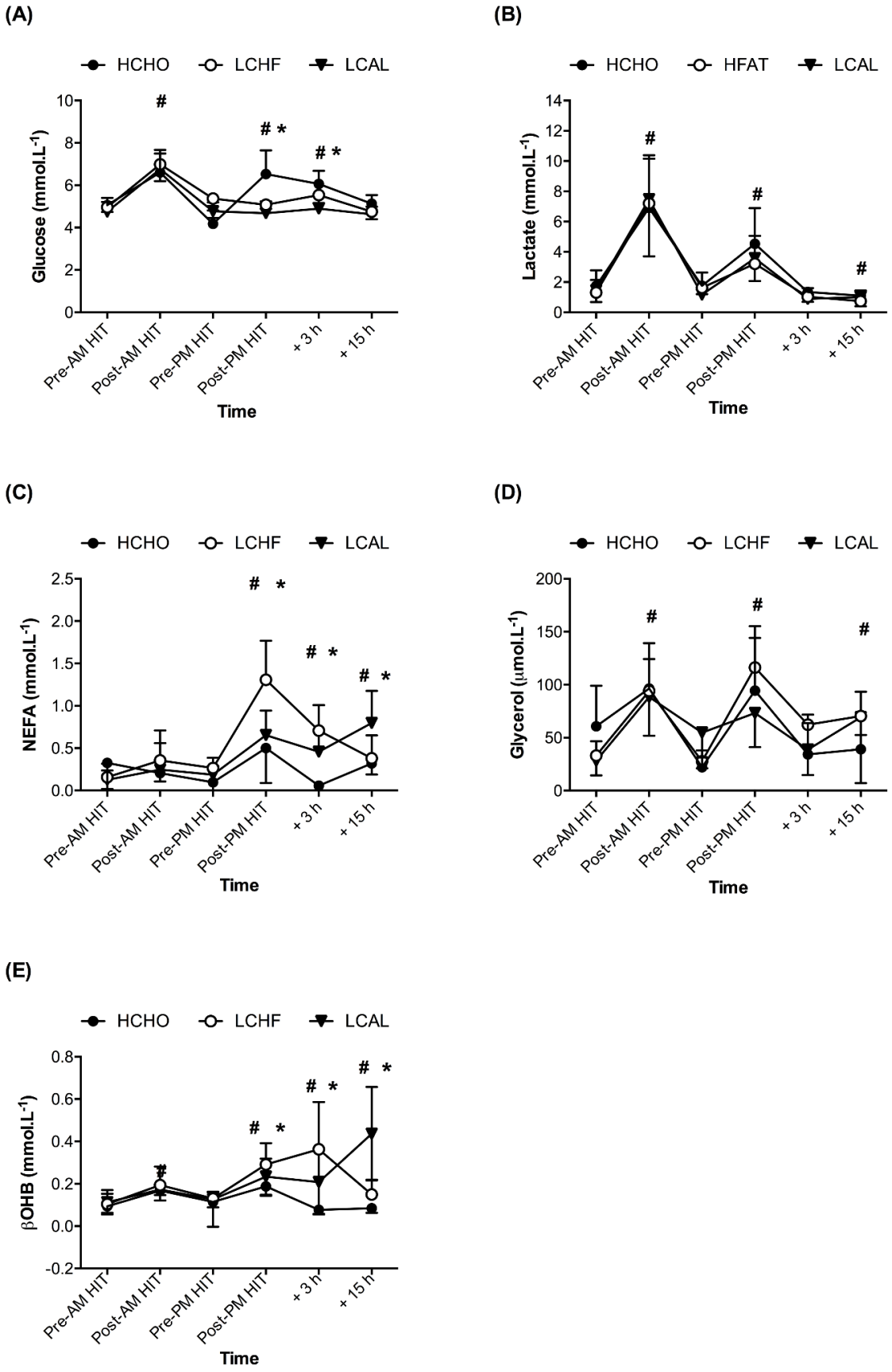

Figure 5.3 Plasma glucose (A), lactate (B), NEFA (C), Glycerol (D) and β-OHB (E) before and after the AM HIT and PM HIT protocols. # denotes significant difference from Pre-AM HIT, P<0.05. * denotes significant difference between conditions, P<0.05

5.4.3 Muscle glycogen: Exercise induced significant decreases (P<0.01) in muscle glycogen immediately post-PM HIT, though no differences were apparent between HCHO, LCHF, and LCAL at this time-point (see Figure 5.4). However, in accordance with the provision of CHO after the PM HIT exercise protocol in HCHO, muscle glycogen re-synthesis was observed such that glycogen concentration was higher in HCHO compared with both LCHF (P =0.028) and LCAL (P = 0.002) were observed at 3-h and 15-h post exercise.

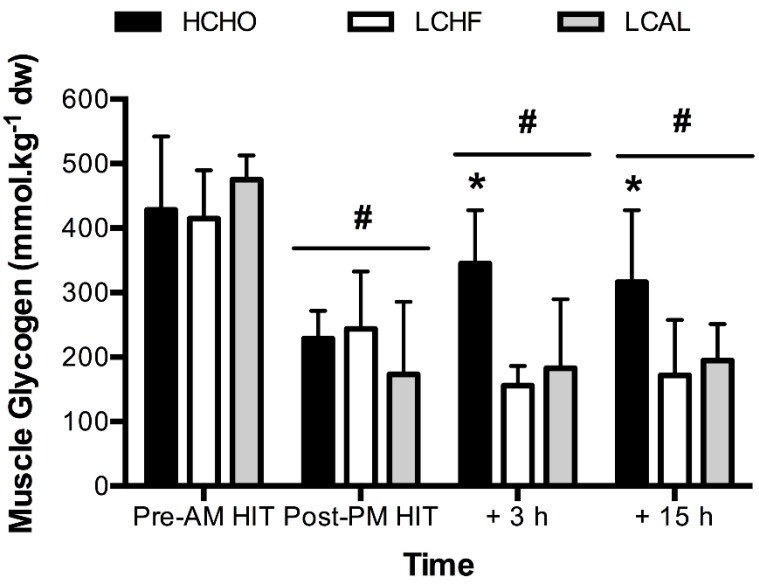

Figure 5.4 Skeletal muscle glycogen content before the AM HIT and after the PM HIT exercise protocols. # denotes significant difference from Pre-AM HIT, (P<0.05). * denotes significant difference between conditions (P<0.05).

5.4.4 p38MAPK Phosphorylation: Exercise induced significant increases in p38MAPK (P=0.037) however, there were no differences (all P>0.05) in this response between HCHO, LCHF and LCAL trials (see Figure). At 15-h post PM HIT p38MAPK had returned back to baseline levels in all three experimental conditions (see Figure 5.5).

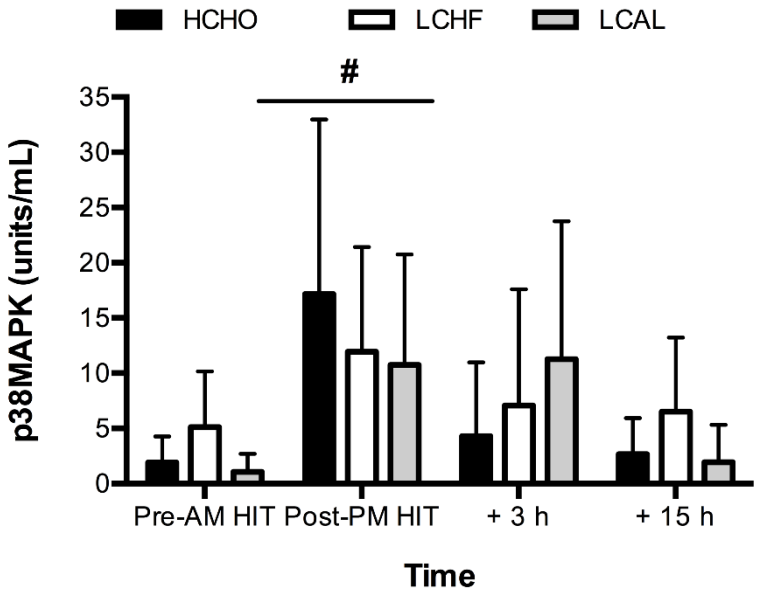

Figure 5.5 Skeletal muscle phosphorylated p38MAPK signalling before the AM HIT and after the PM HIT exercise protocols. # denotes significant difference from Pre-AM HIT, (P<0.05).

5.4.5 Gene expression*:* Exercise increased the expression of PGC-1α (P<0.001), p53 (P=0.01), CPT1 (P<0.001) and CD36 (P=0.01) however, there were no differences (all P>0.05) between LCHF, HCHO and LCAL trials (see Figure 5.6). In contrast, the exercise-induced increase (P=0.001) in PDK4 mRNA was greater in LCHF and LCAL versus HCHO (P=0.004) at 3-h post exercise (see Figure 5.6 G). Similarly, mRNA expression of SIRT1 was significantly greater in LCAL compared to HCHO and LCHF at 15h -post exercise (P=0.01, see Figure 5.6 C). In contrast, neither exercise

(P=0.25) nor diet (P=0.74) significantly affected the expression of Parkin (see Figure -G) or Tfam (P= 0.17 and P=0.38 respectively, see Figure 5.6 D)

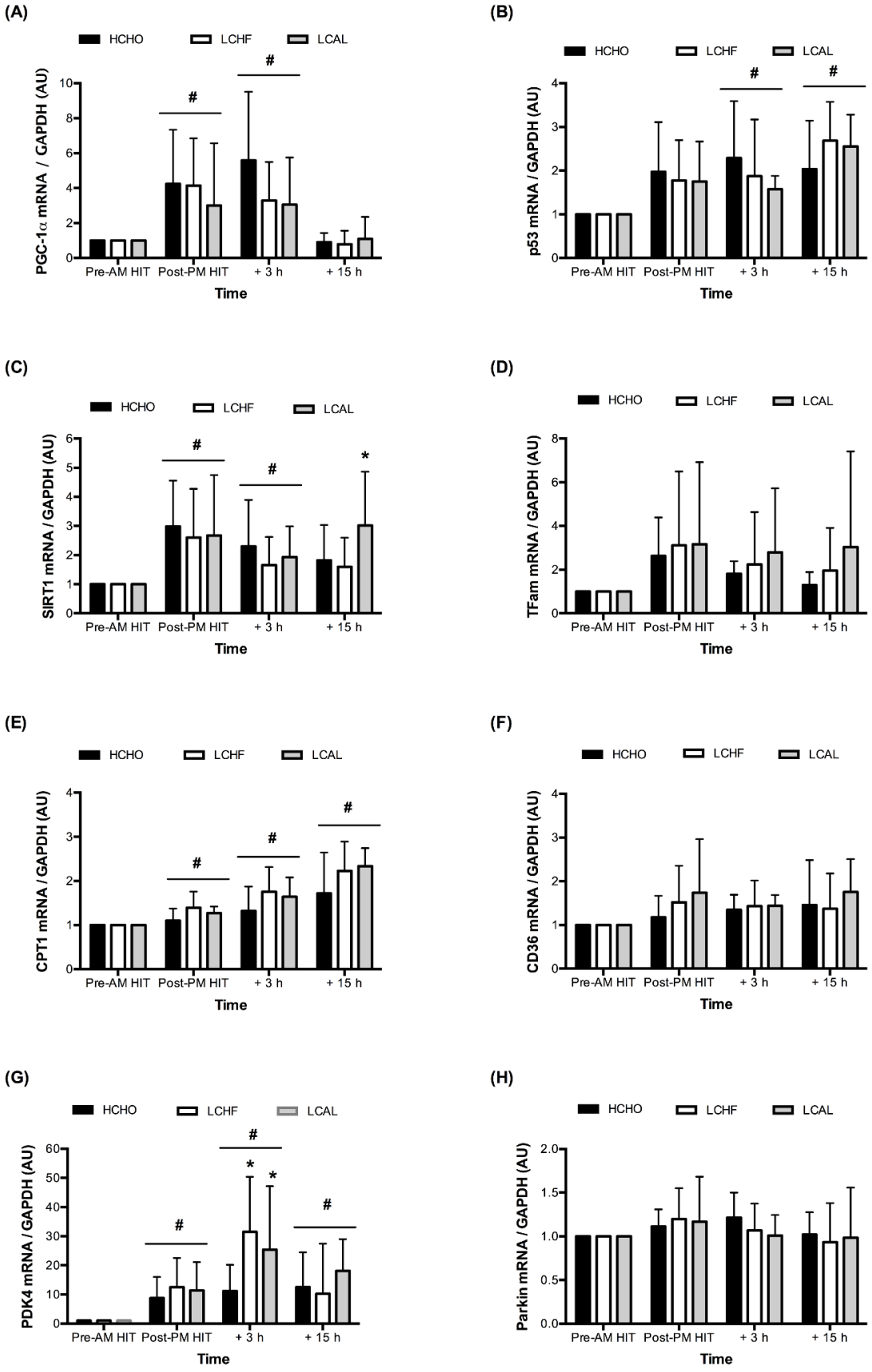

Figure 5.6 (A) PGC-1 α, (B) p53, (C) SIRT1, (D) Tfam, (E) CPT1, (F) CD36, (G) PDK4, and (H) Parkin, (I) CPT1 mRNA before AM HIT exercise and after PM-HIT exercise. # denotes significant difference from Pre-AM HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

5.5 Discussion

The aim of the present chapter was to examine the effects of post-exercise CHO and caloric restriction on the modulation of skeletal muscle cell signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism . In contrast to our hypothesis, we observed that simultaneous post-exercise CHO and energy restriction did not augment cell signalling associated with mitochondrial biogenesis and lipid metabolism when compared with conditions of high energy availability, the latter achieved through increased dietary CHO or fat intake. In recovery conditions where post-exercise muscle glycogen concentration is maintained within the range of 200-350 mmol.kg^{-1} dw, our data therefore suggest that short-term periods of acute CHO and energy restriction (i.e. <24 hours) does not potentiate the metabolic signalling pathways that regulate the hallmark adaptations associated with endurance training.

In accordance with Chapter 4 and the original train-low investigations (Hansen et al. 2005; Yeo et al. 2008; Morton et al. 2009), we employed a twice per day exercise protocol (considered representative of real-world practices often adopted by elite endurance athletes), whereby multiple training sessions are completed on the same day with limited recovery time between sessions (Fudge *et al.*, 2006). Having adhered to a high CHO diet in the day prior to all three experimental trials and after consuming a standardised high CHO breakfast, we observed no differences in cardiovascular strain, RPE, substrate availability or oxidation during the AM-HIT session. In accordance with the provision of CHO intake between training sessions during the HCHO trial, we subsequently observed higher rates of CHO oxidation during the PM-HIT when compared with both the LCHF and LCAL trials, likely reflective of the higher pre-exercise muscle glycogen availability and the provision of exogenous CHO

during exercise. Nonetheless, despite higher circulating NEFA levels in LCHF compared with the LCAL trial, we observed comparable lipid oxidation rates between the LHCF and LCAL trials, both of which were significantly higher than HCHO. Such data are in agreement with classical studies demonstrating that in high-intensity exercise conditions, elevations in circulating free fatty acid (FFA) availability does not readily translate to increased rates of whole body lipid oxidation (Romijn et al. 1995), owing to limitations in FFA uptake across the mitochondrial membrane during conditions of high glycolytic flux (Sidossis et al. 1997). Despite differences in energy availability and substrate utilisation between trials, it is noteworthy that subjects did not report increased ratings of perceived exertion during PM HIT, thus demonstrating that in relation to the chosen exercise intensities studied here, the capacity to complete consecutive high-intensity training sessions in close proximity was not compromised.

In relation to upstream cell signalling, we observed comparable p38MAPK phosphorylation immediately post PM-HIT in all three trials. Despite the potentially increased pre-exercise muscle glycogen availability and utilisation during HCHO (owing to the consumption of CHO between sessions), such a comparable signalling response is not unexpected given that the absolute post-exercise glycogen concentration (i.e. approximately 200 $mmol.kg^{-1}$ dw) was similar between trials. In accordance with comparable signalling through key upstream kinases, we subsequently observed similar magnitudes in mRNA expression of PGC-1α, p53, and CPT1 in the initial 3 hours of recovery after PM-HIT. Nonetheless, despite the sustained CHO and energy restriction for the subsequent 12 hour recovery period, we also observed that neither energy or CHO availability had no further modulatory effect on gene expression (with the exception of the nutrient sensitive gene SIRT1). Such data contrast with those of Pilegaard et al. (2005) who observed that 8 hours of post-

exercise CHO restriction (0.5 g.kg^{-1} of CHO) enhances the expression of PGC-1α, CPT1 and CD36 mRNA (when assessed in the time-scale of recovery ranging from 8-24 hours post-exercise) when compared with recovery conditions where 5 g.kg^{-1} of CHO was consumed in the initial 8 h post-exercise period. Such discrepancies between studies are likely explained by the absolute glycogen concentrations achieved by the chosen feeding protocol. Indeed, these authors suppressed muscle glycogen concentrations to ~440 vs 300 mmol.kg^{-1} dw at 8-h post-exercise in their high CHO trial compared to low CHO in contrast to the comparable changes observed here. When considered this way, our data lend further support for the glycogen threshold hypothesis (Impey et al. 2018) surmising that the acute cell signalling (Gejl *et al.*, 2017) and chronic training adaptations (Hansen *et al.*, 2005; Morton *et al.*, 2009; Yeo *et al.*, 2008) associated with "train-low" protocols typically only present when absolute glycogen concentration is depleted to <350 mmol.kg^{-1} dw. As such, the acute manipulation of both CHO and energy availability in relation to the exercise protocols and training status of the participants studied here was likely not sufficient to elicit post-exercise metabolic conditions that could be considered indicative of 'true' train-low conditions. Indeed, in previous studies from our laboratory where we utilised a "sleep low" manipulation of muscle glycogen (as achieved via evening glycogen depleting exercise followed by overnight CHO and energy restriction), we observed that differences in muscle glycogen availability of 400 versus 100 mmol.kg^{-1} dw was associated with enhanced expression of PGC-1α, CPT1 and Tfam mRNA. Future studies should therefore adopt experimental designs and feeding protocols which more readily achieve distinct differences in acute and chronic glycogen availability.

In summary, we provide novel data by demonstrating that in conditions where post-exercise muscle glycogen concentration is maintained within the range of 200-350

mmol.kg^{-1} dw, short-term periods of acute CHO and energy restriction (i.e. <24 hours) does not potentiate skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism.

Chapter 6

The effects of post-exercise CHO and caloric restriction on markers of bone turnover, inflammation and appetite regulation

6.1 Abstract

Purpose: To examine the effects of post-exercise CHO restriction and caloric restriction on markers of bone turnover, inflammation and appetite regulation. **Methods:** Nine males completed a twice per day exercise model (3.5 h between sessions) comprising a morning and afternoon high-intensity interval (HIT) (8 x 5-min at 85% $\dot{V}O_{2peak}$) running protocol. In a repeated measures crossover design, runners exercised under three different dietary conditions consisting of 1) high CHO availability (HCHO: ~ 5030 kcals, CHO ~12.6, protein ~ 2.3, and fat ~0.6 $g.kg^{-1}$ per whole trial period), 2) reduced CHO but high fat availability (LCHF: ~ 5021 kcals, CHO ~3, protein ~ 2.3, and fat ~3 $g.kg^{-1}$ per whole trial period), or 3) reduced CHO and reduced energy availability (LCAL:~ 2714 kcals, CHO ~3, protein ~ 2.3, and fat ~0.4 $g.kg^{-1}$ per whole trial period). **Results:** βCTX responses to exercise were significantly lower across all time points in the post-exercise period in HCHO (P=0.035) compared to LCHF and LCAL. Both AM-HIT (P=0.001) and PM-HIT (P=0.005) significantly increased P1NP responses but there was no difference between trials (P=0.633). IL-6 responses to exercise were significantly higher in LCAL (P = 0.016) post PM-HIT compared to LCHF and HCHO. Repeated exercise bouts increased IL-6 at 3-h post-exercise in all three trials (P = 0.009). Circulating leptin levels were significantly lower (P = 0.04) in LCAL compared to HCHO in the post exercise sampling period. There was no difference in the short-term response of ghrelin to feeding (P= 0.408) with increases following both AM-HIT (P = 0.001) and PM-HIT (P = 0.025) in all three trials. **Conclusion:** Consuming CHO before, during and after HIT running attenuates circulating β-CTX concentrations in the hours after exercise, effects that are independent of energy availability. In contrast, energy availability (but not CHO availability) modulates the regulation of post-exercise circulating leptin and IL-6 concentrations.

Keywords: βCTX, IL-6, Caloric restriction, bone responses

6.2 Introduction

The concept of training with reduced CHO availability (the so-called train low paradigm) in an attempt to promote oxidative adaptations of skeletal muscle is now becoming an accepted component of nutritional periodization for elite athletes (Burke *et al*. 2018; Stellingwerff *et al*. 2019). Although various models of CHO periodization exist (see Burke et al. 2018 and Chapter 2), the practice of training twice per day is most relevant for elite runners owing to typical training practices of training multiples times per day (Fudge *et al.* 2006). Whilst the adaptive responses of skeletal muscle are apparently dependent on the absolute range of muscle glycogen concentration (see Impey *et al.* 2018 and Chapter 5), it is also possible that the acute and transient fluctuations in both CHO and energy availability in the twice per day training model can exert regulatory effects on other physiological systems, many of which are associated with Relative Energy Deficiency in Sport (RED-S) syndrome (Mountjoy *et al.*, 2014). As such, whilst train-low and/or energy restriction may be beneficial for certain aspects of training adaptation, the conscious or unconscious (as a default of the athlete's habitual training structure and daily pattern of feeding) manipulation of CHO and energy availability may have negative implications for the acute regulation of bone turnover (Ihle and Loucks, 2004), immune function (Nieman, 2007), endocrine function (Loucks *et al.*, 1998; Hagmar *et al.*, 2013) and appetite regulation (Considine *et al.*, 1996), the result of which could impair athletic performance if performed chronically.

The effects of energy availability on bone turnover is especially pertinent for runners owing to the increased risk of stress fractures in this population (Bennell *et al.*, 1996;

Townsend, 2016 unpublished observations). Indeed, prolonged and intense weight bearing exercise increases bone resorption (as evident by increases in C terminal telopeptide of type 1 collagen (β-CTX)) by 40-45% for up to four consecutive days (Peake *et al.*, 2015). Early research evidenced that both acute (Grinspoon *et al.*, 1995) and chronic (Grinspoon *et al.*, 1997) dietary energy restriction is associated with impaired bone remodelling and reduced bone formation. Interestingly, feeding a mixed macronutrient meal prior to exercise attenuates markers of bone breakdown (Scott *et al.*, 2012), thus suggesting energy and/or CHO availability influences the interaction between training and bone turnover. Support for the latter has been provided by those studies demonstrating that CHO ingestion before (de Sousa et al. 2014) and during (Sale et al. 2015) exercise also attenuated the exercised induced increase in bone breakdown when compared to a placebo.

In relation to energy availability, values below 30 $kcal.kg^{-1}$ fat free mass (FFM).day^{-1} is associated with the suppression of key metabolic hormones such as insulin and leptin (Hilton & Loucks, 2000). Indeed, in severely energy restricted (10kcal/kg FFM) individuals in a short-term (4-days) energy deficit, significant suppression of plasma leptin levels are observed (Hilton & Loucks, 2000), the result of which can affect appetite regulation (Considine *et al.*, 1996) and endocrine responses (Hagmar *et al.*, 2013). Such responses are also associated with disruptions in bone turnover (Zanker & Cooke, 2004) and the regulation of bone mass (Thomas & Burguera 2002). Prolonged endurance exercise also increases circulating inflammatory cytokines such as IL-6 (released from both muscle and immune cells) in the post-exercise recovery period (Hennigar *et al.*, 2017), the magnitude of which correlates with increases in markers of bone breakdown (Sale *et al.*, 2015). IL-6 is thought to respond to the glycogen status of the muscle during exercise, and as such commencing exercise with

sufficient glycogen availability attenuates the exercise-induced increases in circulating IL-6 (Steensberg *et al.*, 2001). This is of particular importance in those instances where athletes train multiple times per day (such as the original twice per day train low models) given that recovery could be considered sub-optimal in between training sessions. When taken together, such data clearly highlight that acute fluctuations in energy availability in close proximity to the exercise stimulus can affect acute bone turnover, the result of which may lead to chronic adaptations in bone mass. Nonetheless, it is not yet known whether it is the total energy availability or more specifically, CHO (and indeed glycogen) availability per se that is the dominant factor in regulating many of the aforementioned parameters. The aim of the current chapter was to therefore investigate the effects of post-exercise CHO and calorie restriction on markers of bone turnover, inflammation and appetite regulation. In using an identical research design as the previous chapter, we aimed to test the hypothesis that CHO availability exerts regulatory roles on such parameters that are independent of energy availability.

6.3 Methods

6.3.1 Subjects: Nine trained male runners volunteered to participate in the study (mean ± SD: age, 22 ± 2.1 years; body mass, 71 ± 6.7 kg; height, 175.9 ± 6 cm; $\dot{V}O_{2peak}$, 57 ± 4.6 $ml \cdot kg^{-1} \cdot min^{-1}$). All subjects gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. None of the subjects had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment over the course of the testing period. Subjects were instructed to refrain from any strenuous physical activity, alcohol and caffeine consumption in the 48h prior to each experimental trial. The study was approved by the ethics committee of Liverpool John Moores University.

***6.3.2 Design*:** In a repeated measures, randomised, cross-over design separated by 7 days, subjects completed a twice per day exercise model under three different dietary conditions, consisting of either high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best practice nutrition) or alternatively, reduced CHO but high fat availability (LCHF), or reduced CHO and reduced calorie availability (LCAL). All three dietary conditions were matched for protein content, LCHF and HCHO matched for total energy intake, and LCHF and LCAL matched for CHO intake. The twice per day exercise model comprised a morning (9-10 am) high-intensity interval (AM-HIT) training session (8 x 5-min at 85% $\dot{V}O_{2peak}$) followed by an afternoon (130-230 pm) training session consisting of the same high-intensity interval exercise bout (PM-HIT). To promote training compliance during the AM-HIT protocol in all three trials, subjects adhered to a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery between the HIT sessions and in the recovery period upon completion of the PM-HIT exercise protocol until the subsequent morning, subjects adhered to either the HCHO, LCHF, or LCAL feeding

protocol. An overview of the experimental design and nutritional protocols are shown in Figure 6.1.

***6.3.3 Preliminary testing*:** At least 7-10 days prior to the first main experimental trial, subjects performed a combined running economy and maximal incremental running test to volitional fatigue on a motorised treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany). As such, both peak oxygen uptake and individual running speeds for the subsequent experimental trials could be determined as outlined in Chapter 3. These measurements were recorded via breath-by-breath gas measurements obtained continuously throughout both tests using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US), with heart rate (Polar, Kempele, Finland) also recorded continuously during exercise.

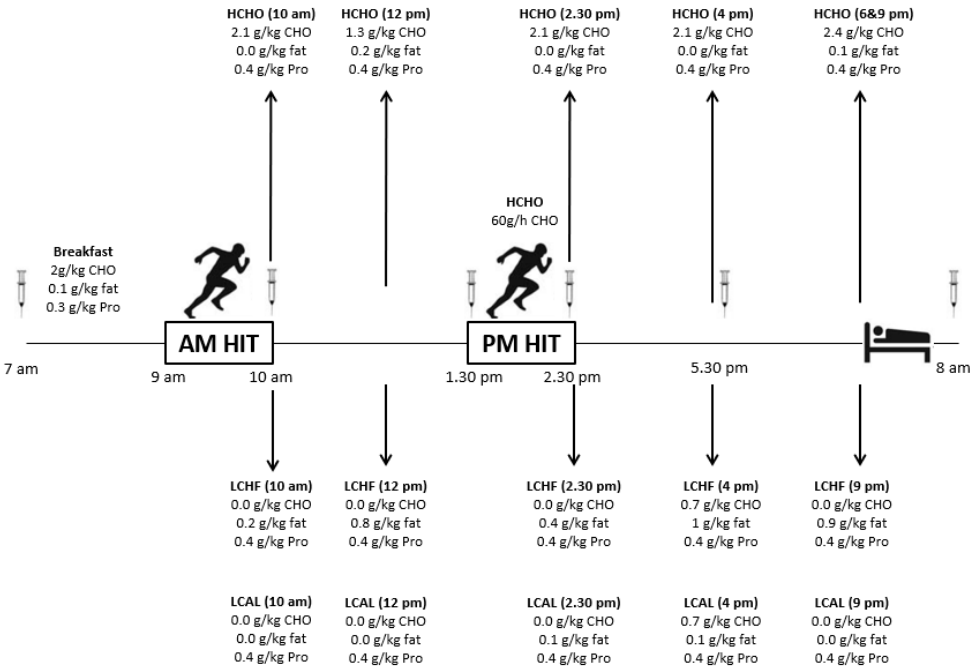

Figure 6.1 Overview of the experimental protocol employed in each trial. HIT = 8 x 5-mins running at a workload equal to 85% $\dot{V}O_{2peak}$ interspersed by 1-min recovery.

6.3.4 Experimental protocols:

***6.3.4.1 AM HIT protocol*:** In the 24-h preceding each main experimental trial, subjects consumed a standardised high CHO diet in accordance with typical nutritional recommendations (8 g.kg^{-1} CHO, 2 g.kg^{-1} protein, and 1 g.kg^{-1} fat). On the morning of each experimental trial, subjects reported to the laboratory at ~7 am where a venous blood sample was collected from an antecubital vein in the anterior crease of the forearm. Subjects were then given a standardised high-CHO breakfast (2 g.kg^{-1} CHO, 0.3 g.kg^{-1} protein, and 0.1 g.kg^{-1} fat), and at 2-h post-prandial. Subjects were then fitted with a heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the first high intensity interval running (AM-HIT) protocol which lasted ~1-h. The HIT protocol consisted of 8 x 5-min bouts running at a velocity corresponding to 85% $\dot{V}O_{2peak}$ interspersed with 1-min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm up and cool down at a velocity corresponding to 50% $\dot{V}O_{2peak,}$ and a further venous blood sample was obtained immediately upon completion of the protocol. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, Borg, 1973) were obtained upon completion of each HIT bout. In order to determine substrate utilisation during exercise (Jeukendrup and Wallis, 2005), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2-mins of each 5-min interval.

***6.3.4.2 PM HIT protocol*:** During the 3.5 h recovery period between the AM HIT and PM HIT protocols, subjects consumed either the HCHO (3.5 g.kg^{-1} CHO, 0.8 g.kg^{-1} Protein, 0.2 g.kg^{-1} Fat), LCHF (0 g.kg^{-1} CHO, 0.8 g.kg^{-1} Protein, 1.2 g.kg^{-1} Fat), or

LCAL (0 g.kg^{-1} CHO, 0.8 g.kg^{-1} Protein, 0.1 g.kg^{-1} Fat) feeding protocols (the pattern and frequency of feeding is shown in Figure 1). Following the recovery period, another venous blood sample was obtained immediately prior to commencing the afternoon HIT exercise protocol. After a 10-min warm up at a velocity corresponding to 50% $\dot{V}O_{2peak}$, subjects subsequently commenced the PM HIT protocol. During exercise, subjects also consumed 60 g.h^{-1} of CHO (SiS GO Istonic Gels, Science in Sport, Blackburn, UK) in HCHO whereas no form of energy was consumed in the LCHF or LCAL trials. Again, water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were also collected for the last 2-mins of each 5-min interval throughout the exercise trial (CPX Ultima, Medgraphics, Minnesota, US) and substrate utilisation again determined according to Jeukendrup and Wallis (2005). Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, 9) were obtained at the end of each HIT bout. Upon completion of the PM HIT protocol until sleep, subjects consumed either the HCHO (6.4 g.kg^{-1} CHO, 1.3 g.kg^{-1} Protein, 0.3 g.kg^{-1} Fat), LCHF (0.8 g.kg^{-1} CHO, 1.3 g.kg^{-1} Protein, 2.3 g.kg^{-1} Fat), or LCAL (0.8 g.kg^{-1} CHO, 1.3 g.kg^{-1} Protein, 0.1 g.kg^{-1} Fat) feeding protocols where the pattern and frequency of feeding is shown in Figure 6.1 . Venous blood samples were also collected immediately post- and at 3 h and 15 h post completion (i.e. ~8 am and in a fasted state) of the PM-HIT exercise protocol. The total energy intake across the whole trial period (i.e 7 am – 9 pm) in HCHO was: ~12.6 g.kg^{-1} CHO, ~2.3 g.kg^{-1} Protein and ~0.6 g.kg^{-1} Fat, in LCHF was: ~3 g.kg^{-1} CHO, ~2.3 g.kg^{-1} Protein and ~3 g.kg^{-1} Fat (where both trials were matched for total energy intake), and in LCAL was ~3 g.kg^{-1} CHO, ~2.3 g.kg^{-1} Protein and ~0.4 g.kg^{-1} Fat (where CHO intake was matched with HFAT).

6.3.5 Energy Availability

Energy availability was estimated for each experimental trial as calculated from: energy intake minus energy expenditure during AM-HIT and PM-HIT exercise / fat free mass (FFM) (Mountjoy *et al.*, 2014). Whilst FFM was not directly measured, we based estimates of FFM on the assumption that all participants were ~15% body fat (as based on unpublished data from our laboratory examining male participants of similar body mass, training history and maximal oxygen uptake, Impey *et al.*). The total energy expenditure from both AM-HIT and PM-HIT in HCHO was 1474 ± 107 kcal, in LCHF was 1429 ± 142 kcal and in LCAL was 1455 ± 190 kcal. Estimated energy availability across the whole trial period in HCHO was 58 ± 5.3 $kcal.kg^{-1}$ FFM, in LCHF was 59 ± 6.4 $kcal.kg^{-1}$ FFM, and in LCAL was 20 ± 4.1 $kcal.kg^{-1}$ FFM.

6.3.6 Blood sampling and analysis: Venous blood samples were collected and analysed for plasma insulin, β-CTX, P1NP, adiponectin, leptin, and ghrelin as previously described in Chapter 3, Section 3.0.

6.3.7 Flow Cytometry: Plasma IL-6 was determined using Flow Cytometry as previously outlined in Chapter 3, Section 3.8.3.

6.3.8 Statistical analysis: All data were analysed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA) using a two-way repeated-measures general linear model, where the within factors were time and condition. Post hoc LSD tests were used where significant main effects and interactions were observed in order to locate specific differences between time points and conditions). The area under the curve (AUC) was also calculated for all variables measured. All data in text, figures and tables are presented as mean ± SD, with P values ≤ 0.05 indicating statistical significance.

6.4 Results

6.4.1 Plasma insulin

Consistent with CHO feeding before and after PM HIT, plasma insulin concentration was significantly higher (P=0.001) immediately post, 3-h post, and 15-h post in HCHO compared with LCHF and LCAL. Additionally, insulin concentration significantly changed over time (P<0.001) such that significant differences from Pre- AM HIT were evident immediately post (P<0.001), and 3-h after completion of the PM HIT session (P=0.024). Insulin AUC was also significantly greater in HCHO compared with the LCHF and LCAL trial (P<0.001).

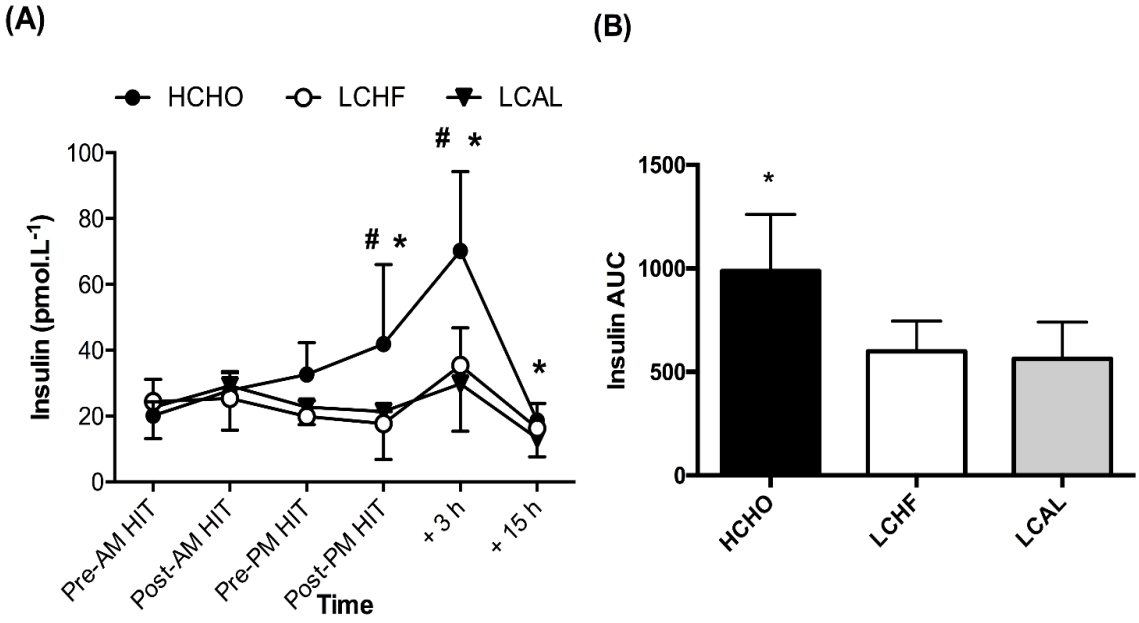

Figure 6.2 (A) Plasma insulin before and after the AM-HIT and PM-HIT protocols and (B) AUC for insulin. # denotes significant difference from Pre- AM-HIT , P<0.05. * denotes significant difference between conditions, P<0.05

6.4.2 Bone responses

Exercise induced significant increases in P1NP (P<0.01) following both AM-HIT (P=0.011) and PM-HIT (P=0.005), then decreasing at 15-h post-exercise (P=0.002). No differences were apparent between trials (P=0.633). Exercise induced significant reductions in βCTX (P<0.001) following AM-HIT (P<0.001). Levels were then significantly increased following PM-HIT (P<0.001) concomitant to increases in formation. βCTX was significantly lower immediately post- and 3-h post-exercise in HCHO compared to HFAT and LCAL (P=0.035). AUC analysis showed no differences between the three feeding conditions for P1NP (P=0.9) or βCTX (P=0.5)

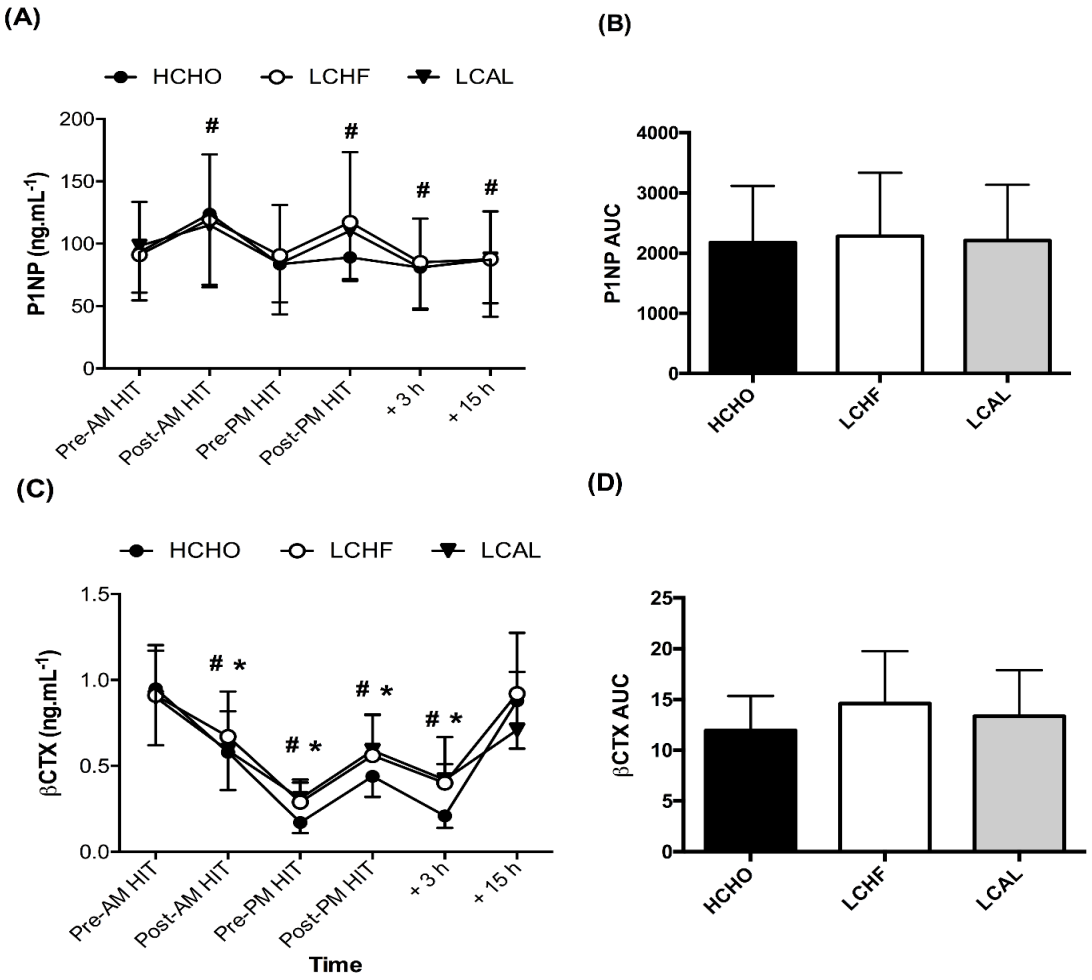

Figure 6.3 (A) Plasma P1NP concentrations and (B) AUC. (C) Plasma βCTX concentrations and (D) AUC before AM-HIT exercise and after PM-HIT exercise. # denotes significant difference from Pre-AM-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

6.4.3 Appetite hormone responses

Whilst exercise did not affect circulating leptin concentration (P=0.72), leptin was significantly lower (P=0.041) in LCAL compared to HCHO in the post exercise sampling period.. There was a tendency for differences in leptin concentration between HCHO and LCHF (P=0.057) though no differences were apparent between LCAL and LCHF (P=0.27). Exercise significantly reduced ghrelin concentration to comparable levels in all three trials following both AM-HIT (P = 0.001) and PM-HIT (P = 0.025), with no significant differences between conditions (P=0.408). Ghrelin concentration returned to baseline levels at 17-h post PM-HIT in all conditions (P = 0.392). Neither exercise (P=0.524) nor dietary condition (P=0.156) significantly affected adiponectin concentration throughout the sampling period.

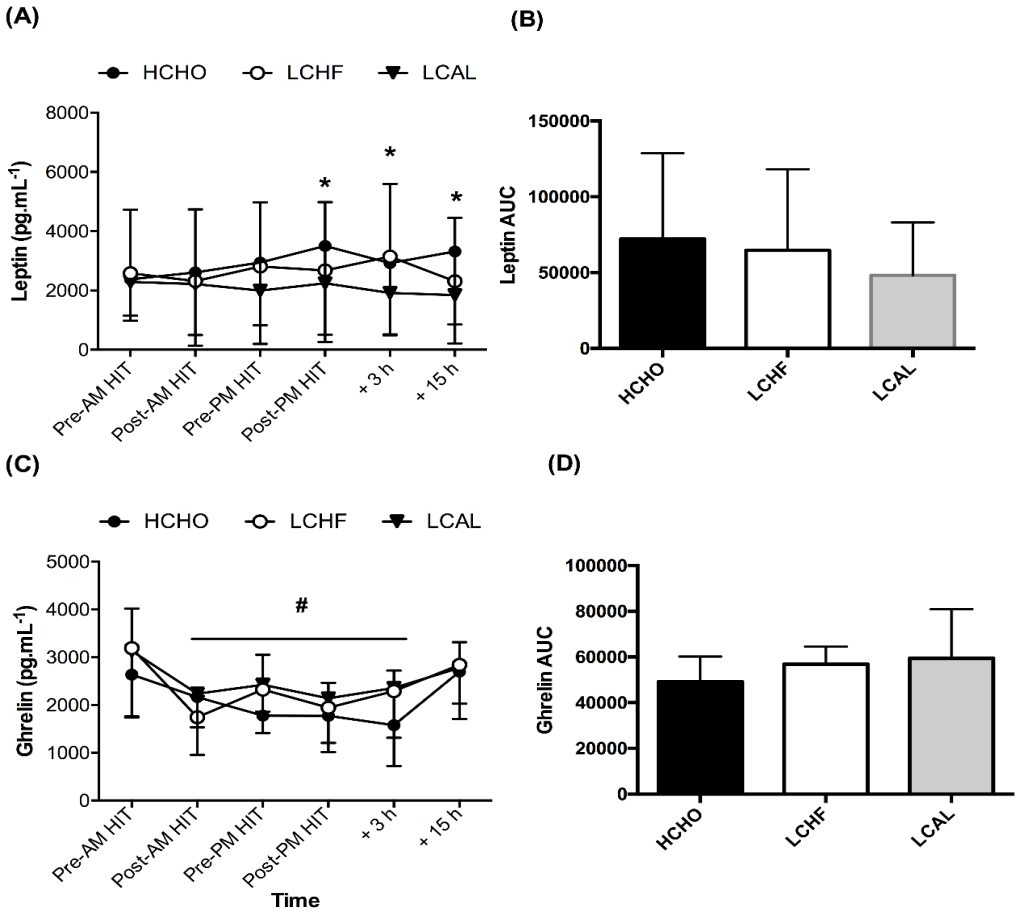

Figure 6.4 (A) Plasma Leptin and (B) AUC. (C) Ghrelin concentrations and (D) AUC before AM-HIT exercise and after PM-HIT exercise. # denotes significant difference from Pre- AM-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

6.4.4 Interleukin-6 (IL-6) responses

Immediately post PM-HIT (P=0.016) and at 3-h post PM-HIT (P=0.009) IL-6 was significantly elevated compared to baseline. This increase in was significantly higher in LCAL (P =0.016) compared to LCHF and HCHO. There were no differences in post-exercise elevations in IL-6 between LCHF and HCHO (P=0.145). Plasma IL-6 concentrations returned back to baseline levels at 15-h post-PM-HIT in all three experimental trials. AUC analysis showed no differences between the three feeding conditions (P=0.58).

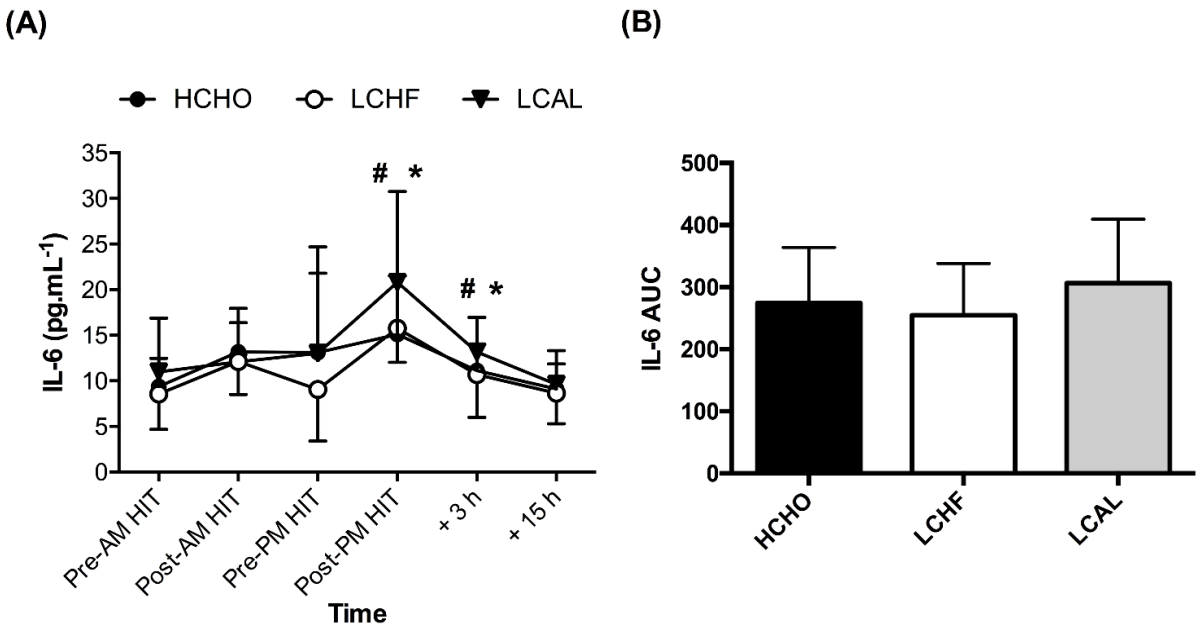

Figure 6.5 Plasma IL-6 concentrations before AM-HIT exercise and after PM-HIT exercise (n = 7). # denotes significant difference from Pre- AM-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

6.4.5 Adiponectin responses

Neither exercise (P = 0.524) or condition (P = 0.156) significantly affected adiponectin levels throughout the sampling period.

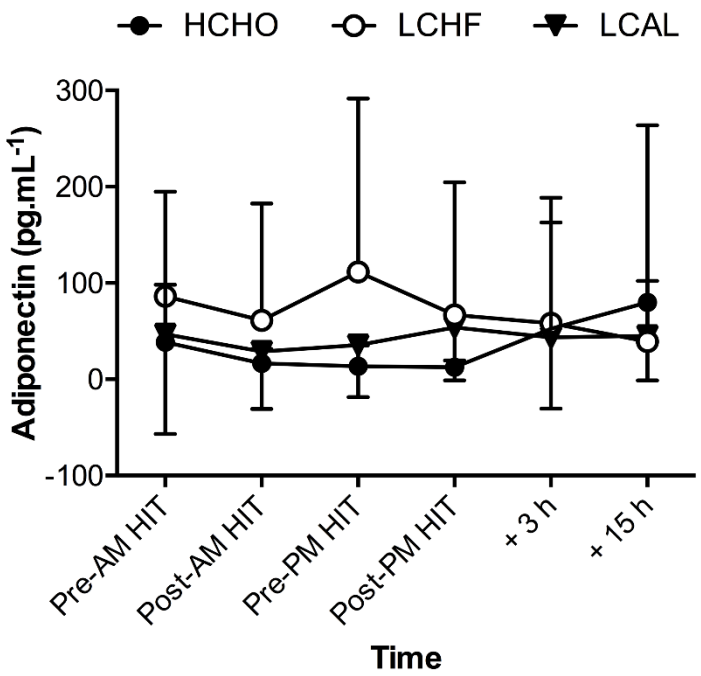

Figure 6.6 Plasma Adiponectin concentrations before AM HIT exercise and after PM HIT exercise.

6.5 Discussion

The aim of the current chapter was to investigate the effects of post-exercise CHO and calorie restriction on markers of bone turnover, inflammation and appetite regulation. Confirming our hypothesis, we provide novel data by demonstrating that consuming CHO before, during and after HIT running attenuates circulating β-CTX concentrations in the hours after exercise, effects that are independent of energy availability. In contrast, energy availability (but not CHO availability) modulates the regulation of post-exercise circulating leptin and IL-6 concentrations. From a practical perspective, such data demonstrate that the acute within day fluctuations in both energy and CHO availability that are inherent to twice per day training protocols may contribute to the development of symptoms associated with the RED-S syndrome.

In accordance with the previous study designs in chapters 4 and 5, we employed the twice per day exercise model that is considered representative of the typical training structures of elite runners (Fudge *et al.,* 2006; Stellingwerff 2014). The AM-HIT protocol caused a significant decrease in bone turnover in the recovery period post-AM-HIT under all three experimental conditions, as indicated by decreases in P1NP and β-CTX concentrations to levels below baseline. Such comparable responses to the initial morning training session is unsurprising owing to the similar CHO intakes in both the 24 h control diet and breakfast consumed prior to the session. In contrast, subsequent ingestion of CHO in the 3-h recovery period prior to PM-HIT caused a greater suppression in β-CTX compared with those conditions where energy was restricted (LCAL) or matched by consuming the isocaloric high fat diet (LCHF). Similarly, when CHO was ingested during PM-HIT and in the subsequent 3-h recovery period, there was a greater suppression in β-CTX immediately post- and 3-h

post-exercise. Since no differences were observed in β-CTX between LCHF and LCAL, it would appear that it is the energy from CHO *per se* rather than overall energy availability which is important in minimising bone resorption following a training session. In agreement with previous studies, bone formation marker P1NP was less responsive to the acute exercise bouts when compared to the bone resorption marker, β-CTX (Scott *et al.*,2010; Scott *et al.,* 2012). This suggests that intense training could potentially lead to an uncoupling or imbalance in bone turnover, favouring increases in bone resorption. If these effects were repeated over subsequent training sessions, (which is likely to be the case for elite endurance athletes), this could lead to detrimental effects on bone mass and health (Ihle & Loucks, 2004) thus increasing the risk of stress fracture injuries.

The finding that CHO availability attenuates circulating β-CTX are similar to previous researchers who also observed that consuming an 8% CHO solution at a rate of 0.7g/kg/h immediately before, and every 20-mins during a prolonged bout of running (120-mins at 70% of max) induced a greater reduction in β-CTX compared with a placebo solution (Sale *et al.,* 2015). Furthermore, when examining the effects of feeding during the post-exercise recovery period, the same research group also demonstrated that a mixed CHO and protein recovery drink immediately following an exhaustive run significantly suppressed β-CTX when compared to a placebo solution (Townsend *et al.,* 2017). Interestingly, these authors demonstrated that this suppression was greater when the high CHO solution was ingested immediately upon completion of the running protocol rather than delayed feeding (2-h post-exercise). This rapid response is particularly important where recovery time may be short between training sessions and where there is limited time for food consumption. Findings from the latter authors also demonstrate that bone formation was at its highest

and resorption at its lowest at ~3-4-h post-exercise when CHO was ingested immediately (Townsend *et al.*, 2017). Consistent with these observations, we also observed that participants commenced PM-HIT (~3-h following AM-HIT) with lower concentrations of β-CTX in HCHO compared to LCHF and LCAL. Therefore, since the real-world practices of elite endurance athletes typically constitute multiple training sessions per day often with only 2-4-h recovery between sessions, immediate CHO feeding following the first session is perhaps the most important focus. Future studies should now examine the dose response relationship by which CHO ingestion exerts such regulatory roles.

The potential mechanisms for suppression of bone resorption by CHO feeding are still not fully understood, however previous research suggests that the short-term changes in bone turnover may be due to the direct effects of hypoglycaemia on bone cells or changes in the concentration of regulatory hormones such as insulin (Clowes *et al.*, 2002). Previous research from Clowes et al. (2002) employed a hypoglycaemic clamp technique where glucose was either lowered to 2.5 mmol/L or maintained at 5 mmol/L. Data demonstrated no alterations in bone turnover marker P1NP under the euglycemic condition, however when glucose was clamped at 2.5 mmol/L, P1NP was suppressed. It is likely that these changes are mediated in part by circulating insulin concentrations. Indeed, in the current study insulin concentrations were significantly higher during the post exercise recovery period (following both AM-HIT and PM-HIT) in HCHO compared to LCHF and LCAL, corresponding to the suppression of β-CTX in this trial. A decline in insulin may also act to reduce circulating leptin concentrations, as has been observed in response to acute short-term energy deficit (Hilton & Loucks, 2000).

Although leptin has been suggested as a potential mediator to bone turnover responses (Thomas & Burguera, 2002; Holloway *et al.*, 2002), CHO availability did not significantly alter circulating leptin levels in the current study. This is in agreement with findings from Sale et al. (2015) who also observed that CHO feeding during exercise suppressed β-CTX post-exercise, effects that were independent of changes in leptin. In agreement with previous findings demonstrating leptin suppression with low energy availability (Hilton & Loucks, 2000), we observed significant reductions in circulating leptin concentrations independent of exercise in the 15-h following exercise in LCAL compared to HCHO and HFAT. Consistent with the estimate of energy availability studied here, Koehler and colleagues (2016) also reported reduced levels of leptin following both cycling based exercise and at rest under conditions of 4-days low energy availability (15 kcal.kg^{-1} FFM/day) when compared with 4-days normal levels of energy availability (40 kcal.kg^{-1} FFM/day), and also reported no changes in circulating ghrelin. Chan et al. (2003) reported that a reduction in circulating leptin is directly linked to the suppression of growth hormone IGF-1 and the thyroid axes, which may serve as a metabolic signal of starvation and energy conservation. Indeed, research also indicates that muscle protein synthesis is reduced at energy availabilities of 30 kcal.kg^{-1} FFM/day (Areta *et al.*, 2013). When considered this way, our data demonstrate that total energy availability (but not CHO availability) is the more dominant factor in modulating components of appetite regulation when training under conditions representative of the twice per day train low model.

Prolonged strenuous exercise is often characterized by increases in circulating levels of the inflammatory cytokine IL-6 with levels typically peaking immediately post-exercise (Fischer, 2006). Interestingly, CHO feeding during running attenuates post-exercise IL-6 and a strong correlation exists between changes in IL-6 and β-CTX in

the post-exercise period (Sale et al. 2015). As such, the latter authors postulated that IL-6 may regulate bone turnover via stimulating osteoclastogenesis and bone resorption. In the present chapter, we observed that AM-HIT did not significantly increase circulating IL-6 levels above baseline in any of the three experimental trials. This is likely due to participants commencing AM-HIT under conditions of high CHO and high energy availability in all three dietary conditions, consistent with the notion that the release of IL-6 from exercising muscle is related in part to pre-exercise glycogen availability (Steensberg *et al.,* 2001). However, the repeated bout of high intensity running in PM HIT significantly increased circulating IL-6 in all three experimental conditions, the magnitude of which was greater in LCAL compared to LCHF and HCHO.

Whilst the effects of both endogenous (Steensberg *et al.,* 2001; Keller *et al.,* 2001; Chan *et al.,* 2004) and exogenous (Nieman *et al.,* 1998; Nieman *et al.,* 2003; Febbraio *et al.,* 2003) CHO availability on regulation of exercise-induced IL-6 production is well documented, the present data could also suggest that energy availability plays a more dominant regulatory role in mediating IL-6 responses, as compared with CHO availability *per se*. In contrast, an alternative interpretation is that high fat availability in the LCHF trial (despite also being low in absolute CHO) may have suppressed IL-6 production to levels that were comparable to the HCHO trial. In this regard, Badenhorst et al. (2016) examined the effects of isocaloric diets containing high CHO and low fat, or moderate CHO and high fat on IL-6 responses to exercise over a 7-day endurance training block in runners. Serum IL-6 concentrations were measured post-exercise on day 7, and although levels increased following exercise, there were no significant differences between the two dietary conditions. Additionally, there is also evidence to suggest that ω-3 polyunsaturated fatty acids may suppress IL-6

concentrations post-exercise (lengthening contractions of the elbow flexors) and thus reduce the muscle damaging effect of training (Phillips *et al.*, 2003; DiLorenzo *et al.*, 2014). When taken together, these data suggest that both macronutrient composition and energy availability may regulate IL-6 responses to exercise, the result of which may have implication for the acute regulation of bone turnover.

In summary, we provide novel data by demonstrating that nutritional interventions that promote high CHO availability before, during, and in recovery from exercise appear to be of greater importance for the acute regulation of bone turnover when compared with energy intake *per se.* In contrast, energy availability (from both CHO and fat) appears a more influential factor in regulating both IL-6 and leptin responses in recovery from exercise. In an attempt to develop optimal approaches to CHO periodisation that reduce the risk of developing negative consequences associated with RED-S, future studies should now examine the long-term implications of the twice per day train low protocol when performed as part of a chronic training programme.

Chapter 7

Synthesis of findings

The aim of this chapter is to provide an overview of the experimental findings in relation to the original aims and objectives set out in Chapter 1. A general discussion is then provided whereby specific attention is given to how the data presented have advanced our understanding of the effects of macronutrient and energy availability on metabolic responses to endurance exercise training. A discussion of the practical implications of the present data is also provided as well an overview of experimental limitations and directions for future research. Due to the aims of Study 2 changing during the course of the PhD, discussion of additional measures which could have also been included to contribute to the field are also provided.

7.1 Achievement of aims and objectives

The primary aim of this thesis was to determine the effects of macronutrient and energy availability (with a specific emphasis on manipulation of CHO and fat) on the regulation of molecular signalling pathways associated with exercise-induced mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. A secondary aim was to examine the effects of such feeding strategies on markers of bone turnover, inflammation and appetite regulation given the relevance of such parameters in contributing to symptoms of RED-S.

Objective 1 **- To examine the effects of post-exercise CHO restriction but high fat feeding on skeletal muscle cell signalling and gene expression associated with the regulation of mitochondrial biogenesis, lipid metabolism, and MPS (Study 1, Chapter 4).**

It was established in Study 1 that although feeding high fat in the recovery period post-exercise increased the expression of genes involved in lipid transport and oxidation,

high fat availability exerted no modulatory effect on the acute signalling pathways regulating mitochondrial biogenesis. High fat feeding also suppressed the activity of p70S6K1 and increased the expression of PDK4. These findings indicate that although high fat feeding increases the potential for lipid oxidation, feeding of this type may be detrimental to the regulation of MPS and muscle remodelling in the post-exercise recovery period, and may negate the ability to oxidise CHO as a fuel source during high intensity exercise. As such, these data are suggestive of maladaptive responses for training adaptation if high fat feeding strategies are employed in the long-term. In contrast to the findings from previous investigators, restricting CHO availability in the post-exercise recovery period had no modulatory effect on the expression of those genes associated with regulatory roles in mitochondrial biogenesis when combined with high fat feeding and where overall energy intake was matched between HCHO and HFAT trials.

Objective 2 **- To examine the effects of post-exercise CHO and caloric restriction on skeletal muscle cell signalling and gene expression associated with the regulation of mitochondrial biogenesis and lipid metabolism (Study 2, Chapter 5).**

Having identified no differences in the expression of genes involved in the regulation of mitochondrial biogenesis between energy matched HCHO and HFAT conditions in Study 1, Study 2 assessed whether the enhanced mitochondrial biogenesis responses typically observed when "training-low" is due to CHO restriction *per se*, or rather due to a reduction in energy availability. However, the data presented in this study indicated no differences between reduced CHO availability and reduced energy availability on the expression of those genes with putative roles in mitochondrial

biogenesis. Similar to Study 1, Study 2 also highlighted that the restriction of CHO both with and without energy restriction augments PDK4 expression, thus suggesting that acute periods of train-low may impair the ability to use CHO as a fuel source, thereby potentially causing decrements in performance if performed long-term. Ultimately, these data demonstrate that under conditions where muscle glycogen is maintained within the range of 200-350 mmol.kg^{-1} dw, short-term periods of acute CHO and energy restriction (i.e. <24-h) does not potentiate skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism. As such, we suggest a potential critical level of muscle glycogen which must be reached in order to augment the muscle adaptive responses to training that occur under "true" train low conditions.

Objective 3 **- To examine the effects of post-exercise CHO restriction and caloric restriction on markers of bone turnover, inflammation and appetite regulation (Study 3, Chapter 6).**

Whilst the acute manipulations of CHO and energy availability did not likely achieve absolute glycogen concentrations sufficient to constitute "true" train-low conditions, it is possible that such alterations in dietary intake may regulate other aspects of physiological function, many of which are associated with symptoms of RED-S. Accordingly, the data presented in Study 3 demonstrates that the acute within day manipulation of CHO availability during twice per day training exerts regulatory effects on bone turnover, effects that are independent of energy availability. Additionally, training with reduced energy availability during this experimental model exerts regulatory roles on both IL-6 and leptin, effects that appear independent of CHO availability.

7.2 General discussion of findings

Endurance training results in a number of physiological and metabolic adaptations which function to delay the onset of fatigue and thus ultimately increase exercise capacity. Functionally, these adaptations manifest as an increase in maximal oxygen uptake and the classic rightward shift in the lactate threshold curve (Holloszy and Coyle, 1984). The most prominent adaptation to endurance exercise training is often considered as the increase in the size and number of mitochondria (i.e. mitochondrial biogenesis) within the muscle (Holloszy, 1967) which essentially permits a closer matching between ATP requirement and production via oxidative metabolism. The adaptive response of muscle mitochondria is also accompanied by increases in capillary density, the abundance of substrate transport proteins, and an increase in metabolic enzyme activity. Furthermore, endurance training increases the capacity for skeletal muscle to store glycogen and triglycerides, thereby increasing substrate availability. The molecular mechanisms underpinning these adaptive responses to endurance training are now becoming increasingly well understood. Upon the onset of muscle contraction, the accumulation of multiple metabolic signals generated during exercise (i.e., increased AMP/ATP ratio, Ca^{2+} flux, lactate, hypoxia and energy availability) initiates a cascade of events that activate or suppress specific signalling pathways that regulate gene expression and protein translation. The dynamic fluctuation in content and subcellular location of metabolites activates regulatory cell signalling kinases that converge on nuclear and mitochondrial transcription factors, and co-activators to induce a co-ordinated up-regulation of both nuclear and mitochondrial genomes.

7.3 Macronutrient / energy availability and muscle adaptive responses

Given the well documented effects of high CHO availability on promoting endurance exercise performance, traditional nutrition guidelines for elite endurance athletes typically advocate diets high in daily CHO intake. While this is important from a performance perspective, it has now become increasingly apparent that deliberately restricting CHO intake before, during, and after training sessions is associated with beneficial muscle adaptive responses to exercise. Accordingly, the concept of deliberately training with low CHO availability (termed the 'train-low' paradigm) has now become increasingly popular within athletic circles. Nevertheless, despite advances in the literature, the precise mechanisms underpinning these augmented adaptive responses when training with reduced CHO availability still remain unclear. The mRNA data reported in Chapters 4 and 5 indicated that the expression of genes with roles in the regulation of mitochondrial biogenesis was not different between the feeding conditions employed, and an exercise induced expression was present independent of CHO and/or energy availability when twice per day exercise was performed. This conflicted with findings from many previous investigations whereby the expression of many of these genes is augmented when CHO (and energy) availability is reduced. Indeed, in such conditions, increased mRNA expression has been demonstrated using a variety of the different 'train-low' methodologies including contralateral leg models (Hansen *et al.,* 2005; Pilegaard *et al.,* 2002), exercising in the fasted state (Cluberton *et al.,* 2005; van Proeyen *et al.,* 2011), twice per day training (Pislander *et al.* 2013; Yeo *et al.* 2008), sleep low models (Bartlett *et al.* 2013; Lane *et al.* 2015), and an amalgamation of some of these strategies (Impey *et al.,* 2016). Where no differences were observed between dietary conditions in mRNA expression in Study 1 and Study 2a or in AMPK-α2 activity in Study 1, the chosen exercise

modality and muscle biopsy site may have contributed to this in part. Indeed, when compared to other lower extremity muscles, there is a reduced absolute muscle fibre recruitment from the vastus lateralis (VL) during walking and running (Jensen *et al.*, 2012). In addition, muscle fibre recruitment from the VL appears reduced during running compared to exercising at similar relative intensities during cycling based exercise (Arkinstall *et al.*, 2004). As such, future studies should also include muscle biopsies from the gastrocnemius muscle during running based protocols and combine both running and cycling exercise modalities when using twice per day train-low models.

In addition to the role of CHO and energy availability on the muscle adaptive responses to exercise, there has been a growing interest over the last few decades on the potential of high fat feeding protocols for enhancing endurance adaptations and performance. In this regard Study 1 (Chapter 4) aimed to also investigate the role of post-exercise high fat availability on regulating the cell signalling and gene expression associated with mitochondrial biogenesis, lipid metabolism, and MPS when CHO was low. The post-exercise high fat feeding protocol ($3.5g/kg^{-1}$ fat) employed in this study was successful in elevating lipid oxidation, FFA availability and the mRNA expression of CPT1 and CD36 when compared to HCHO, thus highlighting that acute alterations in fat availability alter the muscle adaptive responses involved in the regulation of lipid transport and oxidation in response to exercise. Thus, if performed in the long term, feeding of this type may increase the ability to utilise lipids as a fuel source. Although there is potential for this to subsequently 'spare' muscle glycogen, it is possible that high fat feeding may induce suppressive effects on CHO metabolism, the latter typically required to fuel endurance events. Indeed, data presented in both Study 1 and Study 2 demonstrate significantly higher post-exercise expression of

PDK4 when both CHO and energy availability are reduced, which if performed long-term could lead to impaired high intensity exercise performance through suppression of PDH.

When examining the effects of post-exercise high fat feeding on MPS responses, Study 1 also demonstrated a suppressive effect on p70S6K1 activity at 3-h post-exercise compared to when participants consumed CHO during recovery. It is likely that the suppression observed here is mediated through the direct effects of fat that are independent of CHO and energy availability. Indeed, previous research has demonstrated a role of circulating FFAs in attenuating MPS in human skeletal muscle (Stephens *et al.,* 2015) and reducing p70S6K1 activity in rat liver (Kimball *et al.,* 2015). These data suggest that if performed in the long-term, feeding of this type may have a negative effect on muscle remodelling and MPS in the post-exercise period, which may ultimately be detrimental to performance. It is possible that the type of fat (i.e. saturated or unsaturated) may contribute to the suppression of MPS post-exercise. Indeed, data from McGlory et al. (2016) demonstrated a suppressive effect of fish oil on PKB and p70S6K1 activity when compared to coconut oil over 8-weeks, thus suggesting that high fat availability may impair or perhaps enhance sensitivity to anabolic signalling. It is therefore important for future studies to determine the precise mechanisms by which this suppression occurs, and the associated effects of high fat feeding on long term muscle adaptive responses.

7.4 Glycogen Threshold Hypothesis

When taken together, combined data from this thesis and previous investigations suggest that the augmented signalling and transcriptional responses associated with 'train-low' appear to be glycogen dependent and are particularly apparent when absolute pre-exercise muscle glycogen concentrations are ≤300 mmol.kg^{-1} dw (Impey *et al.* 2018). In contrast, when 'train-low' sessions are commenced with markedly higher pre-exercise muscle glycogen concentrations (400-500 mmol.kg^{-1} dw) skeletal muscle markers of training adaptation are not augmented (Gejl *et al.,* 2017). Such data suggest the presence of a muscle glycogen threshold, whereby a critical absolute level of glycogen must be surpassed in order to induce the augmented cell signalling responses associated with the 'train-low' model (Pilegaard *et al.,* 2002). In chapters 5 and 6, participants commenced the first high intensity exercise bout with higher concentrations of muscle glycogen (~400-450 mmol.kg^{-1} dw and ~ 450-500 mmol.kg.$^{-1}$ dw respectively) having adhered to a high CHO diet the day before, and being provided with a high CHO breakfast on the morning of each experimental trial. The changes in muscle glycogen across the sampling period of each trial in Study 1 and Study 2a for each individual participant is outlined in Figure 7.1 below. Although no muscle biopsy was obtained prior to the afternoon exercise bouts in either Study, it is likely that the concentrations of muscle glycogen available prior to this session were at concentrations higher than, or at the upper end of the proposed glycogen threshold in all feeding conditions. In both Study 1 and Study 2a, average muscle glycogen concentration was suppressed to comparable levels (~250 mmol.kg.$^{-1}$ dw) immediately-post afternoon exercise under all feeding conditions despite CHO being restricted in the 3-h prior to commencing this second exercise bout in the low CHO and low energy trials. It would appear from Figure 7.1 that participants in both studies

likely commenced the second exercise bout with muscle glycogen concentrations of ~300-400 mmol.kg^{-1} dw and presented with 'moderate' levels of muscle glycogen for the majority of the trial period in all nutritional conditions. Indeed, previous research has demonstrated attenuated (Wojtaszewski *et al.,* 2003) or abolished (Bartlett *et al.,* 2013) activation of cell signalling pathways when muscle glycogen concentrations remain above 300 mmol.kg^{-1} dw post-exercise. As such it is likely that despite restricting CHO and/or energy in the 3-h recovery period between exercise bouts, the higher baseline levels of muscle glycogen in Study 1 and Study 2a attenuated any further increases in mRNA expression. In the same way, given that elevating glycogen in the recovery period to levels >400 mmol/kg dw can attenuate post-exercise gene responses (Pilegaard et al. 2005), it is possible that the dietary manipulations studied here did not constitute those conditions considered as "true" train (or recover) low or

train high but rather, the majority of the signalling environment was in a "train-medium" state.

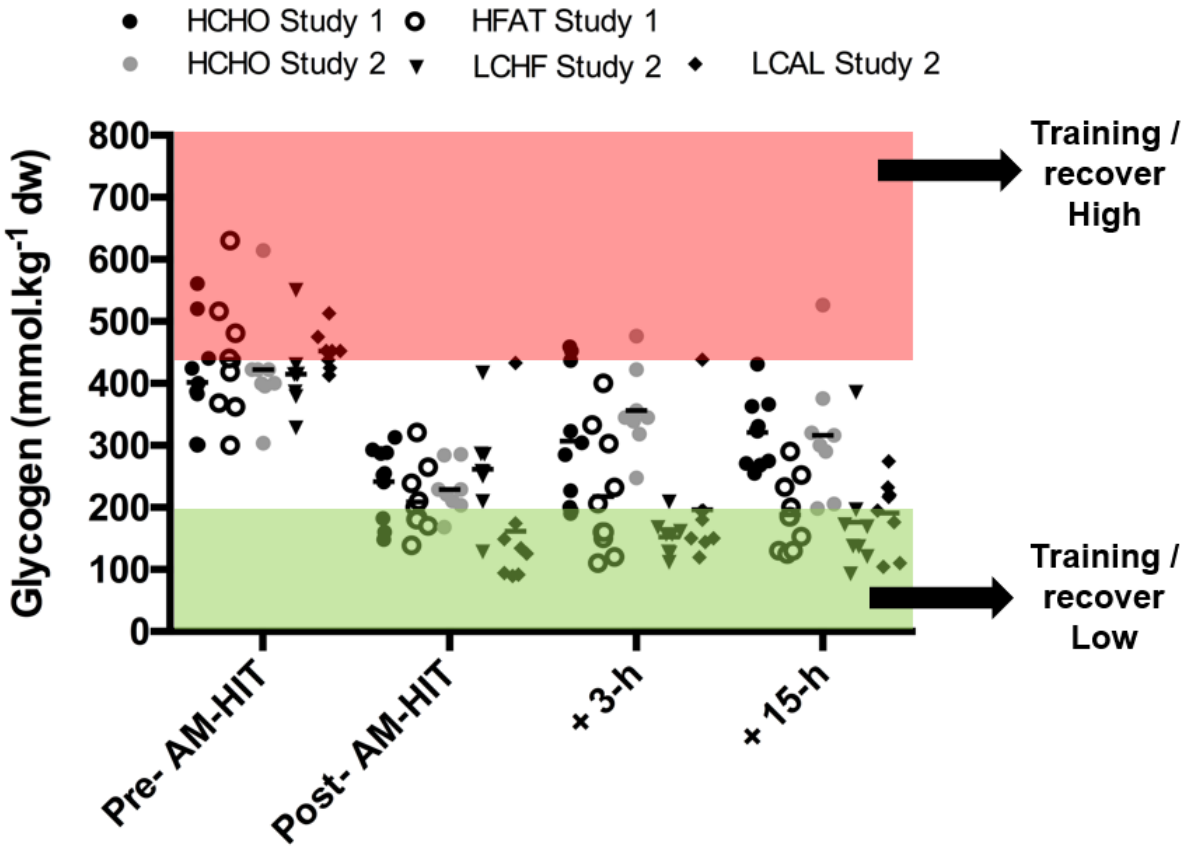

Figure 7.1 Muscle glycogen concentration for each individual participant across experimental trials in Study 1 and Study 2 highlighting the theoretical muscle glycogen threshold. The green shaded area represents concentrations of muscle glycogen which must be reached in order enhance the cell signalling responses associated with increased muscle adaptive responses to exercise when 'training-low' and 'recovering-low'. The red shaded area represents the high levels of muscle glycogen concentration typically associated with CHO loading advised for high intensity endurance performance. Examination of individual data highlights that subjects may not have spent enough time exercising in or recovering in conditions which represent those conditions considered as true train-low signalling environments.

7.5 Macronutrient / energy availability and RED-S

Since low energy availability can have a widespread effect on many physiological functions with a reduction in metabolic and hormonal function (Burke and Lundy, 2018), there is potential for this to increase the risk of injury and illness and impair endurance performance. As such, providing adequate energy availability is a key concept in sports nutrition in order to minimise the development of associated RED-S syndrome.

Stress fractures can lead to months of missed training and restricted weight bearing activity in elite endurance populations (Matheson et al., 1987; Ranson et al., 2010), therefore if a 'podium' level athlete sustained a stress fracture injury during an Olympic year for example, this could have huge negative implications for their planned training programme. As such, it is of importance to use appropriate nutritional strategies to maximise bone health and minimise any time lost from training. The estimated energy available in the LCAL trial in Study 2b (Chapter 6) was suboptimal compared to the energy expended during exercise, and CHO intakes were suboptimal compared to recommended guidelines for elite endurance athletes (Burke *et al.,* 2011). It is highly important that athletes match energy intakes with energy expenditures as closely as possible, as bone turnover processes are negatively affected even with slight deficits in energy (Ihle and Loucks, 2004). It is apparent from Study 2b that bone turnover was rapidly impacted by a single bout of high intensity exercise as indicated by reductions in β-CTX and P1NP concentrations immediately post-exercise in all three experimental trials. Average post-exercise β-CTX concentrations presented in Study 3 were ~2 times higher than the non-active population (Glover *et al*., 2008; Glover *et al*., 2009; Jenkins et al., 2013; Michelsen et al., 2013), and average P1NP

concentrations ~ 3 times higher. Feeding CHO in the 3-h recovery period between exercise bouts was significant in reducing β-CTX concentrations compared to LCAL and LCHF, thus indicative of the need for CHO intake as soon as possible after a training session to minimise bone breakdown.

The release of the inflammatory cytokine IL-6 has the potential for both beneficial and detrimental effects following exercise. Indeed, beneficial adaptations resulting from exercise induced IL-6 occur when the exercise bout is followed by a recovery period (Hennigar *et al.,* 2017), whereas detrimental effects may occur when high intensity exercise bouts are performed excessively without adequate rest or nutrient availability. Data from Study 2b (Chapter 6) highlight significant increases in plasma IL-6 concentrations following the second exercise bout, which are markedly higher when both CHO and energy availability are low. This indicates that even acute periods of caloric restriction can promote IL-6 release when recovery is short between training sessions. This agrees with previous findings reporting repeated bouts of exercise are associated with a more pronounced increase in plasma IL-6 concentrations compared to a single bout of exercise (Nielsen *et al.,* 1996; Ronsen *et al.,* 2002). When taken together, these findings suggest that if 'train-low' sessions are performed over consecutive days, long-term immune function may be negatively impacted. Furthermore, Clarkson and Hubal (2002) also suggest that repeated IL-6 elevations may result in a catabolic effect thus leading to impaired muscle function, which if repeated over time could impact negatively on performance.

7.6 The future of nutrition for endurance sports: big picture thinking

While the current body of available research demonstrates a beneficial effect of different 'train-low' methodologies (i.e. twice per day training, fasted training, restricting CHO post-exercise and sleep low) on augmenting the muscle adaptive responses to exercise, it is common for elite endurance populations to practice an amalgamation of these strategies rather than any one in isolation. Due to the high energy expenditures of endurance athletes it is likely that these adaptive responses are due to transient periods of both CHO and calorie restriction due to multiple training sessions per day as well as longer term periods of sub-optimal energy availability. While this may be of benefit for certain aspects of training adaptation, exercise capacity and exercise performance (Cochran *et al.* 2015; Marquet *et al.* 2016), longer term periods of low energy availability are associated with markers of RED-S in endurance populations (Mountjoy *et al.*, 2014). Indeed, data presented here demonstrates that even acute bouts of endurance exercise training undertaken under conditions of low CHO and low energy availability (in a twice-per-day training model) may be negative for bone turnover and the inflammatory response to repeated exercise bouts. Suggesting that if undertaken over a longer period of time, training of this type may increase the risk of injury and illness as well as having potential catabolic effects. An overview of the overall effects of macronutrient and energy availability on the metabolic responses to exercise based on findings from this thesis are displayed in Figure 7.2.

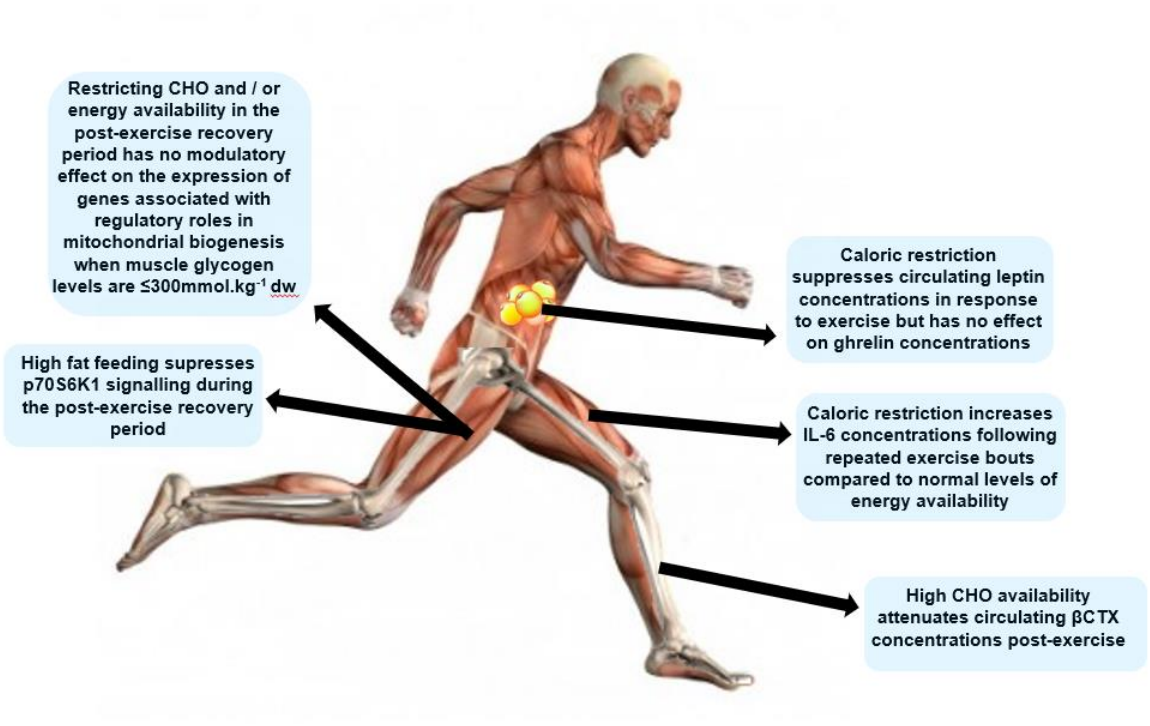

Figure 7.2 Integrated outline of the combined effects of macronutrient and energy availability on the metabolic responses to endurance exercise training.

When taken together, although these findings suggest a muscle glycogen threshold of ≤ 300 mmol.kg^{-1} dw is necessary to facilitate increases in muscle cell signalling responses when undertaking 'train-low' sessions, it is apparent that post-training meals should subsequently provide appropriate CHO to facilitate recovery based on the intensity of training and the time available for recovery prior to the next training session. Based on the findings of this thesis as a whole, it is apparent therefore that the optimal nutritional strategy for an endurance athlete looking to enhance the muscle adaptive responses to training should include CHO restricted sessions whereby muscle glycogen availability can be low, however, CHO should then be consumed as soon as possible post-exercise if the recovery period is short between sessions. In this way, the adaptive response from low CHO and/or energy availability can be augmented while the risk of increased bone breakdown will be minimised, and more positive bone

turnover balance will be facilitated. It would seem sensible for athletes to avoid prolonged periods of low CHO availability in order in to reduce the potentially detrimental effects on overall bone mass (Ihle & Loucks, 2004) and/or bone injury risk, so by manipulating nutrition and 'train-low' sessions in this way, this can be avoided. Consuming energy dense nutrients between multiple training sessions (energy from both CHO and from fat) is also necessary to augment appetite hormone concentrations and minimise the inflammatory responses to multiple training sessions, thus minimising illness risk, and the risk of catabolic effects of training.

7.6.1 Practical applications

For the elite endurance athlete, a nutritionist's philosophy is often focussed around three key areas; 1) facilitating the adaptation to training, 2) reducing the risk of illness, and 3) reducing the risk of injury. All three of these meaning that an athletes training can be more effective, and that less time is lost to training due to negative factors such as illness and injury. As such, the key findings from this thesis can be implemented as part of a day to day periodised plan for endurance athletes to focus on all three of these areas. Figure 7.2 shows an example of a potential weekly plan on CHO intake created for an endurance runner based around these three factors using the key findings from this thesis.

SESSION	AM TRAINING	BREAKFAST	LUNCH	SNACKS	PM RUN	DINNER
MON	NO CHO	HIGH CHO	HIGH CHO	HIGH CHO	NO CHO	HIGH CHO
TUES	NO CHO	HIGH CHO	HIGH CHO	HIGH CHO	MODERATE CHO	HIGH CHO
WED	NO CHO	HIGH CHO	HIGH CHO	HIGH CHO	NO CHO	HIGH CHO
THURS	NO CHO	HIGH CHO	HIGH CHO	HIGH CHO	MODERATE CHO	HIGH CHO
FRI	NO CHO	HIGH CHO	HIGH CHO	HIGH CHO	NO CHO	HIGH CHO
SAT	NO CHO	HIGH CHO	HIGH CHO	HIGH CHO	NO CHO	HIGH CHO
SUN	NO CHO	HIGH CHO	NO PM SESSION	NO PM SESSION	NO PM SESSION	NO PM SESSION

Figure 7.3 An example periodised weekly plan for an endurance runner based on the combined findings from this thesis. Where red boxes represent no CHO/energy intake, orange boxes represent moderate CHO/energy intake, and green boxes represent high CHO/energy intake.

When taken together, by following this plan, an endurance athlete can be advised to focus on three key aims of nutrition, for example:

1) Facilitating the adaptive response to training – Undertaking the morning training sessions in a fasted state, whereby muscle glycogen levels will be lower in order to increase the cell signalling associated with mitochondrial biogenesis. Recovering from a morning training session with high CHO containing meals where recovery time is short in order to reduce the potential catabolic effect of 'training-low'.

2) Minimising the risk of injury – Feeding with high CHO in the recovery time between training sessions where recovery time is short in order to minimise bone breakdown and facilitate a more positive bone turnover.
3) Minimising the risk of illness – Feeding energy dense meals (containing both CHO and fat) between the two training sessions when recovery time is short in order to minimise the inflammatory responses associated with suppressed immune function.

A previous case study from Stellingwerff et al. (2012) stated 'Low CHO availability training had a large degree of variability according to individual tolerance and acclimatisation. Feedback from the athletes indicated that morning fasted training was physiologically much easier to integrate than the more strenuous reduced glycogen training'. As such it would appear most effective to include 'train-low' sessions in the morning, but also to vary this training across a training block. In a real world setting, the example in figure 7.3 could be used for part of a training block whereby some CHO restricted sessions are included, however, as progression moved towards competition, more CHO/energy fuelled sessions should be included than CHO/energy restricted sessions in order to ensure PDH activity is not suppressed and athletes still have the ability to oxidise CHO as a fuel source during competition.

The complexity of practical 'train-low' models is further exacerbated by the observations that many endurance athletes 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; Morton, unpublished observations). 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. Such data suggest

that the adaptive responses and performance improvements observed following chronic periods of 'train-low' may also be to a change in body composition – something which should be further explored in future studies.

7.6.2 Future research questions

Based on the current findings from this thesis, there are several other research studies/ideas which could be conducted in order to help inform future applied practice in endurance sports.

Initially within this thesis, the aims of Study 2 also included the assessment of p70S6K1 and AMPK-α2 activity in order to follow on from some of the key findings in Study 1. Given that AMPK-α2 activity was unchanged following exercise in Study 1 - likely due to the intensity of the steady state exercise protocol, it is likely that with the higher intensity exercise protocol utilised in Study 2 combined with CHO and energy restriction there would have been a change in the signalling in response to exercise. Similarly, it would have been interesting to see if any changes were observed in p70S6K1 activity in response to a more intense protocol in combination with low CHO and high fat availability and if this was any different to those changes observed following steady state exercise. As such future studies would also benefit from measuring the activity of these signalling kinases when using similar twice per day protocol designs.

Having suggested a potential muscle glycogen threshold at which the augmented cell signalling and transcriptional responses to exercise with low CHO availability occur, future studies should now explore this concept further. Studies should identify the ideal signalling environment for facilitating training adaptations while maintaining the

desired workload for the session by employing a protocol to 'grade' muscle glycogen concentrations to different levels between trials.

Having identified impaired p70S6K1 signalling in response to high fat feeding in Study 1 (Chapter 4), future work should now look at free living muscle protein turnover (synthesis and breakdown) across a training day as well as examining the acute signalling mechanisms by which changes occur. Further measures of insulin, PKB and mTOR activity could be obtained in order to add to the overall picture of where in the mTOR pathway fat may be having an effect. It would also be of benefit to examine this chronically over a 3-6 week training period and determine the functional effects of longer-term high fat feeding on direct measures of MPS.

Though Study 2a (Chapter 6) was novel in terms of the real-world high-intensity repeated bout protocol employed, future studies should now examine the effects of repeated exercise bouts over consecutive days, or multiple training sessions per week on metabolic hormones, inflammatory cytokine responses, and bone turnover markers when combined with low levels of CHO and/or energy availability. It would also be relevant to determine how these responses differ using different exercise modalities across the day, i.e. running based exercise in the morning followed by cycling based exercise in the afternoon and/or with the addition of resistance-based sessions, as is often common practice in endurance sports such as triathlon.

Future studies in this area would also benefit from using DEXA as an accurate indication of FFM, and doubly labelled water as an accurate indication of total energy expenditure across a study period in order to accurately determine energy availability. It would also be of benefit to examine/track bone turnover responses chronically over periods of interest in elite endurance populations across different phases of an athletic

season in order to determine if there are any periods of increased injury risk i.e. while training heavily in the season where energy availability is likely to be low. In this way, findings could inform applied practice by highlighting key timepoints where energy availability needs to be increased to minimise the risk of bone mass loss and stress fracture type injuries. Tracking bone turnover across a season in this way across full squads would also allow comparison between turnover in males and females.

In future studies (both applied and lab based), further measures such as testosterone, IGF-1, and hepcidin should also be incorporated to give an overall picture of energy availability and RED-S syndrome responses that are relevant to the endurance athlete. These research studies should be undertaken in both male and female athletes to compare REDS-s related responses.

7.7 Implications for methodological design

Protocol design - As discussed previously, it is likely that the participants in Studies 1 and 2 (Chapter 4 and 5) did not exceed the potential 'muscle glycogen threshold' that is required to fully activate the regulatory cell signalling pathways during exercise. However, since no muscle biopsy was obtained prior to the afternoon exercise bout in either study it is difficult to determine what level of muscle glycogen concentration was available prior to commencing afternoon HIT. Additionally, the time-course of mRNA production following exercise is not well characterised and it is possible that time-course of biopsies adopted here (i.e. post, 30h and 15-h post PM HIT) was not optimal to capture peak responses. Additionally, while measuring mRNA content is often used to demonstrate the presence of signalling pathways, this does not

necessarily translate into increased protein abundance (Hornberger *et al.,* 2016). Similarly, while the use of p70S6K1 is a useful marker of signalling within the mTOR pathway, this does not necessarily indicate the presence of MPS despite previous investigations highlighting the requirement of mTOR signalling in MPS responses (Dickinson *et al.,* 2011). Future studies should now assess post-translational modifications and the biological/functional significance of these changes chronically when performed over a longer-term training period to gain better understanding of the precise mechanisms underpinning the muscle adaptive responses which occur in response to training and nutritional stimuli. Indeed, this would give a better indication of how to potentially periodise some of these strategies within an athletes training programme across a cycle for maximum benefit. In study 2b, no significant changes were displayed with leptin in response to exercise, something which is unusual in comparison to previous research. It is likely that the blood sampling frequency here wasn't enough to pick up these changes in circulating levels, and as such further samples taken during exercise and in the hours following each bout would have been useful. More subjective measures of appetite could also have been included as part of a questionnaire given to participants to gain better understanding of the psychological implications of each of the diets.

Participants – The participants used for the studies in this thesis were recreationally active male subjects who were free from injury and currently participating in running based activity twice per week. Because of this factor, the training status of individual participants may undoubtedly differ due to the specific nature of their training history (i.e. running, cycling, triathlon etc) and that provides altered adaptive stimuli (i.e. frequency and intensity). As such, it is unclear whether the findings from this thesis

would be translatable to elite endurance populations given the physiological and metabolic differences that would exist. As this thesis contained large amount of cell-signalling analysis, with training history potentially impacting upon this (Coffey et al., 2006), future studies should pay particular attention to the inclusion criteria for subject recruitment so as to minimise the effect of training status upon cell signalling responses. In addition, only male participants were recruited for the studies in this thesis. It is possible that the responses observed would differ if female participants were included, thus future research should also look to include females for studies of this type.

Dietary interventions – Although all food was provided to participants in both studies in order to try to maximise compliance, there were still some issues with consumption of the foods chosen in places. For example, the high CHO diets contained large volumes of both food and fluid in order to meet the CHO amounts required, and as such there were some GI issues during the afternoon HIT session. Similarly, some of the meals in the high fat diet contained foods such as oily fish and coconut oil which some participants found difficult to consume immediately following high intensity exercise, so in places these meals were consumed more slowly. Future studies in real world endurance populations should look to make any dietary interventions more specific to each participant based on individual preference and foods they are used to consuming on a day to day basis. Due to the nature of the studies, it was difficult to blind which experimental trial was which, and as such participants were aware of which was the low CHO and low energy trial. Although RPE did not significantly differ between trials, participants in some cases did arrive to the laboratory for the low CHO trials with the perception they would fatigue more. In Study 2b (Chapter 6), energy availability was estimated by calculating the difference between the prescribed

energy intake, and the energy expenditure during AM HIT and PM HIT exercise. Although all food was administered to participants across each trial, limitations also exist when using indirect calorimetry to assess energy expenditure during high intensity exercise (Jeukendrup and Wallis, 2005). Additionally, the use of indirect calorimetry does not permit the assessment of lipid oxidation from peripheral versus intramuscular stores, as has been examined previously (Hulston *et al.,* 2010). Furthermore, energy availability was estimated from fat free mass (FFM) with the assumption that all participants were ~15% body fat. Since participants were not assessed for body composition using dual energy X-ray absorptiometry (DEXA) prior to participating in the study, estimations of energy availability have obvious limitations.

7.9 Conclusions

In summary, we provide novel data by demonstrating that in conditions where post-exercise muscle glycogen concentration is maintained within the range of 200-350 mmol.kg^{-1} dw, short-term periods of acute CHO and energy restriction (i.e. <24 hours) does not potentiate skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism. In addition, promotion of high CHO availability before, during and in recovery from exercise appears to be of greater importance for the acute regulation of bone turnover when compared with energy intake *per se.* In contrast, energy availability appears a more influential factor in regulating both IL-6 and leptin responses in recovery from exercise as opposed to CHO availability *per se*. Future studies should now examine the potential presence of a muscle glycogen threshold as an important regulator of skeletal muscle adaptations

to endurance training. Additionally, the long-term implications (in relation to RED-S) of the acute within day fluctuations in both CHO and energy availability that occur when training twice per day should now be examined when performed as part of a periodised training programme.

Chapter 8

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Appendix 1

	Time	Food
Breakfast	8am	Coco pops (60g) with 200ml semi skimmed milk 2 slices white toast with butter and jam Glass of orange juice(400ml) & protein drink (with 200ml water)
Snack	10.30-11am	Jaffa cakes (6 cakes) Lucozade original (1 bottle)
Lunch	13.00-13.30	Ham/chicken sandwich Muller rice x 2 Banana Jaffa cakes x5
Snack	15.00	Jaffa cakes (6 cakes) Lucozade original (1 bottle)
Dinner	18.00	Chicken breast Pasta (220g) and tomato sauce (100g) Apple juice (200ml)
Snack	21.00	Coco pops (60g) with 200ml semi skimmed milk Protein drink (in 200ml water)

Appendix 2

Meal	Time	HIGH (CHO, PRO, FAT) Description	Nutrition CHO (g)	Pro (g)	Fat (g)	LOW (PRO & FAT) Description	Nutrition CHO (g)	Pro (g)	Fat (g)
Breakfast	07:00	Coco pops (100g)	85	5	2.5	Coco pops (100g)	85	5	2.5
		Whole milk (200ml)	9.4	6.5	7.2	Whole Milk (200ml)	9.4	6.5	7.2
		Orange juice (200ml)	21	1	0	White toast x2 (80g)	38	6.4	1.5
		White toast x2 (80g)	38	6.4	1.5	Strawberry jam (15g)	9	0	0
		Strawberry jam (15g)	9	0	0	Orange juice (200ml)	21	1	0
		Total	**162.4**	**18.9**	**11.2**	**Total**	**162**	**18.9**	**11.2**
During HIT	09:00-10:30	Water only	0	0	0	Water only	0	0	0
		Total	**0**	**0**	**0**	**Total**	**0**	**0**	**0**
Post-HIT	10:30	GO bar x2	52	9.2	3.2	Whey protein drink (250ml)	0	20	0
		CHO and protein drink	50	20	0	Peanut butter (1 tbsp)	2.4	3.7	7.5
						Avocado (60g)	1.1	1.1	11
		Total	**102**	**29.2**	**3.2**	**Total**	**3.5**	**24.8**	**18.5**
Lunch	12:00	Chicken breast (125g)	0	29	8	Salmon (240g)	0	48	38
		Broccoli (40g)	0.5	1.5	0	Broccoli (40g)	0.5	1.5	0
		Flavoured brown rice (250g)	57.5	5	6	Butter (10g)	0	0	8.1
		Bread roll x1	30	7	2	Coconut oil (14g)	0	0	14
		Total	**88**	**42.5**	**16**	**Total**	**0.5**	**49.5**	**46.1**
During SS	13:30-14:30	CHO gels (1g/min)	60	0	0	WATER ONLY	0	0	0
		Total	**60**	**0**	**0**	**Total**	**0**	**0**	**0**
Post SS	15:00	CHO bar x2	52	9.2	3.2	Whey protein drink (250ml)	0	20	0
		CHO and protein drink (500ml)	50	20	0	Peanut butter (1tbsp)	2.4	3.7	7.5
						Avocado (60g)	1.1	1.1	11
		Total	**102**	**29.2**	**3.2**	**Total**	**3.5**	**24.8**	**18.5**
Dinner	16:00	Turkey breast (125g)	0	40	4.5	Mackerel (200g)	0	43	49.8
		Broccoli (40g)	0.5	1.5	0	Broccoli (40g)	0.5	1.5	0
		Flavoured brown rice (250g)	57.5	5	6	Butter (10g)	0	0	8.1
						Coconut oil (14g)	0	0	14
		Bread roll x1	30	7	2	**Total**	**0.5**	**44.5**	**57.9**
		Total	**88**	**45.5**	**12.5**				
Dessert	17:00	Muller rice	31	6.8	4.9	Cheddar cheese (50g)	0	12.7	17.4
		Apple juice (330ml)	35	0.7	0				
		Banana x1	23	1	0	Red Leicester (50g)	0	12.45	17.25
		Total	**89**	**8.5**	**4.9**	**Total**	**0**	**25.15**	**34.65**
Snack	21:00	Protein drink with 200ml water	0	20	0	Walnuts (50g)	1.6	7.4	34
		Jaffa cakes (5 cakes)	43	3	5	Almonds (50g)	4.7	13.6	24.7
		White toast x2 & jam	47	6.4	1.5				
		Total	**90**	**29.4**	**6.5**	**Total**	**6.3**	**21**	**58.7**
		TOTAL	**781.4**	**211**	**57.5**	**TOTAL**	**176.3**	**209**	**253.5**
		kcal = 4378				**kcal = 4320**			

Appendix 3

Meal	Time	LOW CHO LOWCAL LCAL Description	Nutrition CHO (g)	Pro (g)	Fat (g)
Breakfast	07:00	Coco pops (100g)	85	5	2.5
		Whole milk (200ml)	9.4	6.5	7.2
		Orange juice (200ml)	21	1	0
		White toast x2 (80g)	38	6.4	1.5
		Strawberry jam (15g)	9	0	0
		Total	**162.4**	**18.9**	**11.2**
During HIT	09:00-10:30	Water only	0	0	0
		Total	**0**	**0**	**0**
Post-HIT	10:30	Whey protein drink (250ml)	0	30	0
		Total	**0**	**30**	**0**
Lunch	12:00	Chicken breast (125g)	0	29	8
		Broccoli (40g)	0.5	1.5	0
		Total	**0.5**	**30.5**	**8**
During HIT2	13:30-14:30	WATER ONLY	0	0	0
		Total	**0**	**0**	**0**
Post HIT2	14:30	protein drink (500ml)	0	30	0
		Total	**0**	**30**	**0**
Dinner	16:30	Chicken breast (110g)	0	30	8
		Broccoli (40g)	0.5	1.5	0
		Flavoured brown rice (250g)	57.5	5	6
		Total	**58**	**31.5**	**10.5**
Snack	20:30	Whey protein drink (250ml)	0	30	0
		Total	**0**	**30**	**0**
		TOTAL kcal = 2714	**220.9**	**175.9**	**29.7**

Meal	Time	HIGH CHO HIGH CAL Description	HCHO Nutrition CHO (g)	Pro (g)	Fat (g)
Breakfast	07:00	Coco pops (100g)	85	5	2.5
		Whole milk (200ml)	9.4	6.5	7.2
		Orange juice (200ml)	21	1	0
		White toast x2 (80g)	38	6.4	1.5
		Strawberry jam (15g)	9	0	0
		Total	**162.4**	**18.9**	**11.2**
During HIT	09:00-10:30	Water only	0	0	0
		Total	**0**	**0**	**0**
Post-HIT	10:30	Lucozade original (500ml)	86	0	0
		CHO and protein drink	75	30	0
		Total	**161**	**30**	**0**
Lunch	12:00	Chicken breast (100g)	0	25	8
		Broccoli (40g)	0.5	1.5	0
		Flavoured brown rice (250g)	57.5	5	6
		Lucozade original (250ml)	43	0	0
		Total	**101**	**31.5**	**14**
During HIT2	13:30-14:30	CHO gels (1g/min)	60	0	0
		Total	**60**	**0**	**0**
Post HIT2	14:30	Lucozade original (500ml)	86	0	0
		CHO and protein drink (500ml)	75	30	0
		Total	**161**	**30**	**0**
Dinner	16:00	Chicken breast (100g)	0	25	8
		Flavoured brown rice (125g)	30	2.5	3
		Lucozade original (500ml)	86	0	0
		Banana x1	23	2	0
		Total	**139**	**29.5**	**11**
Snack	18:00	Lucozade original (250ml)	43	0	0
		Jaffa cakes (6 cakes)	51	3	0
		Total	**94**	**3**	**5**
Snack	20:30	Protein drink with 200ml water	0	30	0
		Jaffa cakes (5 cakes)	43	3	5
		Lucozade original (250ml)	43	0	0
		Total	**86**	**33**	**5**
		TOTAL kcal=5030	**964.4**	**175.9**	**46.2**

Meal	Time	LOW CHO HIGH CAL LCHF Description	Nutrition CHO (g)	Pro (g)	Fat (g)
Breakfast	07:00	Coco pops (100g)	85	5	2.5
		Whole Milk (200ml)	9.4	6.5	7.2
		White toast x2 (80g)	38	6.4	1.5
		Strawberry jam (15g)	9	0	0
		Orange juice (200ml)	21	1	0
		Total	**162**	**18.9**	**11.2**
During HIT	09:00-10:00	Water only	0	0	0
		Total	**0**	**0**	**0**
Post-HIT	10:00	Whey protein drink (250ml)	0	30	0
		Avocado (60g)	1.1	1.1	11
		coconut oil (10g)	0	0	10
		Total	**1.1**	**31.1**	**21**
Lunch	12:00	Salmon (100g)	0	25	15
		Broccoli (40g)	0.5	1.5	0
		Butter (10g)	0	0	8.1
		Coconut oil (40g)	0	0	40
		Walnuts (25g)	0.8	3.7	17
		Total	**1.3**	**30.2**	**60.1**
During HIT 2	13:30-14:30	WATER ONLY	0	0	0
		Total	**0**	**0**	**0**
Post HIT 2	14:30	Whey protein drink (250ml)	0	30	0
		coconut oil (20g)	0	0	20
		Avocado (60g)	1.1	1.1	11
		Total	**1.1**	**31.1**	**31**
Dinner	16:30	Mackerel (200g)	0	21	25
		Butter (10g)	0	0	8.1
		Coconut oil (40g)	0	0	40
		Flavoured brown rice (250g)	57.5	5	6
		Walnuts (25g)	0.8	3.7	17
		Total	**58.3**	**29.7**	**76.1**
Snack	20:30	Walnuts (50g)	1.6	7	34
		Cheddar cheese (50g)	0	13	17
		Red Leicester (50g)	0	12	17
		Total	**1.6**	**32.4**	**68**
		TOTAL kcal = 5021	**225.4**	**173**	**207.4**

Appendix 4

BASIC SCIENCES

Postexercise High-Fat Feeding Suppresses p70S6K1 Activity in Human Skeletal Muscle

KELLY M. HAMMOND[1], SAMUEL G. IMPEY[1], KEVIN CURRELL[2], NIGEL MITCHELL[2], SAM O. SHEPHERD[1], STEWART JEROMSON[3], JOHN A. HAWLEY[1,4], GRAEME L. CLOSE[1], LEE D. HAMILTON[3], ADAM P. SHARPLES[1], and JAMES P. MORTON[1]

[1]Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UNITED KINGDOM; [2]English Institute of Sport, Loughborough University, Loughborough, UNITED KINGDOM; [3]Health and Exercise Sciences Research Group, University of Stirling, Stirling, UNITED KINGDOM; and [4]Centre for Exercise and Nutrition, Mary MacKillop Health Research Institute, Australian Catholic University, Victoria, AUSTRALIA

ABSTRACT

HAMMOND, K. M., S. G. IMPEY, K. CURRELL, N. MITCHELL, S. O. SHEPHERD, S. JEROMSON, J. A. HAWLEY, G. L. CLOSE, L. D. HAMILTON, A. P. SHARPLES, and J. P. MORTON. Postexercise High-Fat Feeding Suppresses p70S6K1 Activity in Human Skeletal Muscle. *Med. Sci. Sports Exerc.*, Vol. 48, No. 11, pp. 2108–2117, 2016. **Purpose**: This study aimed to examine the effects of reduced CHO but high postexercise fat availability on cell signaling and expression of genes with putative roles in regulation of mitochondrial biogenesis, lipid metabolism, and muscle protein synthesis. **Methods**: Ten males completed a twice per day exercise model (3.5 h between sessions) comprising morning high-intensity interval training (8 × 5 min at 85% $\dot{V}O_{2peak}$) and afternoon steady-state (SS) running (60 min at 70% $\dot{V}O_{2peak}$). In a repeated-measures design, runners exercised under different isoenergetic dietary conditions consisting of high-CHO (HCHO: 10 g·kg^{-1} CHO, 2.5 g·kg^{-1} protein, and 0.8 g·kg^{-1} fat for the entire trial period) or reduced-CHO but high-fat availability in the postexercise recovery periods (HFAT: 2.5 g·kg^{-1} CHO, 2.5 g·kg^{-1} protein, and 3.5 g·kg^{-1} fat for the entire trial period). **Results**: Muscle glycogen was lower ($P < 0.05$) at 3 h (251 vs 301 mmol·kg^{-1} dry weight) and 15 h (182 vs 312 mmol·kg^{-1} dry weight) post-SS exercise in HFAT compared with HCHO. Adenosine monophosphate-activated protein kinase α2 activity was not increased post-SS in either condition ($P = 0.41$), although comparable increases (all $P < 0.05$) in PGC-1α, p53, citrate synthase, Tfam, peroxisome proliferator-activated receptor, and estrogen-related receptor α mRNA were observed in HCHO and HFAT. By contrast, PDK4 ($P = 0.003$), CD36 ($P = 0.05$), and carnitine palmitoyltransferase 1 ($P = 0.03$) mRNA were greater in HFAT in the recovery period from SS exercise compared with HCHO. Ribosomal protein S6 kinase activity was higher ($P = 0.08$) at 3 h post-SS exercise in HCHO versus HFAT (72.7 ± 51.9 vs 44.7 ± 27 fmol·min^{-1}·mg^{-1}). **Conclusion**: Postexercise high-fat feeding does not augment the mRNA expression of genes associated with regulatory roles in mitochondrial biogenesis, although it does increase lipid gene expression. However, postexercise ribosomal protein S6 kinase 1 activity is reduced under conditions of high-fat feeding, thus potentially impairing skeletal muscle remodeling processes. **Key Words:** AMPK-α2, PGC-1α, p53, GLYCOGEN, MITOCHONDRIAL BIOGENESIS

Traditional nutritional strategies for endurance athletes have largely focused on ensuring high-CHO (HCHO) availability before, during, and after each training session (2). However, accumulating data from our laboratory (7,29) and others (12,16,17,23,39) have demonstrated a potent effect of CHO restriction (the so-called train-low paradigm) in augmenting the adaptive responses inherent to endurance training. Indeed, reduced CHO availability before (33), during (1), and after (32) training sessions augments the acute cell signaling pathways and downstream gene expression responses associated with regulating training adaptation. Accordingly, reduced CHO availability during short-term periods of endurance training augments markers of mitochondrial biogenesis (16,29,39), increases both whole body (39) and intramuscular lipid metabolism (17), and also improves exercise capacity and performance (16,24). In the context of nutrient–gene interactions, it is therefore apparent that the

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acute molecular regulation of cell signaling processes provides a theoretical basis for understanding the molecular mechanisms underpinning chronic training adaptations.

In addition to manipulation of CHO availability, many investigators have also demonstrated a modulatory role of high-fat availability in augmenting components of training adaptation (10). For example, the acute elevation in circulating free fatty acid (FFA) availability during exercise regulates key cell signaling kinases and transcription factors that modulate the expression of genes regulating both lipid and CHO metabolism (31,40). In addition, 5–15 d of high-fat feeding increases resting intramuscular triglyceride (IMTG) stores (38), hormone-sensitive lipase (38), carnitine palmitoyltransferase 1 (CPT1) (15), adenosine monophosphate-activated protein kinase α2 (AMPK-α2) activity (38), and protein content of fatty acid translocase (FAT/CD36) (11). Such adaptations undoubtedly contribute to the enhanced rates of lipid oxidation observed during exercise following "fat adaptation" protocols (10). Taken together, these data suggest carefully chosen periods of reduced CHO, but concomitant high-fat availability may therefore represent a strategic approach for which to maximize both the training-induced skeletal muscle mitochondrial biogenesis and the enhanced capacity to use lipid sources as fuels during exercise.

However, such a feeding strategy is not without potential limitations, especially if performed on consecutive days. Indeed, reduced CHO availability impairs acute training intensity (17,39) and 5 d of high-fat feeding reduces pyruvate dehydrogenase (PDH) activity (35), thus potentially leading to a detraining effect, reduced capacity to oxidize CHO, and ultimately, impaired competition performance (17,39). Moreover, although many endurance training–induced skeletal muscle adaptations are regulated at a transcriptional level, the turnover of myofibrillar (i.e., contractile) proteins are largely regulated through the translational machinery and the mechanistic target of rapamycin complex and ribosomal protein S6 kinase 1 (p70S6K1) signaling axis (28). In this regard, recent data suggest high circulating FFA availability impairs muscle protein synthesis (MPS) despite the intake of high quality protein, albeit examined via lipid and heparin fusion and euglycemic hyperinsulemic clamp conditions (36).

With this in mind, the aim of the present study was to examine the effects of reduced CHO but high postexercise fat availability on the activation of key cell signaling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism, and MPS. In accordance with the original train-low investigations (16,17,29,39), we used a twice per day exercise model whereby trained male runners completed a morning high-intensity interval training (HIT) session followed by an afternoon training session consisting of steady-state (SS) running. Runners completed the exercise protocols under two different dietary conditions (both energy and protein matched) consisting of HCHO availability in the recovery period after both training sessions (i.e., best practice nutrition) or, alternatively, reduced CHO but high-fat availability in the postexercise recovery periods (HFAT). We specifically hypothesized that our high-fat feeding protocol would enhance cell signaling and the expression of those genes with putative roles in the regulation of mitochondrial biogenesis and lipid metabolism but would also impair the activity of MPS-related signaling.

METHODS

Subjects

Ten trained male runners volunteered to participate in the study (mean ± SD; age = 24 ± 1.5 yr, body mass = 75.9 ± 6 kg, height = 177.3 ± 7.2 cm, $\dot{V}O_{2peak}$ = 60 ± 3.6 mL·kg^{-1}·min^{-1}). All subjects gave written informed consent before participation after all experimental procedures and potential risks had been fully explained. None of the subjects had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment during the testing period. Subjects were instructed to refrain from any strenuous physical activity, alcohol, and caffeine consumption in the 48 h before each experimental trial. The study was approved by the ethics committee of Liverpool John Moores University.

Design

In a repeated-measures, randomized, crossover design separated by 7 d, subjects completed a twice per day exercise model under two different dietary conditions (both energy and protein matched) consisting of HCHO availability in the recovery period after both training sessions (i.e., best practice nutrition) or, alternatively, reduced CHO but HFAT. The twice per day exercise model comprised a morning (9:00–10:00 a.m.) high-intensity interval (HIT) training session (8 × 5 min at 85% $\dot{V}O_{2peak}$) followed by an afternoon (1:30–2:30 p.m.) training session consisting of steady-state (SS) running (60 min at 70% $\dot{V}O_{2peak}$). To promote training compliance during the HIT protocol in both the HCHO and the HFAT trials, subjects adhered to a standardized HCHO breakfast before this session. However, during the 3.5-h recovery between the HIT and the SS session and in the recovery period upon completion of the SS exercise protocol until the subsequent morning, subjects adhered to either an HCHO or an HFAT feeding protocol. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre-HIT, immediately post-SS, and at 3 and 15 h post-SS. An overview of the experimental design and the nutritional protocols are shown in Figure 1.

Preliminary Testing

At least 7–10 d before the first main experimental trial, subjects performed a maximal incremental running test to volitional fatigue on a motorized treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany) to determine maximal oxygen uptake. After a 10-min warm-up at a self-selected treadmill speed, the maximal incremental test commenced, beginning

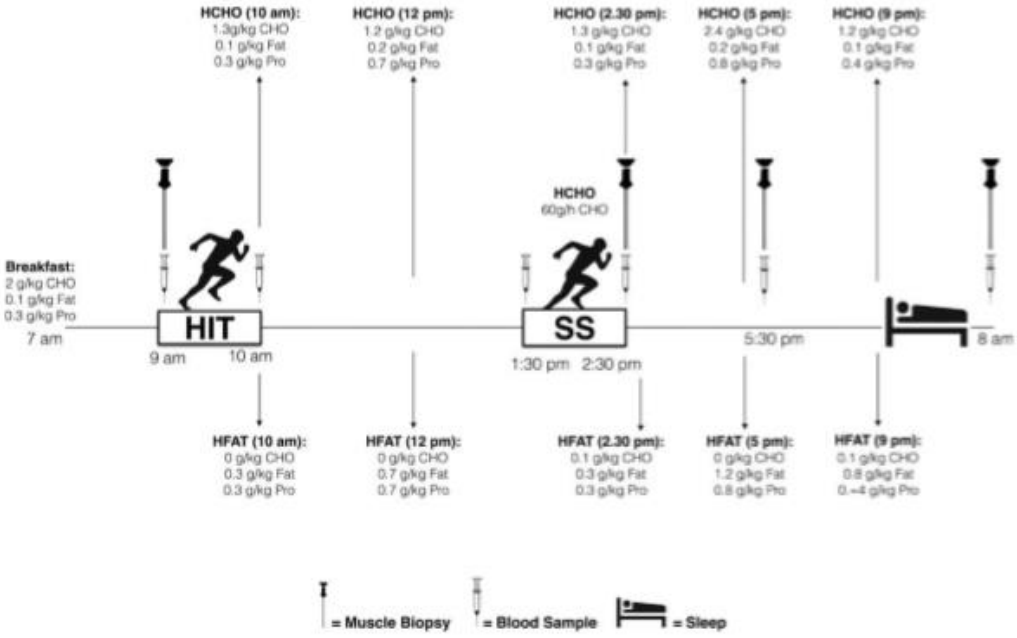

FIGURE 1—Overview of the experimental protocol used in each trial. HIT = 8 × 5 min running at a workload equal to 85% $\dot{V}O_{2peak}$ interspersed by 1 min recovery. SS = 1-h SS running at a workload equal to 70% $\dot{V}O_{2peak}$.

with a 2-min stage at a treadmill speed of 10 km·h^{-1}. Running speed was then increased by 2 km·h^{-1} every 2 min until a speed of 16 km·h^{-1} was reached, after which the treadmill inclined by 2% every 2 min until volitional exhaustion. $\dot{V}O_{2peak}$ was defined as the highest $\dot{V}O_2$ value obtained during any 10-s period and was stated as being achieved by two of the following criteria: 1) HR was within 10 bpm of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. On their second visit to the laboratory (approximately 3 d later), subjects completed a running economy test to determine their individual running speeds for subsequent experimental trials. After a warm-up, the test began with a 5-min stage at a treadmill speed of 8 km·h^{-1} with 1% incline, and speed was then increased by 1 km·h^{-1} every 5 min thereafter. The test was stopped when >90% of the previously determined $\dot{V}O_{2peak}$ was reached. These measurements were recorded via breath-by-breath gas measurements obtained continuously throughout both tests using a CPX Ultima series online gas analysis system (Medgraphics, St. Paul, MN). The test–retest reliability of this system in our laboratory when quantified using 95% limits of agreement is 0.29 ± 2.4 mL·kg^{-1}·min^{-1} (data were compiled from comparison of the oxygen uptake during the HIT protocols in the HCHO and HFAT trials undertaken in the present study). HR (Polar, Kempele, Finland) was also recorded continuously during exercise.

Experimental Protocols

HIT protocol. In the 24-h preceding each main experimental trial, subjects consumed a standardized HCHO diet in accordance with typical nutritional recommendations (8 g·kg^{-1} CHO, 2 g·kg^{-1} protein, and 1 g·kg^{-1} fat). On the morning of each experimental trial, subjects reported to the laboratory at ~7:00 a.m., where they were given a standardized HCHO breakfast (2 g·kg^{-1} CHO, 0.3 g·kg^{-1} protein, and 0.1 g·kg^{-1} fat). At 2-h postprandial, a venous blood sample was then collected from an antecubital vein in the anterior crease of the forearm, and a muscle biopsy sample was taken from the vastus lateralis muscle. Subjects were then fitted with an HR monitor, and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the high-intensity interval running (HIT) protocol, which lasted ~1 h. The HIT protocol consisted of 8 × 5-min bouts running at a velocity corresponding to 85% $\dot{V}O_{2peak}$ interspersed with 1 min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm-up and cooldown at a velocity corresponding to 50% $\dot{V}O_{2peak}$, and a further venous blood sample was obtained immediately

upon completion of the protocol. Water was given *ad libitum* throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. HR was measured continuously during exercise (Polar), and RPE (9) were obtained upon completion of each HIT bout. To determine substrate use during exercise (20), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics) for the final 2 min of each 5-min interval.

SS protocol. During the 3.5-h recovery period between the HIT and the SS protocols, subjects consumed either the HCHO (2.5 g·kg^{-1} CHO, 1 g·kg^{-1} protein, 0.3 g·kg^{-1} fat) or the HFAT (0 g·kg^{-1} CHO, 1 g·kg^{-1} protein, 1 g·kg^{-1} fat) feeding protocols (the pattern and frequency of feeding is shown in Fig. 1). After the recovery period, another venous blood sample was obtained immediately before commencing the afternoon SS exercise protocol. After a 5-min warm-up at a self-selected treadmill speed, subjects subsequently commenced the 60-min SS running protocol at a velocity corresponding to 70% $\dot{V}O_{2peak}$. During exercise, subjects also consumed 60 g·h^{-1} of CHO (SiS GO Istonic Gels; Science in Sport, Blackburn, UK) in HCHO, whereas no form of energy was consumed in the HFAT trial. Water was given *ad libitum* throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were also collected for 5 min at 15-min intervals throughout the exercise trial (CPX Ultima, Medgraphics) and substrate use again determined according to Jeukendrup and Wallis (20). HR was measured continuously during exercise (Polar), and RPE (9) were obtained every 15 min during exercise. Upon completion of the SS protocol until sleep, subjects consumed either the HCHO (3.6 g·kg^{-1} CHO, 1.5 g·kg^{-1} protein, 0.4 g·kg^{-1} fat) or the HFAT (0.2 g·kg^{-1} CHO, 1.5 g·kg^{-1} protein, 2.3 g·kg^{-1} fat) feeding protocols, where the pattern and frequency of feeding is shown in Figure 1. Vastus lateralis muscle biopsies and venous blood samples were also collected immediately postcompletion and at 3 and 15 h postcompletion (i.e., ~8:00 a.m. and in a fasted state) of the SS exercise protocol. The total energy intake across the entire trial period (i.e., 7:00 a.m.–9:00 p.m.) was ~10 g·kg^{-1} CHO, ~2.5 g·kg^{-1} protein, and ~0.8 g·kg^{-1} fat in HCHO and ~2.5 g·kg^{-1} CHO, ~2.5 g·kg^{-1} protein, and ~3.5 g·kg^{-1} fat in HFAT, where both trials were matched for total energy intake.

Blood Sampling and Analysis

Venous blood samples were collected in vacutainers containing ethylenediaminetetraacetic acid or lithium heparin and stored on ice until centrifugation at 1500*g* for 15 min at 4°C. After centrifugation, aliquots of plasma were stored in a freezer at −80°C for subsequent analysis. Samples were later analyzed for plasma glucose, lactate, nonesterified fatty acids (NEFA), glycerol, and β-hydroxybutyrate using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser; Randox, Co., Antrim, UK) as per the manufacturers' instructions.

Muscle Biopsies

Muscle biopsy samples (~50 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10 cm length (Bard Biopsy Systems, Tempe, AZ). Samples were obtained from separate incision sites 2–3 cm apart under local anesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at −80°C for later analysis.

Analysis of Muscle Glycogen

Muscle glycogen concentration was determined according to the methods described by van Loon et al. (37). Approximately 3–5 mg of freeze dried muscle was powdered, and all visible blood and connective tissue were removed. The freeze-dried sample was then hydrolyzed by incubation in 500 μL of 1 M HCl for 3 h at 100°C. After cooling to room temperature for ~20 min, samples were neutralized by the addition of 250 μL 0.12 mol·L^{-1} Tris/2.1 mol·L^{-1} KOH saturated with KCl. After centrifugation at 1500 RCF for 10 min at 4°C, 200 μL of the supernatant was analyzed 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 millimoles per kilogram of dry weight, and intra-assay coefficients of variation were <5%.

RNA Isolation and Analysis

Muscle biopsy samples (~20 mg) were homogenized in 1 mL TRIzol reagent (Thermo Fisher Scientific, Leicestershire, UK), and total RNA was isolated according to manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at optical densities of 260 and 280 nm using a Nanodrop 3000 (Fisher, Roskilde, Denmark). Seventy nanograms of RNA was then used for each polymerase chain reaction (PCR). Samples were ran in duplicate.

Primers

The identification of primer sequences was enabled by Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene), and primers were designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Specificity was ensured using sequence homology searches so the primers only matched the experimental gene with no unintended targets identified for primer sequences. To prevent the amplification of gDNA, primers were ideally designed to yield products spanning exon–exon boundaries. Three or more GC bases in the last five bases at the 3-end and secondary structure interactions (hairpins, self-dimer, and cross dimer) within the primers were avoided so there would be no nonspecific amplification. All primers were between 16 and 25 bp and amplified a product between 141 and 244 bp. All primers were purchased from Sigma (Suffolk, UK), and sequences for each gene are shown in parentheses: peroxisome proliferator-activated γ receptor coactivator (PGC-1) (fwd: TGCTAAACGACTCCGAGAA, rev: TGCAAAGTTCCCTCTCTGCT), tumor suppressor

TABLE 1. HR, RPE, and substrate oxidation responses during the HIT protocol in both the HCHO and the HFAT trials.

	HIT (Bout No.)							
	1	2	3	4	5	6	7	8
HR (bpm)								
HCHO	177 ± 9	181 ± 9	184 ± 8	185 ± 6*	186 ± 6*	185 ± 5	185 ± 5*	186 ± 7*
HFAT	173 ± 10	174 ± 8	180 ± 6	182 ± 7*	182 ± 7*	179 ± 6	182 ± 8*	184 ± 7*
RPE								
HCHO	14 ± 1.4	15 ± 1.5*	16 ± 2.0*	17 ± 1.6*	18 ± 0.9*	18 ± 1.0*	19 ± 0.6*	19 ± 0.7*
HFAT	14 ± 1.6	15 ± 1.8*	16 ± 1.9*	17 ± 1.1*	18 ± 1.2*	18 ± 1.0*	19 ± 0.9*	19 ± 1.03*
CHO oxidation (g·min^{-1})								
HCHO	5.5 ± 2.6	5.1 ± 1.4	4.9 ± 1.4	4.4 ± 1.4	3.9 ± 2.1*	3.7 ± 1.9*	3.5 ± 2.3*	3.8 ± 2.7*
HFAT	5.5 ± 2.7	4.8 ± 1.8	4.5 ± 1.6	4.4 ± 1.5	4.1 ± 2.2*	3.6 ± 1.9*	3.6 ± 2.4*	3.7 ± 2.7*
Fat oxidation (g·min^{-1})								
HCHO	0.00 ± 0.29	0.00 ± 1.2	0.00 ± 1.4	0.06 ± 1.8	0.25 ± 2.2*	0.3 ± 2.5*	0.38 ± 3.1*	0.31 ± 3.5*
HFAT	0.00 ± 0.96	0.00 ± 1.1	0.03 ± 1.4	0.09 ± 1.8	0.19 ± 2.1*	0.3 ± 2.5*	0.37 ± 3.0*	0.34 ± 3.4*

*Significant difference from HIT-1, $P < 0.05$.

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protein (p53) (fwd: ACCTATGGAAACTACTTCCTGAAA, rev: CTGGCATTCTGGGAGCTTCA), mitochondrial transcription factor A (Tfam) (fwd: TGGCAAGTTGTCCAAA GAAACCTGT, rev: GTTCCCTCCAACGCTGGGCA), citrate synthase (CS) (fwd: CCTGCCTAATGACCCCATGTT, rev: CATAATACTGGAGCAGCACCCC), estrogen-related receptor α (ERRα) (fwd: TGCCAATTCAGACTCTGTGC, rev: CCAGCTTCACCCCATAGAAA), peroxisome proliferator-activated receptor (PPAR) (fwd: ATGGAGCAGCCACAG GAGGAAGCC, rev: GCATGAGGCCCCGTCACAGC), PDH kinase, isozyme 4 (PDK4) (fwd: TGGTCCAAGATGC CTTTGAGT, rev: GTTGCCCGCATTGCATTCTT), glucose transporter type 4 (GLUT4) (fwd: TCTCCAACTGGACGAG CAAC, rev: CAGCAGGAGGACCGCAAATA), CPT1 (fwd: GACAATACCTCGGAGCCTCA, rev: AATAGGCCTGACGA CACCTG), fatty acid translocase (FAT/CD36) (fwd: AGGACTTT CCTGCAGAATACCA, rev: ACAAGCTCTGGTTCTTATTC ACA), and GAPDH (fwd: AAGACCTTGGGCTGGGACTG, rev: TGGCTCGGCTGGCGAC).

Reverse Transcriptase Quantitative Real-Time PCR

Reverse transcriptase quantitative real-time PCR amplifications were performed using a QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA). The following reverse transcriptase quantitative real-time PCR cycling parameters were used: hold 50°C for 10 min (reverse transcription/cDNA synthesis), initial denaturation and transcriptase inactivation at 95°C for 5 min, followed by PCR steps, i.e., 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Upon completion, dissociation/melting curve analysis was performed to reveal and exclude nonspecific amplification or primer–dimer issues (all melt analysis presented single reproducible peaks for each target gene, suggesting the amplification of a single product). Changes in mRNA content were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (34), where relative gene expression was calculated as $2^{-\Delta\Delta ct}$ and where C_t represents the threshold cycle. GAPDH was used as a reference gene and did not change significantly between groups or time points studied ($C_t = 24.2 \pm 1$); therefore, a pooled reference gene C_t was used in the relative gene expression equation. Furthermore, to enable calculation of expression values immediately post-exercise and 3-h postexercise, the calibrator condition in the $^{\Delta\Delta}C_t$ equation was assigned to the preexercise condition.

[γ^{-32}P] ATP Kinase Assay

Approximately 10–20 mg of muscle tissue was used for the measurement of p70S6K1 and AMPK-α2 activity, as previously described (27).

Statistical Analysis

All data were analyzed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA). Metabolic (i.e., blood metabolites, muscle glycogen, kinase activity, and mRNA data), physiological, and perceptual responses (i.e., HR, RPE, and oxidation rates) were analyzed using a two-way repeated-measures general linear model, where the within factors were time and condition (HCHO vs HFAT). *Post hoc* LSD tests were used where significant main effects and interactions were observed to locate specific differences between time points and conditions. All data in text, figures, and tables are presented as mean ± SD, with P values ≤0.05 indicating statistical significance.

TABLE 2. HR, RPE, and substrate oxidation during the SS protocol in both the HCHO and HFAT trials.

	Time (min)			
	15	30	45	60
HR (bpm)				
HCHO	166 ± 12	169 ± 11	170 ± 12*	172 ± 12*
HFAT	161 ± 11	165 ± 12	166 ± 10*	168 ± 10*
RPE				
HCHO	13 ± 1.3	14 ± 1.5*	15 ± 1.8	15 ± 1.8*
HFAT	13 ± 1.4	14 ± 1.8*	15 ± 2.1	16 ± 1.4*
CHO oxidation (g·min^{-1})				
HCHO	3.4 ± 0.8	3.1 ± 1.2*,**	3.5 ± 0.9*,**	3.3 ± 0.6*,**
HFAT	2.8 ± 0.5	2.3 ± 0.4*,**	2.1 ± 0.5*,**	2.0 ± 0.6*,**
Fat oxidation (g·min^{-1})				
HCHO	0.00 ± 0.30	0.13 ± 0.30*,**	0.26 ± 0.31*,**	0.35 ± 0.31*,**
HFAT	0.32 ± 0.30	0.55 ± 0.26*,**	0.65 ± 0.31*,**	0.71 ± 0.40*,**

*Significant difference from 15 min, $P < 0.05$.
**Significant difference between conditions, $P < 0.05$.

RESULTS

Physiological responses and substrate use during exercise. Comparisons of subjects' HR, RPE, and substrate oxidation during the HIT and SS protocols are displayed in Tables 1 and 2, respectively. HR, RPE, and lipid oxidation (all $P < 0.01$) all displayed progressive increases during both HIT (see Table 1) and SS exercise (see Table 2), whereas CHO oxidation displayed a progressive decrease ($P < 0.01$) during both exercise protocols. In accordance with identical preexercise feeding in HIT, no significant differences were apparent in any of the aforementioned variables between HCHO and HFAT ($P = 0.06$, 0.19, 0.52, and 0.56, respectively). By contrast, however, during the SS exercise protocol, CHO oxidation was significantly greater in HCHO compared with HFAT ($P < 0.001$), whereas fat oxidation was significantly greater during HFAT compared with HCHO ($P < 0.001$).

Plasma metabolite responses. Plasma glucose, lactate, NEFA, glycerol, and β-hydroxybutyrate all displayed significant changes (all $P < 0.01$) over the sampling period (see Table 3). However, in accordance with the provision of postexercise CHO feeding in the HCHO trial, plasma glucose was significantly higher compared with HFAT ($P < 0.01$), whereas postexercise high-fat feeding in HFAT induced significantly greater plasma NEFA, glycerol, and β-OHB (all $P < 0.01$) in HFAT compared with the HCHO trial.

Muscle glycogen and exercise-induced cell signaling. Exercise induced significant decreases ($P < 0.01$) in muscle glycogen immediately post-SS, although no differences were apparent between HCHO and HFAT at this time point (see Fig. 2A). However, in accordance with the provision of CHO after the SS exercise protocol in HCHO, muscle glycogen resynthesis was observed such that significant differences between HCHO and HFAT ($P = 0.01$) were observed at 3 and 15 h post-SS exercise. Neither exercise ($P = 0.407$) nor dietary condition ($P = 0.124$) affected AMPK-α2 activity at any time point studied (see Fig. 2B). By contrast, p70S6K1 activity was significantly increased 3 h post-SS exercise (30 min postfeeding) ($P < 0.01$), although this increase was suppressed ($P = 0.08$) in HFAT (see Fig. 2C). Furthermore, p70S6K1 activity was significantly reduced at 15 h post-SS exercise when participants were fasted compared with pre-HIT when they were high CHO and protein fed ($P < 0.01$).

Gene expression. Exercise increased the expression of PGC-1α ($P < 0.001$), p53 ($P = 0.032$), CS ($P = 0.05$), Tfam ($P = 0.05$), PPAR ($P < 0.01$), and ERRα ($P = 0.01$). However, there were no differences (all $P > 0.05$) between HFAT and HCHO trials (see Fig. 3A–F). By contrast, the exercise-induced increase ($P = 0.001$) in PDK4 mRNA was greater in HFAT versus HCHO ($P = 0.003$). Similarly, the mRNA expression of CD36 ($P = 0.05$) and CPT1 ($P = 0.02$) was significantly greater in HFAT in recovery from the SS exercise protocol (see Fig. 3). By contrast, neither exercise ($P = 0.12$) nor diet ($P = 0.31$) significantly affected GLUT expression (see Fig. 3).

DISCUSSION

The aim of the present study was to examine the effects of reduced CHO but high postexercise fat availability on the activation of key cell signaling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism, and MPS. When compared with HCHO availability, we observed that postexercise high-fat feeding had no modulatory affect on AMPK-α2 activity or the expression of those regulatory genes associated with mitochondrial biogenesis. Furthermore, although postexercise high-fat feeding augmented the expression of genes involved in lipid transport (i.e., FAT/CD36) and oxidation (i.e., CPT1), we also observed suppression of p70S6K1 activity despite sufficient postexercise protein intake. This latter finding suggests that postexercise high-fat feeding may impair the regulation of MPS and skeletal muscle remodeling processes, thereby potentially causing maladaptive responses for training adaptation if performed long-term.

TABLE 3. Plasma glucose, lactate, NEFA, glycerol, and β-OHB before and after the HIT and SS exercise protocols.

	Pre-HIT	Post-HIT	Pre-SS	Post-SS	3-h Post-SS	15-h Post-SS
Glucose (mmol·L^{-1})						
HCHO	5.2 ± 0.863	7.6 ± 0.74**	4.7 ± 0.96*,**	8.7 ± 1*,**	5.9 ± 0.62**	5.5 ± 0.3*
HFAT	5.4 ± 0.84	7.6 ± 1.03**	5.3 ± 0.34*,**	5.6 ± 0.8*,**	5.7 ± 0.6**	5.1 ± 0.42*
Lactate (mmol·L^{-1})						
HCHO	1.9 ± 0.42	7.8 ± 3.1**	1.5 ± 0.3*,**	1.6 ± 0.46**	1.5 ± 0.16*,**	1.1 ± 0.26**
HFAT	1.8 ± 0.41	7 ± 3.4**	1.1 ± 0.22*,**	1.3 ± 0.35**	0.8 ± 0.21*,**	0.9 ± 0.36**
NEFA (mmol·L^{-1})						
HCHO	0.01 ± 0.02	0.23 ± 0.27**	0.09 ± 0.13**	0.59 ± 0.56*,**	0.09 ± 0.17*,**	0.22 ± 0.22**
HFAT	0.05 ± 0.05	0.25 ± 0.28**	0.32 ± 0.21**	1.42 ± 0.74*,**	0.48 ± 0.27*,**	0.24 ± 0.14**
Glycerol (μmol·L^{-1})						
HCHO	14.8 ± 6.07	92.8 ± 27.1**	22.9 ± 12*,**	50.2 ± 37.4*,**	13.2 ± 4.39*,**	23.4 ± 20.1**
HFAT	12.9 ± 4.82	79.3 ± 25.4**	33 ± 7.3*,**	122.9 ± 57*,**	40.8 ± 12*,**	30.85 ± 12.6**
β-OHB (mmol·L^{-1})						
HCHO	0.07 ± 0.02	0.14 ± 0.04**	0.07 ± 0.02*,**	0.15 ± 0.08*,**	0.08 ± 0.02*,**	0.08 ± 0.03
HFAT	0.07 ± 0.02	0.14 ± 0.04**	0.11 ± 0.05*,**	0.33 ± 0.21*,**	0.29 ± 0.2*,**	0.19 ± 0.017

*Significant difference between conditions, $P < 0.05$.
**Significant difference from pre-HIT, $P < 0.05$.

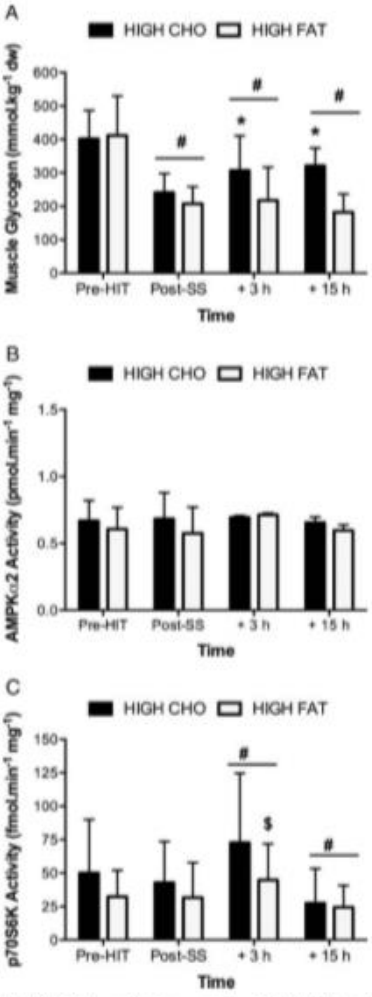

FIGURE 2—Skeletal muscle glycogen content (A), AMPK-α2 (B), and p70S6K1 activity (C) before HIT exercise and after the SS exercise protocol. #Significant difference from Pre-HIT, $P < 0.05$. *Significant difference between conditions, $P < 0.05$. $Difference between conditions, $P = 0.08$.

In accordance with the original train-low investigations examining cycling or knee extensor exercise (16,17,29,39), we also used a twice per day protocol, albeit consisting of morning HIT and afternoon SS running exercise protocol. This model is practically relevant given that many elite endurance athletes (including runners) train multiple times per day with limited recovery time between training sessions (14). Given that reduced CHO availability impairs high-intensity training capacity (17,39), we also chose to schedule the HIT session in the morning period after a standardized HCHO breakfast. As expected, no differences in cardiovascular strain, RPE, substrate use, and plasma metabolite responses were observed between the HCHO and the HFAT trials during the HIT session (see Tables 1 and 3). After completion of the HIT protocol, subjects then adhered to an HCHO or HFAT feeding

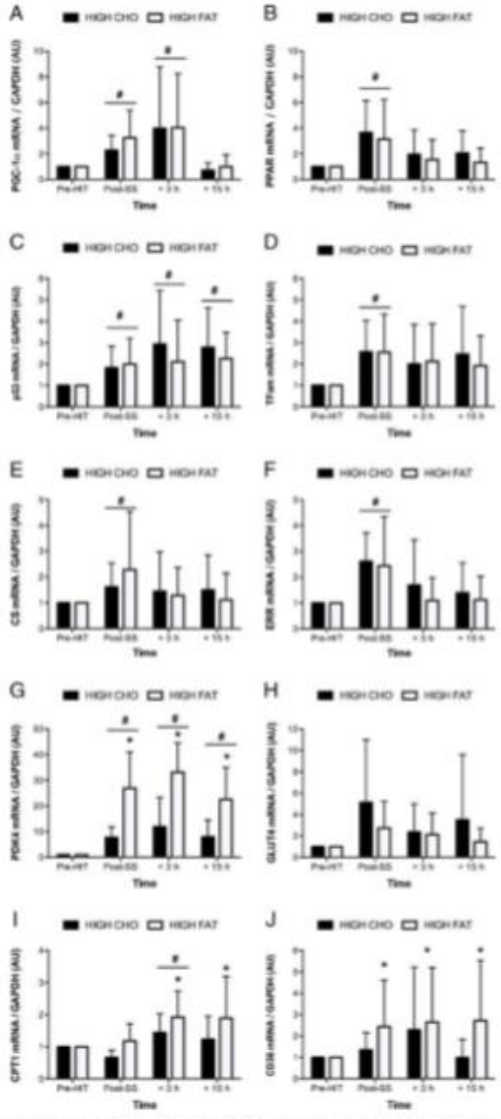

FIGURE 3—PGC-1α (A), PPAR (B), p53 (C), Tfam (D), CS (E), ERRα (F), PDK4 (G), GLUT4 (H), CPT1 (I), and CD36 mRNA (J) before HIT exercise and after the SS exercise protocol. #Significant difference from pre-HIT, $P < 0.05$. *Significant difference between conditions, $P < 0.05$.

protocol in the 3.5 h before commencing the afternoon SS exercise. Given that exogenous CHO feeding during exercise reduces oxidative adaptations even in the presence of reduced preexercise muscle glycogen (29), we also chose to feed exogenous CHO (at a rate of 60 g·h^{-1}) during the afternoon SS protocol during the HCHO trial. Although we did not directly quantify muscle glycogen immediately before SS exercise, plasma metabolite and substrate use during SS exercise were clearly suggestive of differences in both endogenous and exogenous CHO availability between the HCHO and the HFAT trials. Indeed, plasma NEFA, glycerol, β-OHB, and whole body lipid oxidation were all greater during SS exercise undertaken in the HFAT trial compared with the HCHO trial (see Tables 2 and 3). On the basis of comparable muscle glycogen data post-SS exercise (see Fig. 2A) and greater whole body CHO oxidation during the HCHO trial (see Table 2), we also suggest that exercise-induced muscle glycogen use was greater during the SS exercise protocol when completed in the HCHO conditions (7).

Perhaps surprisingly, we observed that our SS exercise protocol did not increase AMPK-α2 activity in either the HCHO or HFAT trial. However, there are likely several physiologically valid reasons to explain the apparent lack of AMPK-mediated signaling. Indeed, exercise-induced AMPK activation is known to be intensity dependent, where >70% $\dot{V}O_{2max}$ is likely required to induce metabolic perturbations sufficient to mediate a signaling response (13). Furthermore, the AMPK response to exercise is attenuated with exercise training (8), an effect that is especially relevant for the present investigation given the trained status of our chosen population and the low plasma lactate observed (approximately 2 mmol·L^{-1}) during SS exercise. Reduced absolute muscle fiber recruitment from the vastus lateralis, when compared with other lower extremity muscles recruited during walking and running (19), or when exercising at similar relative intensities during cycling (4) and where AMPK activation is typically reported (22), could also contribute, in part, to the lack of AMPK signaling observed here. Finally, although exercise-induced AMPK activity is also thought to be regulated, in part, via a glycogen-binding domain on β-subunit of the AMPK heterotrimer (26), it is possible that our runners did not exceed a potential "muscle glycogen threshold" that is required to fully activate the AMPK complex during prolonged endurance exercise (30). Indeed, previous data from our laboratory also using running exercise protocols (6,7) have typically only observed AMPK-related signaling when postexercise whole muscle homogenate glycogen is <200 mmol·kg^{-1} dry weight. Despite previous suggestions that train-low training sessions should be targeted to SS exercise protocols so as to not compromise training intensity (5), our data therefore suggest (at least for AMPK-mediated signaling) that perhaps it is the actual completion of a high-intensity stimulus *per se* (especially in trained athletes) that is really required to create a metabolic milieu that is conducive to augmentation of necessary signaling networks.

In contrast to Yeo et al. (38), we also observed no modulatory effect of postexercise high-fat availability on resting AMPK-α2 activity. Indeed, these authors observed that 5 d of a fat loading protocol increased resting AMPK-α2 activity as well as the exercise-induced phosphorylation of ACCS^{ser221}. Such discrepancies between studies are likely due to the differences in duration of high-fat feeding in that we adopted an acute high-fat feeding protocol (<24 h), whereas the latter authors adopted a 5-d "fat adaptation" protocol that also increased resting IMTG stores. In this regard, it is noteworthy that the magnitude of change in resting AMPK-α2 activity was positively correlated with the elevations in IMTG storage (38).

In contrast to our hypothesis, we also observed comparable two- to threefold changes between trials in the mRNA expression of those genes with key regulatory roles associated with mitochondrial biogenesis. For example, the expression levels of PGC-1α, p53, Tfam, PPAR, and ERRα mRNA were all elevated with similar magnitude and time course in recovery from the SS protocol in both the HCHO and the HFAT trials. Such data conflict with previous observations from our laboratory (7) and others (32) where postexercise CHO restriction (i.e., keeping muscle glycogen low) augments the expression of many of the aforementioned genes. However, in our previous report, we simultaneously adopted a CHO but calorie restriction feeding protocol, whereas the present design incorporated a reduced CHO but isocaloric and protein-matched feeding protocol in our HFAT trial. Given the similarities in metabolic adaptation to both CHO and caloric restriction, such data raise the question whether the enhanced mitochondrial responses observed when "training low" are due to transient periods of CHO restriction, calorie restriction, or indeed a combination of both. This point is especially relevant from an applied perspective given that many endurance athletes present daily with transient periods of both CHO and calorie restriction because of multiple training sessions per day as well as longer-term periods of suboptimal energy availability (14).

In agreement with multiple studies demonstrating a role of both acute elevations in FFA availability (7,23) as well as high-fat feeding protocols (11), we also observed that the postexercise expression levels of PDK4, FAT/CD36, and CPT1 mRNA were elevated in the HFAT trial versus the HCHO trial. However, unlike Arkinstall et al. (4), we did not detect any suppressive effects of high-fat availability on GLUT4 mRNA expression, although a longer and more severe period of CHO restriction used by these investigators (i.e., 48 h of absolute CHO intake <1 g·kg^{-1} body mass resulting in muscle glycogen levels <150 mmol·kg^{-1} dry weight) may explain the discrepancy between studies. Nonetheless, the dietary protocol studied here clearly alters the expression of genes with potent regulatory roles in substrate use and, if performed long term, may increase the capacity to use lipids as a fuel but induce suppressive effects on CHO metabolism (through suppression of the PDH complex), thus potentially limiting high-intensity performance (35). Although we did not directly quantify the signaling mechanisms underpinning these responses (owing to a lack of a

muscle tissue), we suggest both p38MAPK and PPAR-mediated signaling are likely involved. Indeed, using a twice per day exercise model, Cochran et al. (12) also observed enhanced p38MAPK phosphorylation during the afternoon exercise protocol (despite similar preexercise muscle glycogen availability) that was associated with the enhanced circulating FFA availability during the afternoon exercise. Furthermore, pharmacological ablation of circulating FFA availability during exercise suppresses p38MAPK compared with control conditions (40). In addition, FFA-mediated signaling can also directly mediate PPAR binding to the CPT1 promoter thereby modulating CPT1 expression (31).

We also examined the effects of postexercise fat feeding on the regulation of p70S6K activity, a key signaling kinase associated with regulating MPS. In relation to the effects of endurance exercise *per se*, the majority of studies are typically limited to measures of phosphorylation status with some studies reporting increases (25) and others reporting no change. When examined quantitatively using the [γ^{-32}P] ATP kinase assay, our data agree with previous observations from Apro et al. (3), who also reported no change but conflict with recent data from our group where we observed an exercise-induced suppression of p70S6K activity (18). Nonetheless, the exhaustive (a fatiguing cycling HIT protocol) and muscle glycogen depleting (<100 mmol·kg^{-1} dry weight) nature of the latter exercise protocol versus the moderate-intensity nature of the afternoon SS running protocol studied here likely explains the discrepancy between studies.

In relation to the effects of postexercise feeding, we also provide novel data by demonstrating that postexercise high-fat feeding was associated with a suppression of p70S6K activity (albeit $P = 0.08$) at 3 h postcompletion of the SS exercise protocol when compared with the elevated response observed in HCHO (when using both a mean difference and standard deviation of differences of 50 fmol·min^{-1}·mg^{-1}, we estimate a sample size of 12–13 would be required to achieve statistical significance with 90% power, as calculated using Minitab statistical software, version 17). Although we did not measure circulating insulin levels in this study, it is of course possible that the suppressed p70S6K response observed here may be due to reduced upstream insulin-mediated activation of protein kinase B (PKB). Indeed, we recently observed postexercise p70S6K activity to be suppressed in conditions of simultaneous carbohydrate and calorie restriction in a manner associated with reduced insulin and upstream signaling of PKB (18). Alternatively, the suppression of p70S6K observed here may be mediated through direct effects of postexercise high-fat feeding that are independent of CHO availability, energy availability, and insulin. Indeed, Stephens et al. (36) observed infusion of intralipid and heparin to elevate circulating FFA concentrations attenuates MPS in human skeletal muscle in response to ingesting 21 g amino acids under euglycemic hyperinsulemic clamp conditions. Furthermore, Kimball et al. (21) also reported that high-fat feeding impairs MPS in rat liver in a manner associated with reduced p70S6K phosphorylation, an effect that may be induced through sestrin 2 and sestrin 3-mediated impairment of mechanistic target of rapamycin complex signaling. Clearly, further research is required to examine the effects of high-fat feeding on direct measures (and associated regulatory sites) of MPS within the physiological context of the exercising human.

In summary, we provide novel data by concluding that postexercise high-fat feeding has no modulatory affect on AMPK-α2 activity or the expression of those genes associated with regulatory roles in mitochondrial biogenesis. Furthermore, although postexercise high-fat feeding augmented the expression of genes involved in lipid transport and oxidation, we also observed a suppression of p70S6K1 activity despite sufficient postexercise protein intake. This latter finding suggests that postexercise high-fat feeding may impair the regulation of MPS and postexercise muscle remodeling, thereby potentially causing maladaptive responses for training adaptation if performed long-term. Future studies should now examine the functional relevance of the signaling responses observed here, not only in terms of acute MPS but also in terms of chronic skeletal muscle and performance adaptations induced by long-term use of this feeding strategy.

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Appendix 5

Post exercise high-fat feeding increases lipid gene expression but supresses p70S6K1 activity: Implications for training adaptation. Presented as an oral at ECSS for the GSSI Award, 2016, Vienna.

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Introduction

Carbohydrate (CHO) restriction augments training-induced increases in skeletal muscle mitochondrial biogenesis (Bartlett *et al.* 2015). Additionally, high-fat diets enhance the activities of enzymes involved in fat oxidation and transport (Erlenbusch *et al.* 2005). We therefore tested the hypothesis that reduced CHO availability but high fat availability provides an additive effect on the exercise-induced cell signalling pathways and gene expression responses associated with the regulation of mitochondrial biogenesis, lipid gene expression and muscle protein synthesis.

Method

Ten males completed a twice per day exercise model (3.5 h between sessions) comprising morning high-intensity interval (HIT) (8 x 5-min at 85% $\dot{V}O_{2max}$) and afternoon steady-state (SS) running (60 min at 70% $\dot{V}O_{2max}$). In a repeated measures design, runners exercised under different isoenergetic dietary conditions consisting of high CHO (HCHO: 10 CHO, 2.5 Protein and 0.8 Fat, $g.kg^{-1}$ per whole trial period) or reduced CHO but high fat availability in the post-exercise recovery periods (HFAT: 2.5 CHO, 2.5 Protein and 3.5 Fat, $g.kg^{-1}$ per whole trial period). Vastus Lateralis biopsies were obtained pre-HIT, post-SS and at 3 and 15 h post- SS exercise to assess muscle glycogen content, signalling activity of metabolic (AMPK-α2) and protein synthesis (p70S6K) transduction networks and expression of genes associated with mitochondrial biogenesis and lipid metabolism.

Results

Muscle glycogen was lower ($P<0.05$) at 3 h (251 vs 301 mmol.kg^{-1}dw) and 15 h (182 vs 312 mmol.kg^{-1}dw) post-SS exercise in HFAT compared to HCHO. AMPK-α2 activity was not increased post-SS in either condition ($P=0.41$) though comparable 2-3-fold mRNA increases (all $P<0.05$) in those genes associated with mitochondrial biogenesis were observed in both HCHO and HFAT: PGC-1α (3-fold), p53 (2-fold), Tfam (3-fold), PPAR (3-fold) and ERRα (3-fold). In contrast, the mRNA expression of CD36 ($P=0.05$) and CPT1 ($P=0.03$) was 1-2-fold greater in HFAT in the recovery period from SS exercise compared with HCHO. p70S6K activity was higher ($P=0.08$) at 3 h post-SS exercise in HCHO versus HFAT (72.7 ± 51.9 vs 44.7 ± 27 fmol.min^{-1} mg^{-1}).

Discussion

We conclude that post-exercise high fat feeding does not "regulate the regulators" of mitochondrial biogenesis though it does increase lipid gene expression. However, high fat feeding supresses p70S6K1 activity thus potentially impairing post-exercise skeletal muscle remodelling. As such high-fat post-exercise feeding offers no significant metabolic advantages for trained athletic populations.

References

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