The effect of endogenous and exogenous carbohydrate availability on exercise-induced skeletal muscle signalling

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

March 2020

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Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work presented within this thesis is entirely my own.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university.

Parts of the work presented here have been published in peer-reviewed journals and, where this is the case, acknowledgements have been provided.

Abstract

Whilst pre-exercise muscle glycogen concentrations appear to regulate mitochondrial-related cell signalling responses to exercise, the absolute concentrations of muscle glycogen required to potentiate such responses are currently unknown. With this in mind, chapter 4 and 5 of this thesis were designed to systematically titrate muscle glycogen concentrations and determine subsequent mitochondrial-related signalling responses to endurance exercise in an attempt to elucidate the potential existence of a glycogen threshold and provide a better definition of its upper and lower limits. Collectively, using a sleep-low, train-low model, these studies demonstrate that reducing pre-exercise muscle glycogen prior to high-intensity endurance exercise does not confer any benefit to mitochondrial-related signalling and, in some cases, may impair exercise capacity. Nonetheless, these data raise the possibility that obtaining low post-exercise glycogen concentrations (regardless of pre-exercise status) may also be an important factor in regulating mitochondrial-related signalling. Accordingly, given the potential for exogenous CHO to modulate post-exercise muscle glycogen concentrations, chapter 6 examined the effects of exogenous carbohydrate (CHO) feeding during exercise on mitochondrial-related signalling. However, exogenous CHO feeding did not alter muscle glycogen utilisation during exercise and resulted in comparable increases in post-exercise mitochondrial-related signalling. In conclusion, this thesis provides novel data demonstrating that reducing pre-exercise muscle glycogen and/or exogenous CHO restriction during exercise does not confer any additional benefit to exercise-induced mitochondrial related signalling. Practically, these data suggest that the additional stress of CHO restriction may not be required within skeletal muscle that is already subjected to a sufficient metabolic challenge and may be better suited during conditions that do not elicit such cellular perturbations (e.g. prolonged low-intensity exercise completed below lactate threshold).

List of abbreviations

AICAR:	5-Aminoimidazole-4-carboxamide ribonucleotide
ACC:	acetyl-CoA carboxylase
ADP:	adenosine diphosphate
AMP:	adenosine monophosphate
AMPK:	AMP-activated protein kinase
ATF2:	activating transcription factor 2
ATP:	adenosine triphosphate
AU:	arbitrary units
β-HAD:	3-hydroxyacyl-CoA dehydrogenase
B2M:	β2 microglobulin
bp:	base pairs
BCA:	bicinchoninic acid
BSA:	bovine serum albumin
Ca^{2+}:	calcium
CaMKII:	calmodulin dependent protein kinase II
CD36:	cluster of differentiation 36
cDNA:	complimentary DNA
CHO:	carbohydrate
CPT-1:	carnitine palmitoyltransferase 1
COXIV:	cytochrome c oxidase IV
CRE:	cAMP response element
CREB:	cAMP response element binding protein
CS:	citrate synthase
DEPC:	diethylpyrocarbonate
DNA:	deoxyribose nucleic acid
dH^2O:	distilled water
dw:	dry weight
ECL:	enhanced chemiluminescence
FFA:	free fatty acid
g:	gram
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GI:	glycaemic index
h:	hour
HAT:	histone acetyl transferase
HCl:	hydrochloric acid

HDAC: histone deacetylase

hr: heart rate

HRP: horseradish peroxidase

IMCL: intramyocellular

kg: kilograms

KJ: kilojoules

km: kilometres

KO: knock-out

KOH: potassium hydroxide

KCl: potassium chloride

LKB1: liver kinase B1

LDH: lactate dehydrogenase

MEF2: myocyte enhancer factor 2

mg: milligrams

ml: millilitres

min: minute

mmol: millimole

mRNA: messenger RNA

MTC: multiple transportable carbohydrate

MtDNA: mitochondrial DNA

NEFA: non-esterified fatty acid

nm: nanometre

NRF: nuclear respiratory factor

NuGEMPs: nuclear genes encoding mitochondrial proteins

p38 MAPK: p38 mitogen activated protein kinase

p53: tumour suppressor protein 53

PCR: polymerase chain reaction

PDK4: pyruvate dehydrogenase

PGC-1α: peroxisome proliferator-activated receptor [gamma] coactivator-1α

Pi: inorganic phosphate

PPAR: peroxisome proliferator-activated receptor gamma

PPO: peak power output

RER: respiratory exchange ration

RNA: ribonucleic acid

RPE: rating of perceived exertion

RPM: revolutions per minute

RT-qPCR: real time quantitative polymerase chain reaction

SCO2: synthesis of COX 2

SDH: succinate dehydrogenase

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SGLT1: sodium-dependent glucose transporter 1

Ser: Serine

SR: sarcoplasmic reticulum

TCA: tricarboxylic acid

Thr: threonine

Tfam: mitochondrial transcription factor A

UCP3: mitochondrial uncoupling protein 3

W: watt

Wmax: watt max

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Acknowledgements

Firstly, I would like to thank my supervisors Julien Louis, James Morton and Adam Sharples for allowing me the opportunity to pursue my passion in exercise metabolism and nutrition and develop as a scientist over the course of my PhD. I would also like to thank Dan Owens for your time and effort to teach me new laboratory skills and provide direction throughout the project. Your advice and support have been unrivalled throughout.

Secondly, I would like to thank everyone within the sport & exercise science department at LJMU. Their unrelenting work-rate and willingness to help others make this department a special place to work. Most importantly, your friendship is something that I will forever value.

To my family. You have supported me in many ways throughout the entirety of my education and always encouraged me to follow my dreams. I hope this work has given you something to be proud of.

Finally, I want to thank my partner Abigail for her continued support throughout this journey. Thank you for listening to my frustrations, celebrating my successes and your continued belief in me. I could not have done this without you.

Chapter One:

General Introduction

This chapter provides a broad overview of the role of CHO availability on modulating aspects of exercise performance and adaptation and introduces the field of training with low CHO availability.

Since the initial discovery and description of glycogen by Claude Bernard (communicated to Académie des Sciences on 23rd March 1857) over 160 years ago, a significant body of work examining how the storage of carbohydrate as branched polymers effects both metabolism and cellular regulation now exists. Furthermore, the advent of the muscle biopsy technique during the 1960s (Hultman, 1967) paved the way for the examination of carbohydrate metabolism during exercise. Indeed, these early studies (Bergström & Hultman, 1966; Bergström *et al.*, 1967; Hermansen *et al.*, 1967) collectively recognised skeletal muscle glycogen as the predominant energy substrate used during endurance exercise and its importance in regulating the capacity to sustain exercise at a given workload. Later work demonstrated the role of exogenous carbohydrate (CHO) feeding during exercise in extending the capacity to sustain exercise at a given intensity (Coggan & Coyle, 1988; Wright *et al.*, 1991; Widrick *et al.*, 1993; Febbraio *et al.*, 2000) through a potential sparing of muscle glycogen (Bjorkman *et al.*, 1984; Erickson *et al.*, 1987; Stellingwerff *et al.*, 2007) and maintenance of high rates of CHO oxidation as exercise progresses. Collectively, these studies provided the foundations for traditional sports nutrition guidelines that promote high carbohydrate (CHO) availability before, during and after endurance exercise to enhance exercise performance and delay fatigue (American Dietetic Association, 1993).

In addition to its role as a simple fuel source, carbohydrate may also act as a regulatory molecule that is able to modulate the acute molecular response to exercise within skeletal muscle (Philp *et al.*, 2012). More specifically, commencing endurance exercise with low endogenous CHO (defined as a 'train-low' session; Burke *et al.*, 2018) augments key signalling cascades and downstream mRNA expression responses associated with oxidative adaptations within human skeletal muscle. Furthermore, repeated bouts of train-low exercise (3-10 weeks) has been reported to augment selected skeletal muscle markers of training adaptation (Hansen *et al.*, 2005; Yeo *et al.*, 2008; Morton *et al.*, 2009; Hulston *et al.*, 2010), enhance both whole body and intramuscular lipid oxidation (Hulston *et al.*, 2010) and, in some instances, improve exercise capacity and performance (Hansen *et al.*, 2005; Marquet *et al.*, 2016*a*, 2016*b*). Whilst not all studies demonstrate clear positive effects of train-low models on skeletal muscle adaptation (Gejl *et al.*, 2017; Riis *et al.*, 2019) positive outcomes are reported in over 70% of available studies (Impey *et al.*, 2018) and have led to increased recognition amongst athletic populations (Stellingwerff, 2012). In addition to endogenous muscle glycogen, exogenous carbohydrate availability may also play a role in regulating skeletal muscle signalling responses to exercise (Akerstrom *et al.*, 2006; Lee-Young *et al.*, 2006) although current data are contradictory and a more comprehensive understanding of how exogenous CHO regulates alternative signalling pathways in response to exercise is required.

Given the enhanced molecular signalling responses associated with train-low methodologies may, in part, be regulated by muscle glycogen availability, it is prudent to consider the absolute glycogen concentrations required to facilitate these responses and how exogenous CHO feeding during exercise may interact with such responses. In this regard, examination of available train-low studies demonstrate that augmented signalling and transcriptional responses to exercise are particularly apparent when absolute pre-exercise muscle glycogen concentrations are ≤ 300 mmol·kg^{-1} dw (Impey *et al.*, 2018), thus suggesting the potential existence of a muscle glycogen threshold; an absolute concentration of muscle glycogen which must be surpassed in order to augment such responses associated with the train-low model.

However, despite the preliminary evidence for the potential existence of a muscle glycogen threshold (defined as a range of muscle glycogen concentrations that is permissive to facilitating exercise induced cell signalling responses) the use of a systematic approach to induce graded differences in pre-exercise muscle glycogen concentrations is required to provide a better definition of its upper and lower limits. Furthermore, the interaction between endogenous and exogenous carbohydrate availability within the context of the glycogen threshold hypothesis also warrants further investigation in order to develop a more comprehensive understanding of the signalling network that regulates these interactions.

Aims and objectives

The primary aim of this thesis is, therefore, to examine the effects of both endogenous and exogenous CHO availability on the regulation of molecular pathways associated with mitochondrial biogenesis within human skeletal muscle. On this basis, a secondary aim is to investigate the existence of a potential muscle glycogen threshold and provide a better definition of its potential upper and lower limits. These aims will be achieved by completion of the following objectives:

a) To examine the effects of graded reductions in pre-exercise muscle glycogen (below 300 mmol·kg^{-1} dw) on skeletal muscle cell signalling and gene expression responses associated with the regulation of mitochondrial biogenesis and substrate metabolism.
b) To examine the effects of graded reductions in pre-exercise muscle glycogen (between the range of 100-600 mmol·kg^{-1} dw) on skeletal muscle cell signalling and gene expression responses associated with the regulation of mitochondrial biogenesis and substrate metabolism.

c) To examine the effects of graded exogenous CHO intake during exercise on skeletal muscle cell signalling responses associated with the regulation of mitochondrial biogenesis.

Chapter Two:

Literature review

This chapter provides a synthesis of research findings that describe the regulation of carbohydrate storage and its subsequent metabolism during exercise. A review of the literature in relation to muscular adaptation to exercise training, the molecular signals that underpin these adaptations and the modulating role of carbohydrate availability then follows.

Elements of this chapter have resulted in the following publication: Hearris *et al* (2018). Regulation of muscle glycogen metabolism during exericse. Implications for endurance performance and training adaptation. *Nutrients* **10,** 1–21.

2.1 Background

Skeletal muscle tissue demonstrates remarkable plasticity, whereby it possesses an extraordinary ability to undergo major adaptations in response to exercise training. Of these adaptations, an increase in mitochondrial number and content (Holloszy *et al.*, 1970; Varnauskas *et al.*, 1970; Andersen & Henriksson, 1977; Ingjer, 1979; Wibom *et al.*, 1992; Phillips *et al.*, 1996; MacDougall *et al.*, 1998; Perry *et al.*, 2010), known as mitochondrial biogenesis (Hood, 2001), is seen as the major hallmark adaptation, allowing for a higher absolute exercise work capacity. In addition to the observed changes in mitochondrial content, endurance training also induces an increase in the activity and content of proteins within the TCA cycle and ETC as well as those involved in substrate transport and utilisation (Holloszy *et al.*, 1970; Gollnick *et al.*, 1972; Phillips *et al.*, 1996; Talanian *et al.*, 2010). The functional consequence of these adaptations is that exercise in the trained state results in smaller disturbances to cellular homeostasis (i.e. reduced accumulation of ADP, AMP & Pi) resulting in an increased reliance on lipid metabolism, with a proportional down-regulation of CHO metabolism for a given exercise intensity (Hermansen *et al.*, 1967; Karlsson *et al.*, 1972; Henriksson, 1977; Hurley *et al.*, 1986; Coggan *et al.*, 1990; Kiens *et al.*, 1993; Phillips *et al.*, 1996; LeBlanc *et al.*, 2004).

Whilst the signal transduction pathways regulating mitochondrial biogenesis are undoubtedly multi-factorial and highly complex, their activation via specific exercise-induced signals ultimately initiates gene transcription and translation to create new functional proteins (Coffey & Hawley, 2007). Whilst the traditional approach to activate such signal transduction pathways focused on the manipulation of training intensity and duration, it is now apparent that such signalling pathways can be modulated by carbohydrate (CHO) availability (Pilegaard *et al.*, 2002; Wojtaszewski *et al.*, 2003; Steinberg *et al.*, 2006; Yeo *et al.*, 2010; Bartlett *et al.*, 2013) whereby commencing endurance exercise with low CHO availability is now recognised as a potential strategy to augment exercise induced activation of signal transduction pathways that ultimately enhance skeletal muscle adaptation (Impey *et al.*, 2018) and has subsequently gained recognition within athletic training paradigms (Stellingwerff, 2012).

This chapter aims to present a contemporary discussion of our understanding of CHO within the context of skeletal muscle metabolism, signal transduction pathways and exercise performance. This chapter provides an overview of CHO storage followed by a critical review of the well-documented effects of both endogenous and exogenous CHO availability on exercise performance. Finally, this chapter will close by discussing the molecular networks that govern exercise-induced training adaptation and the role of CHO availability in modulating these responses.

2.2 Overview of carbohydrate structure and storage

Carbohydrate is predominantly stored as glycogen in both the liver (~100 g) and skeletal muscle (300-600 g) and demonstrates a relatively small stored energy pool when compared with that of lipids. Within skeletal muscle, glycogen is typically expressed as mmol·kg^{-1} of dry weight (dw) where concentrations in whole muscle homogenate can typically vary from 50 to 800 mmol·kg^{-1} dw, depending on training status, fatigue status and dietary CHO intake (Areta & Hopkins, 2018) (Figure 2.1). Within the classical literature, muscle glycogen is commonly expressed as mmol·kg^{-1} of wet weight (ww), where dry weight values are 4.35 times greater than wet weight values based on an expected 77% water content within the muscle (Areta & Hopkins, 2018). The glycogen granule itself is constructed from a tiered assembly of glucose units, which forms a branched structure via 1:4 and 1:6 α-glycosidic bonds (covalent bonds attached between carbon 1:4 and carbon 1:6; Figure 2.2). Glycogen granules are formed on the protein glycogenin, and can be as large as 42 nm in diameter and consist of up to 55,000 glucosyl units (Graham *et al.*, 2010), although the majority of glycogen granules within skeletal muscle are typically reported to be 25 nm in diameter and comprise of approximately 8 tiers (Marchand *et al.*, 2002). The branched structure of glycogen allows for the dense compartmentalization of glucose and an enhanced surface area for phosphorylase mediated degradation, permitting rapid mobilization in response to elevated energy requirements such as the onset of exercise (Melendez-Hevia *et al.*, 1993).

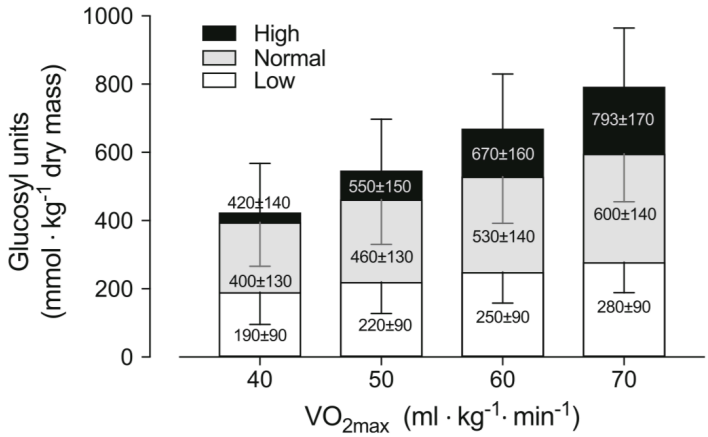

Figure 2. 1. Modelled relationship between variations in muscle glycogen storage of the vastus lateralis according to $\dot{V}O_2max$ status in conditions of low, normal and high carbohydrate availability in individuals of different fitness levels. Taken from Areta & Hopkins (2018)

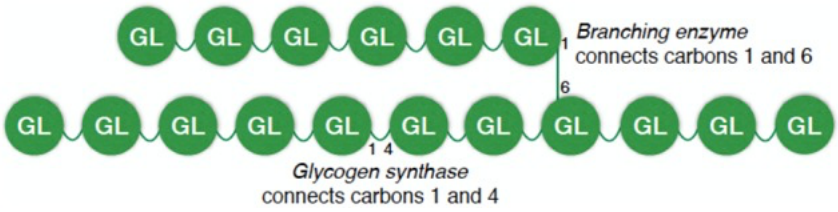

Figure 2. 2. Attachment of glucose units via α-glycosidic bonds. Taken from Murray & Rosenbloom (2018).

Beyond the quantification of muscle glycogen in whole muscle homogenate, it is apparent that glycogen is distributed across different fibre types as well as being located in specific intracellular locations within the muscle cell itself (Figure 2.3). Through the use of histochemical techniques, it is reported that resting glycogen content is not markedly different between type I and II fibres (Essen & Henriksson, 1974; Essén *et al.*, 1975), although biochemical quantification, which provides a more quantitative measure, suggests type II fibres may contain 50-100 mmol·kg^{-1} dw more glycogen than type II fibres (Tsintzas *et al.*, 1995, 1996). Furthermore, the advent of transmission electron microscopy (TEM) techniques has allowed for the quantification of glycogen within three distinct subcellular pools contained within the myofibrils (intra-myofibrillar glycogen, 5-15% of total glycogen pool) between myofibrils (inter-myofibrillar glycogen, 75% of total glycogen pool) and beneath the sarcolemmal region (subsarcolemmal glycogen, 5-15% of total glycogen pool). Within endurance trained muscle, it appears that type I fibres contain 82% more intra-myofibrillar and 31% more subsarcolemmal glycogen than type II fibres, with type II fibres containing 11% more inter-myofibrillar glycogen than type I fibres (Nielsen *et al.*, 2011; Nielsen & Ørtenblad, 2013; Ørtenblad *et al.*, 2013; Gejl *et al.*, 2014) although these fibre type differences do not appear to be present in untrained muscle and may therefore represent training-induced adaptations within skeletal muscle.

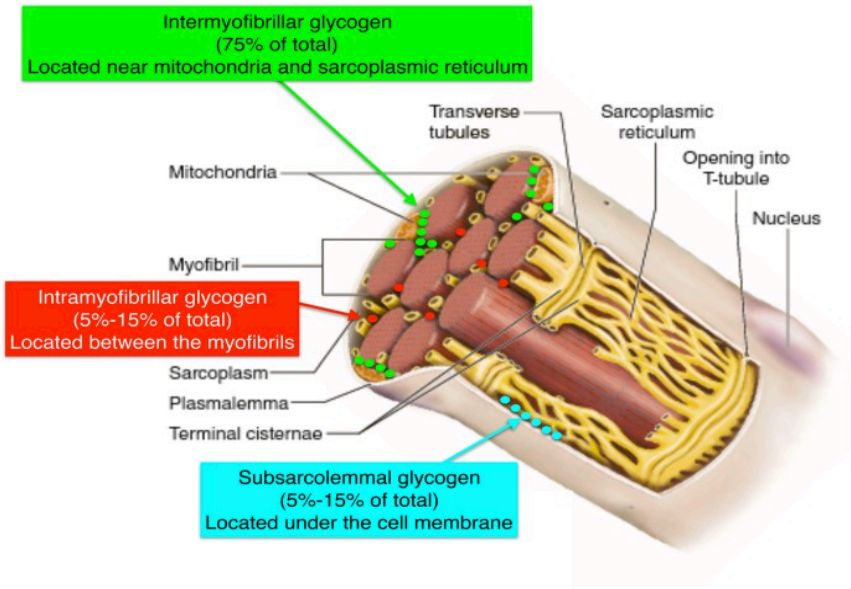

Figure 2. 3. Subcellular glycogen storage within human skeletal muscle. Taken from Murray & Rosenbloom (2018).

2.2.1 Dietary manipulation of skeletal muscle glycogen

At rest, and under habitual dietary conditions, muscle glycogen concentrations within skeletal muscle typically range between 300-600 mmol·kg^{-1} dw (Areta & Hopkins, 2018), although concentrations can be markedly augmented through both exercise and nutritional interventions. The fundamental principles of CHO loading (the process of acutely enhancing endogenous muscle glycogen storage) were originally developed by Scandinavian researchers in the late 1960s where it was identified that a super-compensatory increase in muscle glycogen could be achieved by performing a bout of exhaustive exercise followed by several days of high CHO intake (Bergström & Hultman, 1966; Bergström *et al.*, 1967). However, it was later recognised that such extreme exercise/nutrition regimens are unnecessary, where a simple exercise taper in conjunction with several days of increased dietary CHO intake is sufficient to increase glycogen storage (Sherman *et al.*, 1981). In fact, it is now generally accepted that trained athletes can increase glycogen storage in both type I and II fibres with 24-48 h of high (10 g.kg^{-1} body mass per day) dietary CHO intake and a concomitant reduction in training load (Bussau *et al.*, 2002). From a performance perspective, the general consensus from over 50 years of research suggests that carbohydrate loading can improve both exercise performance (e.g. time taken to complete a set amount of work) by 2-3% and capacity (e.g. time to exhaustion) by 20% when exercise is greater than 90 minutes in duration (Hawley *et al.*, 1997). The enhanced performance effect is likely to be initially mediated by a delay in the time-point at which CHO availability becomes limiting, which is dependent on the maintenance of high rates of CHO oxidation (Karlsson & Saltin, 1971). In addition to providing substrate availability for ATP production, it is now recognized that glycogen availability (especially the

intramyofibrillar storage pool) can directly modulate skeletal muscle contractile function. Indeed, a series of studies from Ørtenblad and colleagues (Ørtenblad *et al.*, 2011; Gejl *et al.*, 2014) have collectively shown preferential utilization of this storage pool during exercise in a manner that also correlates with impaired Ca^{2+} release from the sarcoplasmic reticulum (SR). Consequently, a reduction in SR Ca^{2+} will *per se* lead to reductions in tetanic intracellular free Ca^{2+} (Ørtenblad *et al.*, 2013) and is in line with previous observations of faster decreases in tetanic Ca^{2+} in fibres with low muscle glycogen (Chin *et al.*, 1997).

2.3 Carbohydrate and performance

2.3.3 Pre-exercise carbohydrate ingestion

Carbohydrate ingestion, 1-4 h prior to exercise, can further augment muscle glycogen concentrations by up to 10% (Chryssanthopoulos *et al.*, 2004) and, depending on the time of the exercise bout, promote liver glycogen restoration (Coyle *et al.*, 1985; Casey *et al.*, 2000) which is commonly depleted following an overnight fast. The increase in CHO storage within both storage pools has important performance implications given that muscle and liver glycogen provide an important substrate for muscle contraction during exercise (Baldwin *et al.*, 1975). With this in mind, pre-exercise carbohydrate ingestion appears to enhance exercise performance (Neufer *et al.*, 1987; Sherman *et al.*, 1989, 1991; Wright *et al.*, 1991), when consumed 1-4 h prior to exercise, with the enhanced performance effect associated with a maintenance of blood glucose during the latter stages of exercise when glycogen concentrations become limiting. In addition to its metabolic affects, pre-exercise CHO feeding is also able to attenuate central fatigue through its purported suppression of the fatigue-inducing neurotransmitter, serotonin (Khong *et al.*, 2018). With this in mind, current nutritional guidelines for pre-exercise carbohydrate feeding advise an intake of 1-4 $g.kg^{-1}$ body mass, 3-4 h prior to exercise (Thomas *et al.*, 2018).

2.3.4 Carbohydrate ingestion during exercise

In addition to high endogenous pre-exercise muscle and liver glycogen stores, numerous studies have demonstrated the ergogenic effects of CHO provision during moderate- to high-intensity exercise (Coyle *et al.*, 1983; Wright *et al.*, 1991; Febbraio *et al.*, 2000). Indeed, a systematic review of 61 published performance studies concluded that 82% of studies demonstrated a significant performance benefit of CHO ingestion during exercise (Stellingwerff & Cox, 2014). Furthermore, a meta-analysis of 88 randomised, crossover studies investigating CHO ingestion during exercise demonstrated performance benefits of up to 6% (range -2 to 6%) with CHO supplementation (Vandenbogaerde & Hopkins, 2011). Whilst there are

numerous factors responsible for fatigue during prolonged endurance exercise, CHO feeding during exercise appears to augment exercise performance via multiple mechanisms including muscle (Erickson *et al.*, 1987; Stellingwerff *et al.*, 2007) and liver (Gonzalez *et al.*, 2015) glycogen sparing as well as the maintenance of plasma glucose and high rates of CHO oxidation throughout the latter stages of exercise (Febbraio *et al.*, 2000).

Given the well documented observations that fatigue during exercise coincides with the depletion of muscle glycogen (Bergström *et al.*, 1967) the provision of exogenous CHO during exercise may be able to spare muscle glycogen through an attenuation of the rate of glycogenolysis and subsequently delay fatigue (Bjorkman *et al.*, 1984; Tsintzas *et al.*, 1996). However, the potential sparing of muscle glycogen appears to be fibre type dependent, an effect that is likely regulated by the modality, duration and intensity of the exercise bout undertaken. For instance, during constant pace running at 70% $\dot{V}O_{2max}$, where most type I fibres are recruited and type II fibres are relatively inactive during the early stages (Vollestad *et al.*, 1984), CHO ingestion results in a sparing of muscle glycogen in type I fibres only (Tsintzas *et al.*, 1995). Furthermore, this preferential sparing of glycogen in type I fibres is also seen with exercise durations of between 1.5 – 2 h (Tsintzas *et al.*, 1996). A sparing of muscle glycogen within type II fibres has also been observed with CHO ingestion during exercise (De Bock *et al.*, 2006; Stellingwerff *et al.*, 2007) and these differences are likely to reflect differences in the fibre recruitment patterns of different exercise modalities as well as methodological differences in the quantification of glycogen concentrations within each fibre type.

Exogenous CHO feeding during exercise also promotes the sparing of liver glycogen (Gonzalez *et al.*, 2015) through the suppression of hepatic glucose output (Jeukendrup *et al.*, 1999; Bosch *et al.*, 2007) and thus allows for the maintenance of euglycemia during exercise (Coyle *et al.*, 1986). Given that significant reductions in blood glucose coincides with fatigue during exercise (Levine *et al.*, 1924; Coyle *et al.*, 1986), the maintenance of euglycemia through exogenous CHO feeding appears to delay fatigue by allowing for high rates of CHO oxidation to the maintained. Interestingly, it appears that the utilisation of blood glucose is sufficient to support the maintenance of CHO oxidation even when muscle glycogen stores are depleted (Coyle *et al.*, 1983) and thus provides an important fuel source during the latter stages of exercise (Coyle *et al.*, 1986). Additionally, exogenous CHO feeding during exercise may also delay fatigue induced by central mechanisms through the preservation of euglycemia (Nybo, 2003) and stimulation of oropharyngeal receptors within the oral cavity (Fares & Kayser, 2011).

Whilst it was traditionally accepted that the maximal rates of exogenous CHO oxidation during exercise were limited to approximately 1 $g.min^{-1}$ (Jeukendrup & Jentjens, 2006), primarily due to the saturation of the sodium-dependent glucose transporter (SGLT1) within the gut, it is now acknowledged that exogenous CHO oxidation rates can be increased to 1.8 $g.min^{-1}$ with the addition of fructose to the CHO mixture via its absorption through an alternative transporter, GLUT5 (Jeukendrup, 2014). The ingestion of these multiple transportable CHO (MTC) has been subsequently demonstrated to improve performance when compared with glucose alone (Jeukendrup *et al.*, 2005; Currell & Jeukendrup, 2008; Rowlands *et al.*, 2008; Triplett *et al.*, 2010) although it is generally accepted their use is primarily suited to endurance events lasting longer than 2.5 h (Jeukendrup *et al.*, 2005; Hulston *et al.*, 2009). As such, it is suggested that CHO intake at rates of 30-60 $g.h^{-1}$ should be consumed during events lasting 60-150 min, whereas in events lasting > 2.5 h, 60-90 $g.h^{-1}$ (consisting of glucose/fructose mixtures) is the recommended rate (Jeukendrup, 2014). Practically, these fuelling requirements can be achieved through a combination of solids (e.g. bars), semi-solids (e.g. gels) and fluids (e.g. sports drinks) (Pfeiffer *et al.*, 2010*a*, 2010*b*) given they allow for similar rates of CHO oxidation during exercise.

2.3.5 Glycogen resynthesis after exercise

The pattern of muscle glycogen synthesis following exercise occurs in a biphasic manner (Price *et al.*, 1994), where, following the termination of exercise there is an initial period of rapid resynthesis that occurs independently of insulin and lasts approximately 30-60 min (Jentjens & Jeukendrup, 2003) and is only thought to occur when muscle glycogen is significantly depleted to low levels (< 150 $mmol \cdot kg^{-1}$ dw) (Price *et al.*, 1994). This phase of glycogen resynthesis is characterised by the translocation of GLUT4 to the plasma membrane and increased glycogen synthase activity, allowing for glucose uptake from the circulation and subsequent storage within the glycogen granule. The translocation of GLUT4 to the plasma membrane is thought to be initiated through the activation of the exercise-inducible kinase, AMPK, a signalling protein that is partly regulated through muscle glycogen concentrations (Wojtaszewski *et al.*, 2003), given the glycogen binding domain found within the ß-subunit of AMPK (McBride *et al.*, 2009). The enhanced enzyme activity of glycogen synthase is regulated through its dephosphorylation mediated transformation to its active form in response to reductions in muscle glycogen concentrations (Ivy & Kuo, 1998).

The second phase of muscle glycogen resynthesis occurs at much slower rates (approximately 80% lower) (Jentjens & Jeukendrup, 2003) and is characterised by an increase in the sensitivity of muscle glucose uptake to insulin, which may persist for up to 48 h following exercise

and is dependent on dietary CHO intake and absolute muscle glycogen concentrations (Cartee *et al.*, 1989). Although the precise mechanisms regulating the post-exercise increase in insulin sensitivity are currently unknown, the activation of AMPK appears to be important for the insulin-sensitising role of acute exercise, although this effect is diminished within trained skeletal muscle (Steenberg *et al.*, 2019; McConell *et al.*, 2020).

Following exercise, glycogen synthesis is augmented when CHO is ingested, whereby reported resynthesis rates generally range between 20-50 mmol·kg^{-1} dw h^{-1}, depending on the quantity, type and timing of CHO ingested (Jentjens & Jeukendrup, 2003). Whilst early work suggested that the provision of 0.7 g.kg^{-1} h^{-1} CHO immediately after exercise, eliciting a resynthesis rate of 20 mmol·kg^{-1} dw h^{-1}, was sufficient in maximising muscle glycogen resynthesis it is now apparent that higher intakes are required. Later work from Van Loon (van Loon *et al.*, 2000) demonstrated increasing the rate of CHO ingestion to 1.2 g.kg^{-1} h^{-1} elicited much higher rates of glycogen synthesis (44.8 mmol.kg^{-1} dw h^{-1}) and is now prescribed as the recommended intake to maximise glycogen repletion (Thomas *et al.*, 2018) given that ingestion of 1.6 g.kg^{-1} h^{-1} does not appear to provide any additive benefit (Howarth *et al.*, 2009). Interestingly, in conditions where post-exercise carbohydrate intake is suboptimal (i.e. < 1.2 g.kg^{-1} h^{-1}), the addition of protein appears to augment glycogen synthesis (van Loon *et al.*, 2000) and may provide a viable strategy for athletes who fail to meet the upper limit of CHO intake.

The type of carbohydrate ingested also appears to be of importance given that high glycaemic index (GI) carbohydrates promote markedly higher rates of glycogen resynthesis when compared with low GI carbohydrates (Burke *et al.*, 1993). Furthermore, recent work has also suggested that utilising multiple transportable CHO sources may further augment glycogen synthesis post-exercise. However, the available evidence demonstrates that glucose-fructose or glucose-sucrose mixtures do not further enhance muscle glycogen synthesis over glucose alone, when ingested at rates of 1.2 and 1.5 g.kg^{-1}.h^{-1} respectively (Wallis *et al.*, 2008; Trommelen *et al.*, 2016). Nevertheless, it is apparent that glucose-fructose (sucrose) mixtures enhance the repletion of liver glycogen and minimise gastrointestinal distress when the ingestion rate approaches 1.2 g.kg^{-1}.h^{-1} (Décombaz *et al.*, 2011; Fuchs *et al.*, 2016) and enhance subsequent exercise capacity following both short (4 h) (Maunder *et al.*, 2018) and overnight (15 h) recovery (Gray *et al.*, 2019). Although the provision of CHO in the form of either solid or liquid based sources promotes similar rates of muscle glycogen repletion (Keizer *et al.*, 1987; Reed *et al.*, 1989) it may be preferred to ingest liquid sources during the first hour of recovery as appetite is often supressed post-exercise. Furthermore, the co-ingestion of fat and

protein to post-exercise carbohydrate feedings does not appear to limit glycogen storage when adequate CHO (7 $g.kg^{-1}$) is consumed over 24 h (Burke *et al.*, 1995).

Given the enhanced skeletal muscle blood flow and nutrient sensitivity following exercise, the immediate provision of carbohydrate appears to augment the rate of muscle glycogen resynthesis when compared with delayed (2 h) provision (Ivy *et al.*, 1988) over 4 h of recovery. However, this effect appears to diminish following 8 h of recovery (Parkin *et al.*, 1997) and may be explained by a potential slowing of glycogen resynthesis in the latter stages of recovery with immediate CHO provision, in line with the tightly controlled inverse relationship between muscle glycogen and glycogen synthase activity. During this recovery window, it appears that CHO provision at regular intervals (e.g. 30 min) may augment muscle glycogen resynthesis when compared with extended feeding intervals of 2 h. Although no studies have directly examined these effects, glycogen synthesis rates appear markedly lower (14-25 $mmol.kg^{-1}$ dw h^{-1}) with less frequent CHO provision (Ivy *et al.*, 1988; Reed *et al.*, 1989). Nonetheless, when muscle glycogen resynthesis is measured over 24 h of recovery there appears to be no advantage to increasing the frequency of carbohydrate feedings on muscle glycogen storage (Burke *et al.*, 1996).

2.3.6 Dietary carbohydrate and training capacity

Athletes who undertake daily training sessions likely experience day-to-day fluctuations in skeletal muscle glycogen, whereby repetitive daily training may lead to a reduction in absolute glycogen concentrations if insufficient daily CHO is consumed and subsequently impair training capacity. Costill *et al* was the first to demonstrate that consuming a moderate carbohydrate diet (40-60% of daily energy intake) resulted in progressive glycogen depletion during three consecutive training days (Costill *et al.*, 1971). However, despite reductions in muscle glycogen from 475 to 285 $mmol \cdot kg^{-1}$ dw with three days of training, no increase in fatigue levels were observed. Furthermore, Costill *et al* also demonstrated that, despite a 15% decline in muscle glycogen (562 to 475 $mmol \cdot kg^{-1}$ dw) following 10 days of increased daily training volume, both swimming power and endurance performance were not compromised (Costill *et al.*, 1988). However, those who consumed less daily CHO (5.3 $g.kg^{-1}$) and presented with greater reductions in muscle glycogen (~216 $mmol \cdot kg^{-1}$ dw) were unable to tolerate the increase in training volume and were forced to train at a reduced intensity. In contrast, Kirwan *et al* demonstrated no impairment in training intensity despite marked reductions in muscle glycogen concentrations (354 vs. 523 $mmol \cdot kg^{-1}$ dw) following the consumption of a moderately low CHO diet (3.9 $g.kg^{-1}$) over 5 days of intensified running training (Kirwan *et al.*, 1988). In agreement, Sherman *et al* demonstrated that, despite reductions in muscle glycogen

with a daily CHO intakes of 5 g.kg (324-432 mmol·kg^{-1} dw) exercise time to exhaustion at 80% $\dot{V}O_{2peak}$ was not impaired when compared with higher daily CHO intakes of 10 g.kg^{-1} and higher muscle glycogen concentrations (540-648 mmol·kg^{-1} dw) (Sherman *et al.*, 1993).

Collectively, these data demonstrate that daily CHO intakes of 3-5 g.kg^{-1} per day may result in progressive glycogen depletion during periods of carbohydrate dependent training (i.e. prolonged endurance exercise > 75% $\dot{V}O_{2max}$), whereas intakes of 8-10 g.kg^{-1} per day can minimise or prevent glycogen depletion. Despite this, it appears that progressive reductions in muscle glycogen, within normal resting ranges (Areta & Hopkins, 2018) do not impair the ability to complete the prescribed training workloads in most cases, although depletion beyond these ranges over an extended period may compromise daily training intensity (Costill *et al.*, 1988). Furthermore, there appears no additional benefit of increasing daily CHO intake beyond what is considered sufficient for the given training intensity and volume – a notion previously suggested by Sherman et al. (1981):

> *"Anything above a 'minimal' level of muscle glycogen is unnecessary for performance of a given intensity and duration. More importantly, the practical question may not be how much can I supercompensate, but rather, does my diet contain enough carbohydrate to maintain adequate stores of muscle glycogen on a day-to-day basis for training and performance runs?"* (p. 117).

When taken together, it appears that careful day-to-day periodisation of CHO intake, upon a meal-by-meal basis and in accordance with the demands of the specific training session should be considered in order to ensure that athletes have sufficient CHO availability to allow for the completion of a given workload.

2.4 Endurance training & skeletal muscle adaptation

Skeletal muscle tissue demonstrates remarkable plasticity, whereby it possesses an extraordinary ability to undergo major adaptations in response to exercise training. The first report of biochemical adaptations to endurance training came from the seminal work of John Holloszy, in rat skeletal muscle, who demonstrated an increase in the activity of key respiratory chain enzymes (cytochrome oxidase & succinate oxidase) and a subsequent doubling in the capacity of the mitochondria to oxidise pyruvate (Holloszy, 1967). Holloszy *et al* also reported a two-fold increase in cytochrome c protein content which appears to explain the increase in respiratory chain enzyme activity, allowing for a rise in the capacity to generate ATP via oxidative

phosphorylation. These biochemical adaptations were later reported in human skeletal muscle (Morgan *et al.*, 1971; Hoppeler *et al.*, 1973) in addition to an abundance of other adaptive responses including fibre type shifts towards an oxidative phenotype, angiogenesis and capillarization (Andersen & Henriksson, 1977) and an increased capacity for both glycogen (Hickner *et al.*, 1997; Putman *et al.*, 1998) and lipid storage (Van Loon *et al.*, 2004; Tarnopolsky *et al.*, 2007), providing greater amounts of substrate for utilisation during exercise.

Of these adaptations, the increase in mitochondrial number and content, known as mitochondrial biogenesis (Hood, 2001), is seen as the major hallmark adaptation of endurance training. The metabolic adaptations that accompany mitochondrial biogenesis include increases in the content and activity of enzymes involved in the TCA cycle and electron transport chain (Morgan *et al.*, 1971) as well as those involved in fatty acid transport and oxidation (Talanian *et al.*, 2010), glycolytic metabolism and glucose transport (Morgan *et al.*, 1971; Houmard *et al.*, 1993). The physiological consequences of such adaptations are a tighter coupling of ATP supply with demand and a subsequent reduction in ADP concentrations required to attain the same level of O_2 consumption per gram of muscle (Holloszy & Coyle, 1984). As a result of the enhanced efficiency for energy production of the mitochondria, smaller increases in ADP, AMP and Pi are observed during exercise at the same absolute intensity. As these intracellular constituents play an important role in the allosteric regulation of glycogenolysis and glycolysis, endurance training results in an increased reliance on lipid metabolism, with a proportional down-regulation of CHO metabolism for a given absolute exercise intensity, which is reflected in a lower respiratory exchange ratio (RER) (Hermansen *et al.*, 1967; Karlsson *et al.*, 1974; Phillips *et al.*, 1996). Taken together, it appears that enhanced endurance performance associated with endurance training can be attributed to enhanced resistance to fatigue resulting from a tighter coupling of ATP supply with demand, reduced glycogen depletion and lactate formation and an enhanced ability to transport and oxidise lipids.

2.4.1 Signalling transduction pathways

In accordance with the >100 fold increase in ATP demand (Hochachka & Matheson, 1992) a variety of cellular disruptions occur at the onset of endurance exercise including a rise in cytoplasmic free Ca^{2+}, increased free AMP, an increased ADP/ATP ratio, reductions in muscle glycogen, increased fatty acid availability, lactate accumulation and reactive oxygen species production, all of which appear to be implicated in specific signalling cascades regulating skeletal muscle plasticity (Hawley *et al.*, 2006, 2014) (Figure 2.4). Indeed, these homeostatic perturbations result in the activation of key protein kinases, such as AMP-dependent protein

kinase (AMPK), p38 mitogen activated protein kinase (p38 MAPK) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (Combes *et al.*, 2015; Fiorenza *et al.*, 2018). Upon activation, these kinases converge upon both nuclear and mitochondrial transcription factors and co-activators to augment the transcription of genes that dictate the synthesis of both nuclear and mitochondrial proteins and the subsequent assembly of protein complexes into a functional respiratory chain. Ultimately, this sequence of events results in an expansion of the mitochondrial network within skeletal muscle.

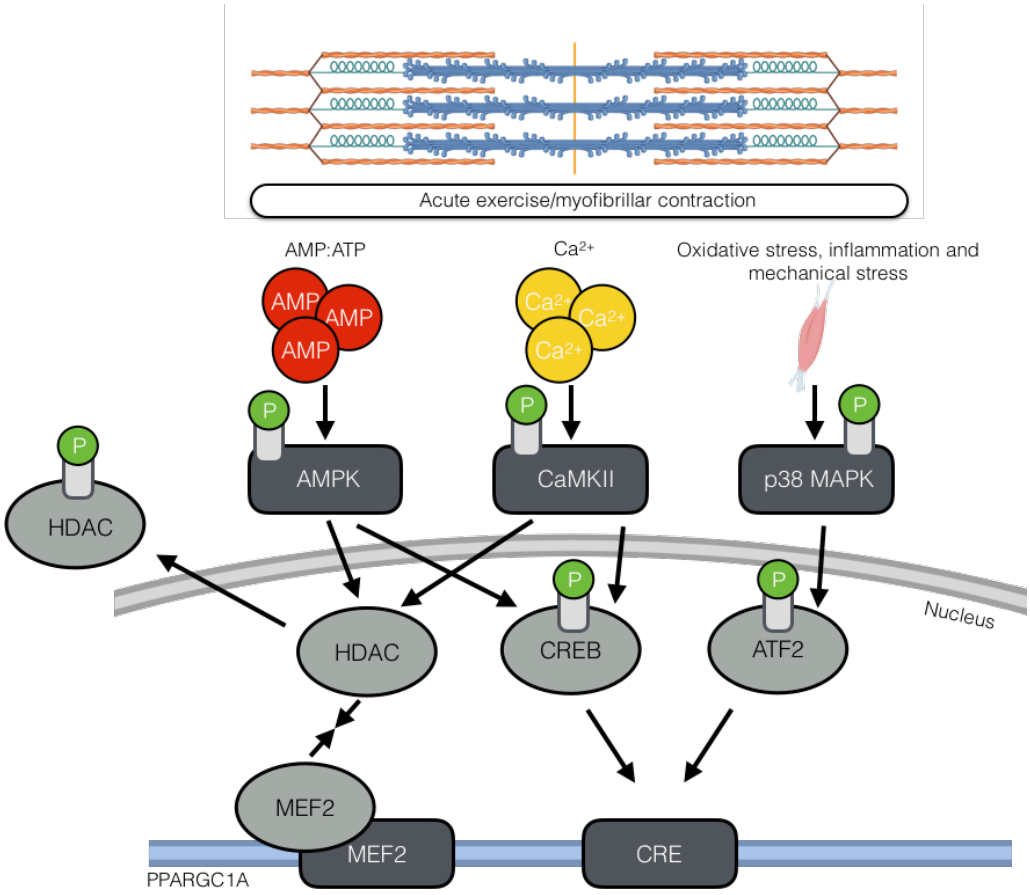

Figure 2. 4. Signalling kinases and their downstream targets. Adapted from Egan *et al* (2010). Acute myofibrillar contraction results in the phosphorylation (P) of AMPK, CaMKII and p38 through molecular sensing of increased ATP turnover, increased calcium release from the SR and mechanical stress, respectively. AMPK and CaMKII phosphorylate class II HDACs, leading to their expulsion from the nucleus and relieving their inhibitory effect on MEF2 transcriptional activity on the PGC-1α promoter. AMPK and CaMKII also phosphorylate CREB, resulting in the activation of CRE sequence of the PGC-1α promoter. p38 MAPK phosphorylates ATF2 and, in turn, acts on the same CRE site, resulting in transcriptional activation. These combined effects on the MEF2 and CRE sequences result in increased PGC-1α promoter activity and increased PGC-1α gene transcription.

2.4.2 Cellular energy status & 5'AMP Activated Protein Kinase (AMPK) signalling

AMPK is a heterotrimeric Ser/Thr kinase comprised of one catalytic α- and two regulatory ß and γ subunits. Both the α- and ß- subunits exist in two isoforms (α1 and α2, ß1 and ß2)

whereas the γ- subunit exists in three (γ1, γ2 and γ3) (Hardie, 2011).The α-subunit contains a Ser/Thr kinase domain which requires phosphorylation from upstream kinases (LBK1 and CaMKII) for complete kinase activity (Hawley *et al.*, 2006). The ß-subunit contains a glycogen-binding domain which allows AMPK to interact with glycogen (McBride & Hardie, 2009) whilst the γ-subunit contains two pairs of Bateman (cystathionine β-synthase [CBS]) domains which appear to play a role in the binding of AMP and ATP (Xiao *et al.*, 2007) (Figure 2.5).

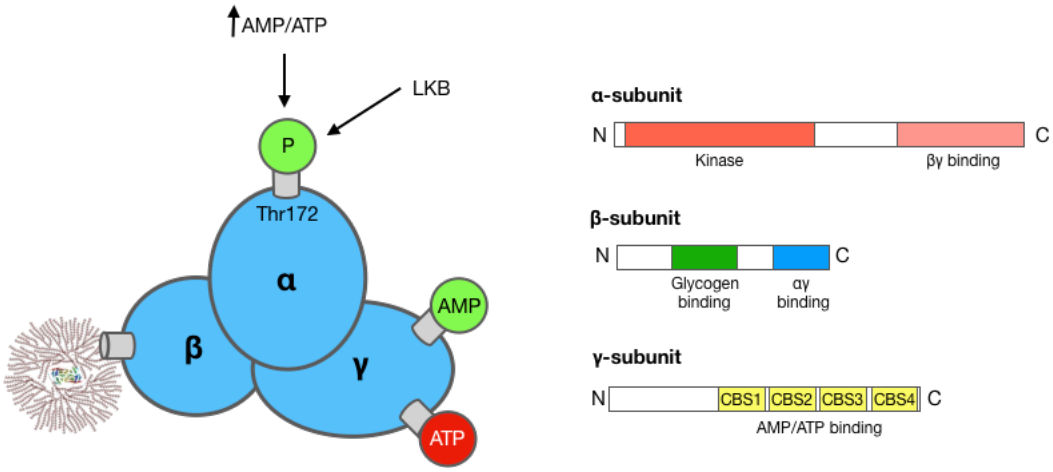

Figure 2. 5. Structure of AMPK, its α, ß and γ subunits and its regulation by P, AMP and ATP.

The binding of AMP to the Bateman domains (primarily at sites 1 & 3) brings about the activation of AMPK via upstream kinases (e.g. LKB1 and CaMKII) (Hawley *et al.*, 2006) whilst also inhibiting its dephosphorylation (Davies *et al.*, 1995), whilst ATP competes for the binding sites and acts to inhibit AMPK activity (Xiao *et al.*, 2011). Under resting conditions, ATP is predominantly bound to the Bateman domains and thus renders AMPK inactive. However, upon the onset of muscle contraction, ATP becomes hydrolysed into ADP and AMP which allow AMP to bind to the Bateman domains and thus activate AMPK (Richter & Ruderman, 2009). Upon activation, AMPK triggers various metabolic processes that increase energy production (e.g. glucose and fatty acid uptake and oxidation; Merrill *et al.*, 1997) whilst concomitantly inhibiting processes that require energy consumption (e.g. protein synthesis; Bolster *et al.*, 2002). Additionally, AMPK appears to enhance fatty acid oxidation through the inactivation of acetyl-CoA-carboxylase-ß (ACCß) and the subsequent reduction in malonyl CoA levels (Saha & Ruderman, 2003). Given the inhibitory effects of malonyl CoA on the FFA transporter carnitine palmitoyltransferase 1 (CPT-1), it appears evident that activation of AMPK facilitates CPT-1 mediated FFA transport into the mitochondria and subsequent increases in lipid oxidation.

In relation to its role in mitochondrial biogenesis, AMPK plays an important role in the signal transduction network regulating training adaptation and can directly phosphorylate the transcriptional co-activator PGC-1α (via Thr^{177} and Ser^{538} residues) (Jager *et al.*, 2007) which controls the expression of both nuclear and mitochondrial genes (Hood *et al.*, 2016). In this regard, chronic activation of AMPK via 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) administration in rat skeletal muscle enhances the protein expression of mitochondrial enzymes citrate synthase (CS) and succinate dehydrogenase (SDH), similar to that observed during endurance training (Winder *et al.*, 2000). The first report of exercise-induced AMPK activation came from rodent skeletal muscle (Winder & Hardie, 1996) and was later confirmed in human skeletal muscle across a range of exercise modalities, including knee extensor (Frøsig *et al.*, 2004) cycling (Chen *et al.*, 2003; Coffey *et al.*, 2006; Little *et al.*, 2011; Kristensen *et al.*, 2015) and running (Bartlett *et al.*, 2013) exercise. Within human skeletal muscle, AMPK is activated during exercise in an intensity-dependent and isoform specific manner. Indeed, it appears that the α-subunit is particularly sensitive to changes in exercise intensity, whereby high intensity exercise provides a more potent stimulus for increased α-subunit activity compared with low intensity exercise. For example, AMPKα2 activity is augmented when exercise intensity is increased from 40 to 80% $\dot{V}O_{2peak}$ (Chen *et al.*, 2003) or from 50 to 75% $\dot{V}O_{2max}$ (Wojtaszewski *et al.*, 2000) whilst augmented $AMPK^{Thr172}$ phosphorylation has been observed following work-matched cycling at 80% $\dot{V}O_{2max}$ compared with 40% $\dot{V}O_{2max}$ (Egan *et al.*, 2010). AMPK also appears to be activated in an isoform-specific manner, where AMPKα2 displays a more pronounced sensitivity and activation compared with AMPKα1, which appears to be relatively unresponsive to either low- or high-intensity exercise (Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000).

Despite increases in the protein content and basal activity of several AMPK subunits following cumulative bouts of exercise training (Frøsig *et al.*, 2004), it is apparent that endurance training attenuates the activation of AMPK during a single bout of criterion exercise (Nielsen *et al.*, 2003; Yu *et al.*, 2003; McConell *et al.*, 2005). The blunted AMPK response following endurance training is likely explained by smaller disturbances in cellular homeostasis (i.e. free AMP, ADP) during exercise (Holloszy & Coyle, 1984) and thus it appears that progressive increases in training intensity are necessary to maintain an adequate training stimulus following endurance training.

Whilst AMPK activity has been shown to be regulated by exercise training, it appears that its activity can also be modulated by carbohydrate availability. The notion for a regulatory role of muscle glycogen on AMPK signalling in human skeletal muscle was first provided by

Wojtaszewski *et al.* (2003) who demonstrated higher basal activity of both AMPKα1 and α2 isoforms under conditions of low muscle glycogen (~160 mmol·kg^{-1} dw), compared with glycogen loaded conditions (~900 mmol·kg^{-1} dw). Importantly, concentrations of both creatine phosphate and adenine nucleotides were similar despite large differences in muscle glycogen concentrations, suggesting that changes in AMPK signalling were modulated by fuel-dependent mechanisms, independent of energy status. Subsequently, commencing exercise with low muscle glycogen appears to also augment AMPK^{Thr172} phosphorylation (Yeo *et al.*, 2010) and the nuclear abundance of AMPKα2 protein (Steinberg *et al.*, 2006) when compared with high muscle glycogen. Alongside muscle glycogen, exogenous CHO availability also appears to modulate exercise-induced AMPK signalling as both fasted exercise (Stocks *et al.*, 2019) and exogenous CHO restriction during exercise (Akerstrom *et al.*, 2006) augment AMPK^{Thr172} phosphorylation and AMPKα2 activity, respectively. These effects are potentially mediated via the presence of the glycogen-binding domain on the ß-subunit (McBride & Hardie, 2009) whereby the physical attachment of glycogen inhibits AMPK activity and translocation to the nucleus.

2.4.3 Mechanical stress & mitogen-activated protein kinase (p38 MAPK) signalling

p38 MAPK, a member of the mitogen-activated protein kinase family, is commonly viewed as a stress inducible kinase due to its activation in response to a range of stimuli such as environmental, oxidative, inflammatory and mechanical stress. Similar to AMPK, p38 MAPK has been identified as an upstream regulator of PGC-1α activity and thus, an important regulatory kinase involved in the signal transduction pathway regulating exercise-induced mitochondrial biogenesis. Indeed, initial data in mice demonstrated p38 MAPK phosphorylation occurs concurrently with increases in PGC-1α mRNA expression, a response that appears to occur through a p38 MAPK mediated increase in PGC-1α promotor activity (Akimoto *et al.*, 2005). Furthermore, it was also demonstrated that expression of a muscle-specific p38 MAPK activator, in transgenic mice, resulted in enhanced expression of both PGC-1α and COXIV in skeletal muscle (Akimoto *et al.*, 2005). Subsequent *in vivo* studies have suggested that p38 MAPK exerts its effects on PGC-1α through myocyte enhancing factor 2 (MEF2) and activating transcription factor 2 (ATF2) binding sites on the PGC-1α promotor (Akimoto *et al.*, 2008).

With this in mind, activation of p38 MAPK has been observed in response to both cycling (Gibala *et al.*, 2009; Egan *et al.*, 2010) and running (Boppart *et al.*, 2000; Bartlett *et al.*, 2012) exercise in human skeletal muscle. Interestingly, p38 MAPK may also be regulated by carbohydrate availability, whereby commencing exercise with low muscle glycogen has been

shown to enhance p38 MAPK phosphorylation (Chan *et al.*, 2004). However, it appears that this response may be specific to nuclear p38 MAPK as p38 MAPK phosphorylation appears unresponsive to carbohydrate availability in both whole muscle homogenate (Yeo *et al.*, 2010; Bartlett *et al.*, 2013; Stocks *et al.*, 2019) and cytosolic fractions (Chan *et al.*, 2004).

2.4.4 Calcium flux & calcium/calmodulin-dependent protein kinase (CaMKII) signalling

Calcium plays an essential role in facilitating the crossbridge interaction between myosin and actin filaments during myofibrillar contraction, during which, calcium oscillations are translated into regulatory signals that modulate the activity of calmodulin-dependent protein kinases (CaMKs). CaMKII is the dominant isoform in human skeletal muscle (Rose et al., 2006) and is recognised as a powerful activator of PGC-1α transcription and mitochondrial biogenesis through MEF2 and cAMP response element binding protein (CREB) phosphorylation. Indeed, CaMKII activates MEF2 via phosphorylation of histone deacetylase (HDAC) proteins, thus removing their inhibition of MEF2 (McKinsey et al., 2000), allowing it to bind to the MEF2 elements in the PGC-1α promotor region and subsequently activate transcription. Similarly, CaMKII can also increase PGC-1α transcription via CREB phosphorylation through the cAMP-responsive elements (CRE) site on PGC-1α (Baar, 2014).

In response to exercise, CaMKII phosphorylation is increased in an intensity-dependent manner (Rose et al., 2006; Egan et al., 2010) and may also be influenced by the pattern of muscle contraction given that CaMKII phosphorylation appears to be augmented with intermittent type exercise (Combes et al., 2015; Fiorenza et al., 2018). To date, it is unclear whether CaMKII phosphorylation is affected by nutrient availability. Although CaMKII phosphorylation appears unresponsive to exercise performed in the fasted state (Stocks et al., 2019) some evidence suggests a potential relationship between kinase phosphorylation and muscle glycogen (Fiorenza et al., 2018). Furthermore, low muscle glycogen (especially glycogen contained within the intramyofibrilliar pool) also impairs the release of Ca2+ from the sarcoplasmic reticulum (Ørtenblad *et al.*, 2011; Gejl *et al.*, 2014) and may act as a potential mechanism whereby carbohydrate availability is able to modulate CaMKII phosphorylation.

2.4.5 Tumor suppressor protein p53

The tumor suppressor protein p53 is a transcriptional regulator that is commonly known for its effects on cell cycle arrest, apoptosis, DNA repair, differentiation and cell senescence (Bartlett *et al.*, 2014). In addition to these primary functions, p53 also plays a vital role in the maintenance of energy homeostasis and mitochondrial biogenesis. The role of p53 in mitochondrial biogenesis is demonstrated through its ability to regulate the transcriptional activity

of synthesis of COX 2 (SCO2), a protein required for the assembly of mitochondrial encoded COX II subunit of the COX complex (Hood *et al.*, 2016). Furthermore, p53 ablation manifests in reduced COX activity, which demonstrates the transcriptional control p53 has in coordinating the expression of both COX subunit I and II. The COX complex, which is made up of 13 subunits in total, plays a vital role in aerobic respiration by accepting electrons from cytochrome C and transferring them to an oxygen molecule. p53 also plays an important role in modulating mtDNA-encoded gene expression and stability through its physical interaction with transcription factor A (Tfam) where it can regulate the binding of Tfam to mtDNA. Furthermore, given the identification of a p53 response element within the promoter region of PGC-1α, p53 also appears to be able to influence the expression of PGC-1α protein whereby PGC-1α expression is reduced within the muscle of p53 KO animals (Saleem *et al.*, 2009).

Activation of p53 appears to occur through AMPK and/or p38 MAPK signalling cascades, resulting in its phosphorylation at serine 15 which is typically linked to increased stability and activity. This is further supported by the fact that phosphorylation of p53 occurs in concert with the classical increases in AMPK and p38 MAPK phosphorylation in response to an acute bout of exercise (Bartlett *et al.*, 2012). In the context of exercise, activation of p53 appears to be mediated by exercise/nutrient interventions that elicit sufficient cellular stress. Indeed, the aforementioned exercise-induced p53 phosphorylation (Bartlett *et al.*, 2013) is augmented under conditions of low muscle glycogen availability whilst cell models that induce glucose deprivation result in p53 phosphorylation via AMPK signalling pathways (Jones *et al.*, 2005). Interestingly, although p53 phosphorylation does not appear to be modulated by exercise intensity within whole muscle homogenate under work-matched conditions (Bartlett *et al.*, 2012) nuclear p53 phosphorylation has been demonstrated to be augmented by exercise intensity (Granata *et al.*, 2017). With this in mind, the subcellular location of p53 may have a distinct effect on the transcription of both nuclear and mitochondrial genes. Indeed, translocation of p53 to both the nucleus (Tachtsis *et al.*, 2016) and mitochondria (Saleem & Hood, 2013) have been reported in response to exercise. Similarly, exercise intensity appears to be important in modulating p53 protein content which correlate with increases in mitochondrial respiration (Granata *et al.*, 2016*a*). Given that these changes are also apparent with increasing training volume (Granata *et al.*, 2016*b*), these data collectively illustrate that increases in p53 content require an augmented stress response via significant metabolic perturbations to homeostasis which can be achieved through modification of nutritional status, exercise volume and intensity or a combination of both.

2.4.6 Peroxisome Proliferator Activated Receptor Gamma Coactivator 1-alpha (PGC-1α)

PGC-1α, a member of the peroxisome proliferator-activated receptor γ coactivator family, is widely recognised as the 'master regulator' of mitochondrial biogenesis and exerts its effects as a transcriptional co-activator for an array of nuclear and mitochondrial transcription factors (Hawley *et al.*, 2014). As a co-activator, PGC-1α does not bind to DNA itself but recruits histone acetyl transferase (HAT) enzymes to a number of different DNA bound transcription factors, including the myocyte enhancer factors 2 (MEF2) and the nuclear respiratory factor 1 and 2 (NRF-1/2), to subsequently modify the chromosome structure to one that favours transcription (Wu *et al.*, 1999; Handschin *et al.*, 2003). In binding with NRF-1 and NRF-2, PGC-1α is able to coordinate the regulation of both nuclear and mitochondrial genomes given that these transcription factors are located in the promoter regions of multiple genes encoding mitochondrial proteins (NuGEMPs).

PGC-1α signalling can be enhanced by either increasing its activity or its protein expression. As such, it appears that increases in PGC-1α activity mediates the initial phase of exercise-induced increases in muscle mitochondrial content given that these increases in mitochondrial enzyme expression occur before an increase in PGC-1α protein (Wright *et al.*, 2007*b*). The activity of PGC-1α appears to be regulated by association with the repressor protein p160myb which can disrupt PGC-1α's association with transcription factors and subsequently inhibit gene transcription (Fan *et al.*, 2004). However, phosphorylation of PGC-1α appears to disrupt this association and allows for the recruitment of PGC-1α to transcription factors. The second strategy to enhance PGC-1α signalling is to enhance its protein expression (Baar & McGee, 2008) which occurs as a result of repeated, transient increases in PGC-1α mRNA as seen with repeated exercise stimuli (Perry *et al.*, 2010). Indeed, a single bout of exercise can induce a ~1.5-10 fold increase in PGC-1α mRNA (Perry *et al.*, 2010), the magnitude of which is dependent on exercise intensity whereby high intensity exercise provides a more potent stimulus for PGC-1α mRNA expression when compared with low intensity exercise (Egan *et al.*, 2010). The transcription of PGC-1α is primarily controlled by proteins binding to either the MEF2 or the cyclic AMP responsive element (CRE) within its promoter (Akimoto *et al.*, 2008) and through the interaction with MEF2, PGC-1α is able to coactivate its own expression (Handschin *et al.*, 2003). However, in order to prevent an excess of PGC-1 within the muscle, MEF2 transcriptional activity is supressed by the transcriptional repressor HDAC5 which is able to modify the local chromosome structure to one that silences transcription (Mckinsey *et al.*, 2001). It appears that AMPK is able to regulate PGC-1α expression by phosphorylating the transcriptional repressor HDAC5 (Mcgee & Hargreaves, 2008), resulting in its removal from the nucleus, via the 14-3-3 transport protein, and allowing for chromatin remodelling

and enhanced transcriptional activity of MEF2. AMPK also appears to directly phosphorylate the CRE protein (Thomson *et al.*, 2008) and subsequently enhance PGC-1α transcription. Furthermore, the activity of AMPK has also been identified as an essential component regulating the cellular shuttling of PGC-1α (Smith *et al.*, 2013) whereby exercise induced AMPK activation promotes its translocation to both the nucleus and mitochondria (Little *et al.*, 2011; Safdar *et al.*, 2011).

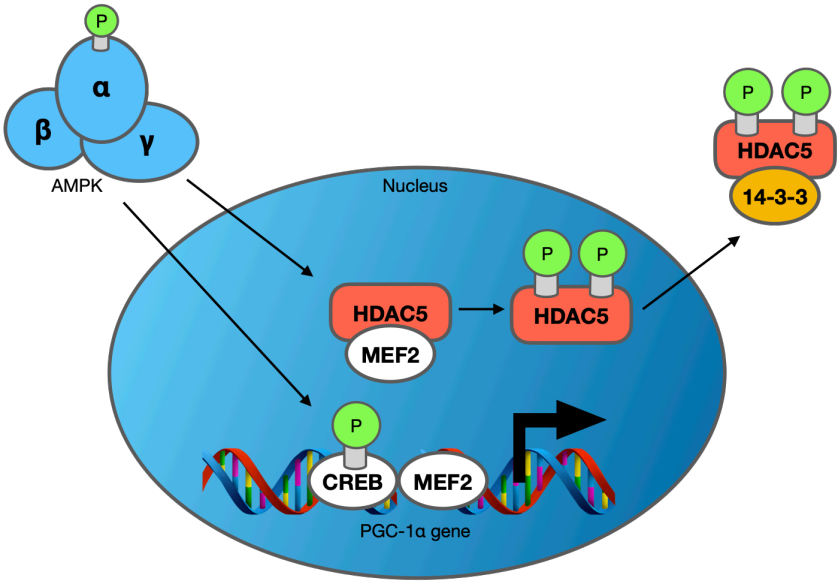

Figure 2. 6. An overview of the signal transduction network regulating PGC-1α activity. AMPK induced phosphorylation of HDAC5 disrupts the interaction between MEF2 and HDAC5 which leads to the removal of HDAC5 from the nucleus by the chaperone protein 14-3-3 and allows MEF2 to bind to the PGC-1α promoter. AMPK can also directly phosphorylate CREB.

In relation to its role within mitochondrial biogenesis, overexpression of PGC-1α protein (~ 10-30 fold) within skeletal muscle results in enhanced expression of mitochondrial enzymes, oxidative capacity and enhanced endurance performance whilst skeletal muscle from PGC-1α transgenic mice have a ~3 fold increase in time to fatigue when electrically stimulated (Lin *et al.*, 2002; Arany *et al.*, 2007). Furthermore, muscle specific overexpression of PGC-1α *in vivo*, induces increases in mitochondrial enzymes and the capacity to oxidise lipids within the mitochondria (Benton *et al.*, 2008). Collectively, these data demonstrate the important regulatory role PGC-1α plays in coordinating mitochondrial biogenesis. Nonetheless, endurance training adaptations (i.e. increases in mitochondrial morphology and function and exercise capacity) can still be achieved to the same extent following muscle specific PGC-1α knock out (KO), demonstrating the multifaceted nature of the signal transduction networks that regulate mitochondrial biogenesis, and downplay the role of PGC-1α as the 'sole' regulatory protein (Rowe *et al.*, 2012).

2.4.7 Time course of molecular events leading to training induced skeletal muscle adaptation

Chronic muscle adaptations to exercise training are attributed to the effects of repeated, acute perturbations in cellular homeostasis and subsequent transient increases in mRNA transcription (Perry *et al.*, 2010) which facilitate an adaptive response via gradual structural remodelling and long-term functional improvements. However, the time course of exercise induced increases in mRNA and protein content are discordant with one another, and changes in mRNA do not always predict changes in protein content (Cochran *et al.*, 2014).

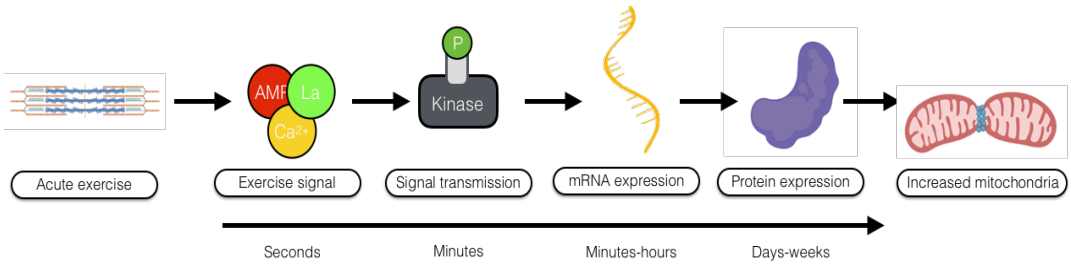

Figure 2. 7. Schematic representation of the time-course of selected contraction-induced physiological, biochemical and molecular responses in skeletal muscle that result in adaptation to training. Acute exercise results in changes in metabolism (e.g. increased ADP, lactate and free Ca^{2+}). These exercise signals subsequently regulate the activity of signalling proteins via phosphorylation in order to control the activity of downstream transcription factors and co-activators to influence gene expression. Over time, transient increases in mRNA expression result in an increase in the synthesis of new proteins.

To date, only two studies (Perry *et al.*, 2010; Egan *et al.*, 2013) have documented the time course of several transcriptional, translational and morphological processes involved in mitochondrial biogenesis. These investigations demonstrate an increase in mRNA in the initial hours following exercise which returns to baseline at 24 h. The exercise-induced increase in mRNA appears to occur before the initial increase in protein content for specific mitochondrial enzymes and transcriptional proteins, including PGC-1α. Following multiple bouts of exercise, and subsequent repeated transient increases in mRNA, the content of various proteins demonstrate a sustained increase before a plateau is reached in the final bouts of training (Figure 2.8). These data also demonstrate that the mRNA response to exercise is attenuated as the muscle adapts and suggest that a continual modification of exercise intensity/duration and/or nutritional status is required to maintain exercise-induced mRNA expression.

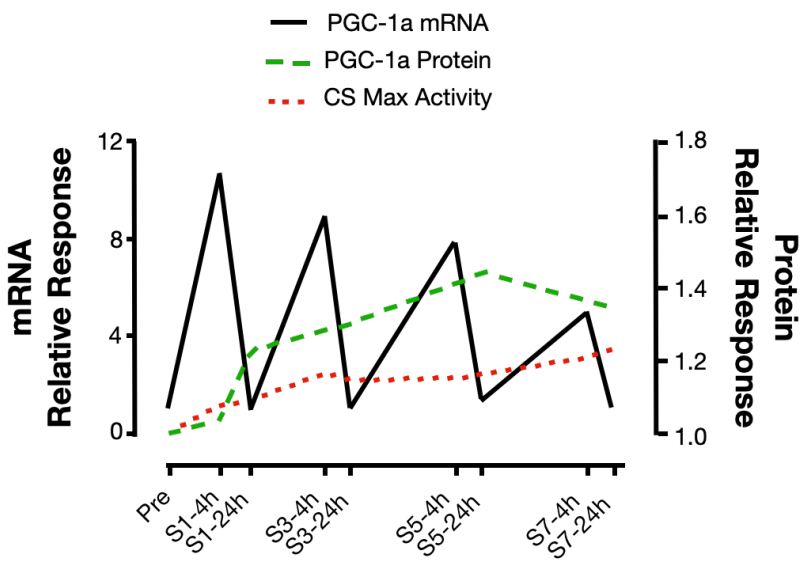

Figure 2. 8. An overview of the time course of PGC-1α mRNA expression and protein content throughout 2 weeks of exercise training. Numbers in brackets along the x-axis represent the training session number. Adapted from Perry *et al* (2010).

2.5 CHO availability modulates training induced skeletal muscle adaptations

Undertaking endurance exercise with restricted CHO availability induces significant metabolic and signalling shifts compared with when exercise is undertaken with high CHO availability. Indeed, at comparable work intensities, exercise with low CHO availability substantially reduces glycogen utilisation (Arkinstall *et al.*, 2004) whilst concomitantly increasing circulatory free fatty acid (FFA) availability (Wojtaszewski *et al.*, 2003) and circulatory catecholamines (Hansen *et al.*, 2005). Consequently, the altered metabolic milieu created through exercising with low CHO availability has a direct impact on molecular signalling events controlling training adaptation, allowing for a compensatory response to such a metabolic challenge.

For instance, AMPK^{Thr172} (Yeo *et al.*, 2010), ACC ^{Ser79} and p53^{Ser15} (Bartlett *et al.*, 2013) phosphorylation, AMPKα2 activity (Wojtaszewski *et al.*, 2003) and its nuclear abundance (Steinberg *et al.*, 2006) are all augmented when exercise is commenced with low CHO availability. However, it is unclear whether commencing exercise with low CHO availability, through a variety of train-low methodologies, modulates the activity of other key signalling kinases such as p38 MAPK or CaMKII (Yeo *et al.*, 2010; Bartlett *et al.*, 2013; Lane *et al.*, 2015; Stocks *et al.*, 2019). In addition to modulating such signalling events, commencing exercise with low CHO availability also augments the mRNA expression of the PGC-1α gene as well as those involved in both mitochondrial biogenesis and substrate utilisation (Pilegaard *et al.*, 2002; Bartlett *et al.*, 2013; Psilander *et al.*, 2013; Lane *et al.*, 2015; Stocks *et al.*, 2019).

When taken together, these data suggest that commencing exercise with low CHO availability primarily augments AMPK-PGC1α mediated signalling to ultimately enhance the mRNA expression of a wide variety of mitochondrial and nuclear encoded genes, although its regulation of alternative signalling cascades is not well understood and requires further understanding.

A variety of acute and chronic train-low interventions to investigate the efficacy of CHO restriction on training adaptations and exercise performance. An overview of specific train-low models is discussed below and relevant experimental details from seminal studies are summarised in Table 2.1.

2.5.1 Twice per day training

On the basis of the molecular evidence derived from acute exercise studies, initial training studies adopted a "training twice every second day versus once daily" model. Using this model, Hansen et al. (2005) subjected seven untrained males to 10 weeks of knee extensor exercise training under conditions of either high or low muscle glycogen (Hansen *et al.*, 2005). Subjects trained both legs according to two different schedules, whereby one leg trained every day (HIGH) whilst the contralateral leg trained twice a day (2 h apart), every other day (LOW) (Figure 2.9). As such, one leg (LOW) commenced every second training session under conditions of low glycogen whilst the other leg performed each session under conditions of high muscle glycogen (HIGH), whilst allowing total work done to be matched for both legs. Following 10 weeks of training, the leg that commenced 50% of training sessions with low muscle glycogen (~ 200 mmol·kg^{-1} dw) demonstrated a superior increase in the maximal activity of both citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD), compared with the contralateral leg. Furthermore, the LOW leg also demonstrated an almost two-fold increase in exercise capacity when compared with the HIGH leg, demonstrating a potent effect of altering substrate availability on exercise-induced adaptation and subsequent performance. Whilst these data are intriguing, the use of a knee extensor model and the clamping of training intensity bear little resemblance to the customary training undertaken by athletic populations and limit the applicability to this cohort.

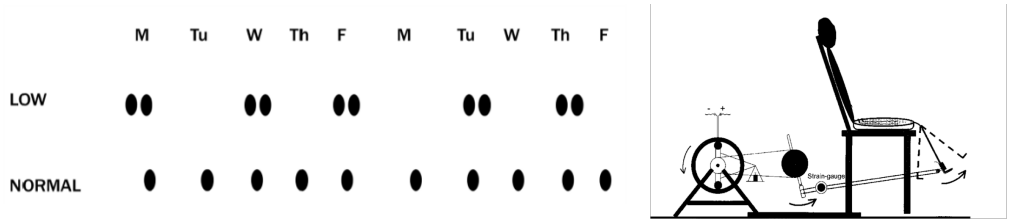

Figure 2. 9. Schematic representation of the twice-per day model used by Hansen *et al* (2005). Black dots represent each individual training session.

Yeo and colleagues employed a similar twice per day model using a cycling protocol representative of real-world practices (Yeo *et al.*, 2008). Similar to that of Hansen (2005), subjects trained according to two different schedules; One group (HIGH) trained once daily (6 days/week for 3 weeks), whereas the other trained twice per day, every other day (LOW), combining classical 'aerobic' training in the morning (100 min steady state cycling at 70% $\dot{V}O_{2peak}$) with high-intensity interval training in the afternoon (HIT; 8x5 min bouts at maximal effort with 1-min recovery between bouts). As such, the LOW group performed the HIT training with reduced muscle glycogen (~250 mmol·kg^{-1} dw) when compared with the HIGH group (~400 mmol·kg^{-1} dw). In support of Hansen's earlier work, these data demonstrate greater increases in the maximal activity of both citrate synthase and β-HAD and protein content of electron transport protein COXIV when 50% of training sessions were commenced with low muscle glycogen. Training with low muscle glycogen also augmented rates of whole body lipid utilisation (Yeo *et al.*, 2008), which appear to be derived primarily from intramuscular triglycerides (Hulston *et al.*, 2010). Interestingly, the superior adaptations observed in the low glycogen training group were achieved despite an inability to maintain the training intensity observed within the high glycogen group (78 vs. 87% *W*max) within weeks 1-2 (Yeo *et al.*, 2008). Notwithstanding this, it appears that the train-low model induces sufficient perturbations to the homeostatic milieu and may even compensate for an initial reduction in training intensity, in order to ultimately drive the observed enhanced training response.

Whilst the aforementioned studies demonstrate a clear effect of training twice-per-day, potentially through the manipulation of endogenous muscle glycogen, it appears that exogenous CHO feeding between exercise sessions (despite muscle glycogen being reduced from the first session) may negate the associated benefits of the twice-per-day model. Indeed, when CHO is fed in the recovery period following the initial exercise session and during the subsequent second exercise bout, the training-induced increases in oxidative enzyme activity (succinate dehydrogenase) observed without exogenous CHO are completely abolished (Morton *et al.*, 2009). Taken together, these data demonstrate that both endogenous glycogen and exogenous

carbohydrate availability exert potential regulatory effects on training induced muscle adaptations, although the interaction between both substrates requires further investigation. Nonetheless, despite the apparent muscle-specific benefits of twice-per-day training, this approach is yet to exert clear performance benefits in whole-body exercise models.

2.5.2 Fasted training

Performing endurance training in the fasted state represents a simpler train-low model where breakfast is consumed following morning training. Although pre-exercise muscle glycogen is not altered as a result of the overnight fast, liver glycogen and circulating glucose remains lower whilst FFA availability is increased compared with when breakfast is fed (Montain *et al.*, 1991; Horowitz *et al.*, 1997). Upon this basis, exercising in the fasted state increases post-exercise AMPK and CREB signalling (Akerstrom *et al.*, 2006; Stocks *et al.*, 2019) and mRNA expression of genes controlling substrate utilisation (PDK4, GLUT4, CD36, CPT-1) and mitochondrial function (UCP3) (Civitarese *et al.*, 2005; Cluberton *et al.*, 2005) compared with when carbohydrate is fed both before and during exercise. In light of this, chronic periods of fasted training elicit similar adaptations to those observed when training with low muscle glycogen. Nybo and colleagues (Nybo *et al.*, 2009) demonstrated that 8 weeks of endurance training (50-90 min of high-intensity intervals at 70-85% $\dot{V}O_{2max}$) in the fasted state enhanced training induced increases in β-HAD activity and the capacity to store muscle glycogen, compared with CHO being fed before and during training. These results were later confirmed by Van Proeyen *et al* (2011) who reported significant increases in the maximal activity of both CS and β-HAD following 6 weeks of fasted endurance training (1-1.5 h cycling at 70% $\dot{V}O_{2max}$) (Van Proeyen *et al.*, 2011). Commencing training in the fasted state also has profound effects on substrate metabolism, whereby chronic periods of fasted training result in a decrease in exercise-induced muscle glycogen breakdown (De Bock *et al.*, 2008) and increases intramyocellular (IMCL) breakdown in type I fibres (Van Proeyen *et al.*, 2011).These data collectively demonstrate that restricting exogenous carbohydrate both before and during exercise enhances post-exercise gene expression and subsequent oxidative enzyme activity following a period of endurance training. However, despite the superior biochemical adaptations with fasted training, the purported metabolic advantage is yet to result in improvements in exercise performance.

2.5.3 Sleep-Low, Train-Low

Using a sleep low, train low model, subjects perform an evening training session to deplete endogenous CHO stores, restrict carbohydrate overnight and complete a fasted training session the subsequent morning. In this way, the total accumulative time spent with reduced

muscle glycogen could extend to 12-14 hours depending on both the timing and duration of training sessions and the sleep period. This model allows overcomes the short time-proximity of the twice-per-day model, while allowing for the manipulation of endogenous CHO stores between sessions. Acute studies utilising the sleep-low, train-low model demonstrate significant activation of signalling kinases (AMPK, p38 MAPK), transcription factors (p53) and expression of a variety of metabolic genes controlling mitochondrial biogenesis and substrate utilisation compared with high glycogen trials (Wojtaszewski *et al.*, 2003; Bartlett *et al.*, 2013; Lane *et al.*, 2015). Bartlett *et al* (2013) demonstrated that this train-low model leads to significant phosphorylation of both ACC and p53 and expression of PGC-1α, COXIV, Tfam & PDK4 genes both at rest and 3 h post-exercise. In contrast, when exercise was commenced with high muscle glycogen and exogenous carbohydrate was provided before, during and after exercise, the aforementioned activation of signalling kinases and gene expression were completely abolished, despite the intense nature of the exercise bout (6 x 3 min at 90% $\dot{V}O_{2max}$). Although these data present two extreme differences in carbohydrate availability, enhanced activation of signalling kinases (Wojtaszewski *et al.*, 2003; Steinberg *et al.*, 2006), and expression of genes that underpin exercise induced training responses (Psilander *et al.*, 2013; Lane *et al.*, 2015) with the sleep low, train low model have also been reported elsewhere. Furthermore, repeated bouts of sleep-low, train-low is the only train-low model shown to enhance performance in trained endurance athletes (Marquet *et al.*, 2016*a*, 2016*b*) although not in all cases (Riis *et al.*, 2019).

2.5.4 Periodized approach and an amalgamation of train-low paradigms

In the real-world training environments of elite endurance athletes, it is likely that an amalgamation of the aforementioned train-low paradigms are practiced (either through default of their current training structure or via coach and sport scientist-led practices), as opposed to undertaking one strategy in isolation (Stellingwerff, 2012). As such, an understanding of the efficacy of amalgamating train-low models and how best to periodize this type of training into an athletes training schedule is warranted. Investigations to date have failed to observe any superior adaptations when a variety of train-low strategies are incorporated into 3-4 weeks of intensified endurance training compared with when adequate carbohydrate is provided for all training sessions. For instance, incorporating periods of fasted and twice-per-day training as well as post-exercise CHO restriction amongst elite race walkers, appeared to offer no performance advantage when compared with traditional practices of undertaking all sessions with high carbohydrate availability (Burke *et al.*, 2017). In agreement, Gejl *et al* (2017) observed similar increases in maximal CS activity and subsequent exercise performance measures (30 min time trial) following 4 weeks of training with periodic CHO restriction (twice-per-day

model) compared with when all sessions were commenced with CHO provision before and after training (Gejl *et al.*, 2017).

Whilst both studies adopted a number of accepted train-low models, examination of the periodisation of these sessions and/or acute muscle glycogen data may provide some explanation for the aforementioned results. For instance, despite Burke *et al* incorporating periods of twice-per-day training it could be questioned whether the model of periodisation was sufficient to induce sufficient glycogen depletion to allow the second daily session to be commenced with low glycogen, given that CHO was fed before and during training (Burke *et al.*, 2017). In support of this, Gejl *et al* reported that muscle glycogen concentrations remained above 400 mmol·kg dw^{-1} following the second session of the twice-per-day model demonstrating the difficulty in inducing sufficient glycogen depletion in well-trained athletes (Gejl *et al.*, 2017). In comparison, others have reported muscle glycogen concentrations of ~100 mmol·kg dw^{-1} following the completion of the twice daily training model (Morton *et al.*, 2009; Yeo *et al.*, 2010) demonstrating that more pronounced glycogen depletion may be required to drive the adaptive responses associated with the train-low model. Taken together, these data suggest the existence of a 'glycogen threshold', a critical absolute level of muscle glycogen concentrations which must be exceeded in order for significant activation of specific cell signalling pathways and subsequent muscular adaptations to occur.

Table 2. 1. Overview of the methodological details and study outcomes of both acute and chronic train-low studies according to the relevant train-low paradigm used

Reference	Subjects	Duration	Exercise Protocol & Glycogen Status (mmol/kg dw)	Skeletal Muscle Adaptations	Exercise Performance Outcomes
Twice per day model					
Hansen et al [9]	7 untrained men	10 weeks 5 days x week	Knee extensor exercise. One leg trained 50% of sessions with low glycogen (LOW) whist the other trained all sessions with high glycogen (HIGH). Second session glycogen in LOW: Pre: 200, Post: 100 mmol/kg dw respectively.	Greater increase in CS activity in LOW condition Increased β-HAD activity in LOW condition only	Improved TTE for knee extensor exercise
Yeo et al [17]	14 trained male cyclists/triathletes	3 weeks 4 x week	100 min steady state cycling (63% PPO) followed by 8 x 5 min intervals at maximal pace either 2 h (LOW) or 24 h (HIGH) later. Pre interval exercise glycogen: (LOW: 256, HIGH: 390). Post exercise glycogen (LOW: 124, HIGH: 229).	Increased CS & β-HAD activity in LOW condition only Increased COXIV protein content in LOW condition only	Similar improvements (10%) in 60 min TT for both groups
Morton et al [18]	23 active men	6 weeks 4 x week	6 x 3 min running (90% VO_{2max}). NORM trained once per day whilst LOW+PLA & LOW+GLU trained twice per day (every other day). LOW+GLU ingested CHO before and during every second training session. Pre exercise glycogen: (LOW: 232 and 253, HIGH: 412 and 387 in gastrocnemius and vastus lateralis respectively). Post exercise glycogen: (LOW: 107 and 176, HIGH: 240 and 262 in gastrocnemius and vastus lateralis respectively).	Greater increase in SDH activity in LOW+PLA compared with LOW+GLU & NORM	Similar improvements in VO_{2max} & YoYoIR2 for all groups

Yeo et al [23]	12 trained male cyclists/triathletes	Acute exercise	100 min steady state cycling (63% PPO) followed by 8 x 5 min intervals at maximal pace either 2 h (LOW) or 24 h (HIGH) later. Pre-interval exercise glycogen: (LOW: 256, HIGH: 390). Post exercise glycogen (LOW: 124, HIGH: 229).	Greater phosphorylation of $AMPK^{Thr172}$ in LOW	N/A
Hulston et al [19]	14 trained male cyclists	3 weeks 6 x week	90 min cycling at 70% VO_{2max} followed by (2 h apart) HIT (8 x 5 min) in LOW group. HIGH group performed alternate days of either steady state or HIT cycling. Acute glycogen status not measured	β-HAD protein content increased in LOW only Increased fat utilisation from muscle triglycerides in LOW only	Similar improvements in 60 min TT for both groups
Cochran et al [22]	10 active men	Acute exercise	HIT cycling (5 x 4-min at 90-95% heart rate reserve) twice per day (separated by 3 h). One group consumed CHO (2.3 g.kg) between sessions (HIGH) whereas the other restricted CHO intake (LOW). Pre PM exercise glycogen: (LOW: 256, HIGH: 390). Post exercise glycogen (LOW: 124, HIGH: 229)	Greater phosphorylation of p38MAPK in LOW following PM exercise Similar increase in PGC-1α & COXIV gene expression	N/A
Cochran et al [20]	18 active men	2 weeks 3 days x week	HIT cycling (5 x 4 min at 60% PPO) twice per day (separated by 3 h). One group consumed CHO (2.3 g.kg) between sessions (HIGH) whereas the other restricted CHO intake (LOW). Acute glycogen status not measured	Similar increase in maximal CS activity & protein content of both CS and COXIV	Greater improvement in 250 kJ TT performance in LOW group.
Fasted training model					
Akerstrom et al [27]	9 active men	Acute exercise	2 h one-legged knee extensor exercise (60% W_{max}) in either a fasted (FAST) or fed (exogenous CHO during) (FED) state. Pre exercise glycogen: (500 mmol/kg dw in both groups). Post exercise glycogen: (300 & 200 in FED and FAST respectively).	Reduced AMPKα2 activity in FED	N/A
Lee-Young et al [49]	9 active men	Acute exercise	120 min cycling (65% VO_{2peak}) exercise in either a fasted (FAST) or fed (exogenous CHO during) state. Pre exercise glycogen: (500 mmol/kg dw in both groups). Post exercise glycogen: (150 & 100 in FED and FAST respectively).	Similar increases in AMPKα2 activity and $AMPK\alpha2^{Thr172}$ & $ACC\text{-}\beta^{Ser222}$ phosphorylation	N/A
De Bock et al [31]	20 active men	6 weeks 3 x week	1 – 2 h cycling (75% VO_{2peak}). One group trained in the fasted state (FAST) with the other consuming CHO before and during exercise (FED). Acute glycogen status not measured	FABPm increased in FAST condition only	N/A
Nybo et al [32]	15 untrained men	8 weeks 3-4 x week	3-6 min of high intensity intervals (70-85% VO_{2max}). Subjects either received CHO or placebo (PLA) during exercise. Acute glycogen status not measured.	Greater increases in β-HAD activity & basal muscle glycogen content in PLA group only.	Similar improvements in peak power, VO_{2max} & 15 min TT performance
Van Proeyen et al [30]	20 active men	6 weeks 4 x week	1 – 1.5 h cycling (70% VO_{2max}). One group trained in the fasted state (FAST) with the other consuming CHO before and during exercise (FED). Acute glycogen status not measured.	CS & β-HAD maximal activity increased in FAST condition only	Similar improvements in 1 h TT performance in both groups

Sleep low model

Pilegaard et al [15]	Study A: 6 active men	Acute exercise	Study A: 1-legged glycogen depleting exercise followed by 2-legged cycling (2 h at 45% VO_{2max}) the subsequent day. Pre-exercise glycogen: (LOW: 337, HIGH: 609). Post exercise glycogen (LOW: 306, HIGH: 423).	Study A: Enhanced gene expression of PDK4, LPL & HKII at rest in LOW only	N/A
	Study B: 6 active men	Acute exercise	Study B: 3 h of 2-legged knee extensor exercise with either normal (NORM) or LOW glycogen. Pre-exercise glycogen: (LOW: 240, HIGH: 398). Post exercise glycogen (LOW: 101, HIGH: 153).	Study A&B: Enhanced gene expression of PDK4 & UCP3 post-exercise in LOW only	N/A
Wojtaszewski et al [36]	8 trained men	Acute exercise	60 min cycling at 70% VO_{2peak} with either LOW or HIGH muscle glycogen (from exercise/diet manipulation the previous day). Pre exercise glycogen: (LOW: 163, HIGH: 909). Post exercise glycogen: (LOW: 150, HIGH: 400).	Increased AMPKα2 activity in LOW only Greater phosphorylation of ACC^{Ser221} in LOW	N/A
Chan et al [37]	8 active men	Acute exercise	60 min cycling (70% VO_{2peak}) with either HIGH or LOW glycogen (achieved by exercise/diet manipulation the previous evening). Pre exercise glycogen: (LOW: 163, HIGH: 375). Post exercise glycogen: (LOW: 17, HIGH: 102).	Greater phosphorylation of p38 MAPK in LOW Enhanced gene expression of IL-6 in LOW	N/A
Steinberg et al [21]	7 active men	Acute exercise	60 min cycling at 70% VO_{2max} with either reduced (LOW) or normal (NORM) muscle glycogen. Pre exercise glycogen: (LOW: 150, HIGH: 390). Post exercise glycogen: (LOW: 17, HIGH: 111).	Greater AMPKα2 activity, phosphorylation of ACC^{Ser221} and nuclear translocation of AMPKα2 in LOW only Enhanced gene expression of GLUT4 in LOW	N/A
Bartlett et al [38]	8 active men	Acute exercise	HIT running (6 x 3 min at 90% VO_{2max}). LOW performed glycogen-depleting cycling the night before and restricted CHO overnight. HIGH consumed high CHO breakfast & CHO during exercise. Pre exercise glycogen: (LOW: 100, HIGH: 500). Post exercise glycogen: (LOW: 80, HIGH: 300).	Phosphorylation of ACC^{Ser79} & $p53^{Ser15}$ in LOW only Enhanced gene expression of PGC-1α, PDK4, Tfam & COXIV in LOW	N/A
Psilander et al [24]	10 trained male cyclists	Acute exercise	6 x 10 min cycling (64% $VO2_{max}$) with either HIGH or LOW glycogen (achieved by exercise/diet manipulation 14 h previously). Pre exercise glycogen: (LOW: 166, HIGH: 478). Post exercise glycogen: (LOW: 130, HIGH: 477).	Enhanced gene expression of PGC-1α in LOW Increased gene expression of PDK4 & COXI in LOW only	N/A
Lane et al [39]	7 trained male cyclists	Acute exercise	Evening bout of high-intensity cycling (8 x 5 min at 82.5% PPO) followed by 120 min steady state cycling (50% PPO) the subsequent morning. LOW group restricted CHO overnight whereas HIGH group consumed high CHO diet (4 g.kg BM). Pre exercise glycogen: (LOW: 349, HIGH: 459). Post exercise glycogen: (LOW: 266, HIGH: 338).	Greater phosphorylation of ACC^{Ser79} post AM exercise in LOW Enhanced gene expression of CD36, FABP3 & PDK4 post AM exercise in LOW	N/A
Marquet et al [40]	21 male triathletes	3 weeks 6 x week	HIT (8 x 5 min cycling at 85% MAP or 6 x 5 min running at individual 10 km intensity) in the evening followed by LIT (60 min cycling at 65% MAP) the subsequent morning. One group consumed CHO between training sessions (HIGH) whereas the other restricted CHO intake (LOW). Acute glycogen status not measured.	N/A	Improved 10 km running TT performance & improved TTE cycling (150% peak aerobic power) in LOW group only

Marquet et al	11 trained male cyclists	1 week 6 x week	HIT (8 x 5 min cycling at 85% MAP) in the evening followed by LIT (60 min cycling at 65% MAP) the subsequent morning. One group consumed CHO between training sessions (HIGH) whereas the other restricted CHO intake (LOW). Acute glycogen status not measured.	N/A	Improved 20 km cycling TT performance in LOW group only
Riis et al	13 trained males	4 weeks 6 x week	HIT (10 x 5 min cycling at 90% HRmax) in the evening followed by LIT (75 min at 65-75% HRmax). LOW group restricted CHO intake overnight whilst HIGH group consumed CHO following evening and prior to morning exercise. Acute glycogen status not measured.	No change in proteins involved in lipid metabolism (ATGL, HSL, CGI-53 or G0S2) in either group.	Similar improvements in 30 min cycling TT for both groups
Recover low model					
Pilegaard et al	9 active men	Acute exercise	75 min cycling (75% VO_{2max}) followed by 24 h recovery with either HIGH or LOW CHO diet. Glycogen was restored to 576 and 348 with HIGH and LOW CHO diets respectively at 24 h.	Gene expression of PDK4, UCP3, LPL & CPT1 remained elevated for 8-24 h with CHO restriction post-exercise.	N/A
Jensen et al	15 male triathletes	Acute exercise	4 h cycling (56% VO_{2max}) followed by 4 h recovery feeding with either HIGH (1 g.kg.h) or LOW (water only) CHO. Post exercise glycogen: (LOW: 234, HIGH: 245). 4 h glycogen: (LOW: 264, HIGH: 444).	Similar gene expression of PGC-1α, Tfam, NRF-1, COXIV, PDK4, LPL, PPAR, UCP3 & GLUT 4 in both groups	N/A
High fat feeding					
Hammond et al	10 active men	Acute exercise	High-intensity running (8 x 5 min @ 85% VO_{2peak}) followed by steady state running (60 min at 70% VO_{2peak}) 3.5 h later. Steady state running was either commenced with high or low (but high fat) CHO availability. Muscle glycogen was similar in both groups (200 mmol/kg dw) post steady state running.	p70S6K activity was supressed with high fat feeding Similar gene expression of PGC-1α, p53, CS, Tfam, PPAR and ERRα in both groups	N/A
Periodised model					
Impey et al [48]	11 amateur male cyclists	Acute exercise	Based on the principle of fuel for the work required. 4 x 30 s HIT cycling (150% PPO) & 45 min steady state cycling (50% PPO) followed by 1 min efforts (80% PPO) until exhaustion with either HIGH or LOW glycogen (by previous exercise/diet manipulation for 36 h previously). HIGH consumed CHO before, during & after exercise whereas LOW consumed leucine enriched protein.	36 h of prior CHO restriction enhanced p53, SIRT1 & Tfam gene expression. CHO restriction before and during exercise induced work efficient AMPK signalling. Post-exercise CHO restriction and keeping glycogen <100 mmol/kg dw reduced p70S6K activity	Exercise capacity (1 min efforts at 80% PPO) enhanced in HIGH trial (158 vs. 100 min)
Burke et al [45]	22 international male race walkers	3 weeks 7 x week	3 weeks of intensified training (race walking, resistance training, cross training). Athletes consumed 3 different diets across the training period: a) high CHO b) LCHF c) periodised CHO intake with periods of low CHO training. Acute glycogen status not measured.	N/A	Similar improvements in VO_{2peak} between all groups Improved 10 km race times in high CHO and periodised CHO groups (no change in LCHF) LCHF diet increased O_2 cost of race walking
Gejl et al [55]	26 elite male endurance athletes	4 weeks 7 x week	4 weeks of intensified training. Athletes either performed all sessions with high CHO availability or followed a periodised model, performing 3 sessions per week with reduced CHO availability. Glycogen content was 400 mmol/kg dw following LOW carbohydrate availability training session.	Similar increase in maximal CS activity No increase in β-HAD activity in either group	Similar improvement in VO_{2max} & 30 min TT performance between groups

2.5.5 The glycogen threshold hypothesis

Given that the enhanced training response associated with train-low models is potentially mediated by muscle glycogen availability, it is prudent to consider the absolute glycogen concentrations required to facilitate this response. With this in mind, the majority of studies that adopt train-low models commence exercise with glycogen concentrations between 100-300 $mmol \cdot kg^{-1}$ dw (Cochran *et al.*, 2010; Yeo *et al.*, 2010; Bartlett *et al.*, 2013; Taylor *et al.*, 2013; Impey *et al.*, 2016). When exercise is commenced with pre-exercise muscle glycogen

concentrations within this range, the activity of key cell signalling kinases, transcription factors and expression of various metabolic genes are augmented (Figure 2.10). Furthermore, in accordance with data derived from acute exercise, selected markers of skeletal muscle adaptation are also augmented when train-low sessions are repeatedly performed below the proposed glycogen threshold (Hansen *et al.*, 2005; Yeo *et al.*, 2008; Morton *et al.*, 2009) whilst failure to induce sufficient glycogen depletion using the twice per day model does not induce an augmented training response (Gejl *et al.*, 2017). Support for the notion of a glycogen threshold is also provided from studies that have fed CHO during exercise. For instance, Akerstrom and colleagues (2006) demonstrated that when glycogen depletion during exercise is attenuated through exogenous CHO provision the activity of AMPK is attenuated. In contrast, exogenous CHO feeding that does not attenuate muscle glycogen depletion, and subsequently allows depletion to low levels (< 200 mmol·kg^{-1} dw) has no impact on AMPK activity. (Lee-Young *et al.*, 2006). Taken together, these data suggest the potential existence of a muscle glycogen threshold, whereby exceeding an absolute level of muscle glycogen concentrations is especially potent in modulating the acute and chronic skeletal muscle adaptations associated with train-low models.

Nonetheless, the potential existence of a glycogen threshold is not to say that adaptation to endurance training is not possible without performing exercise within a specific absolute range of muscle glycogen concentrations. Rather, such a threshold highlights the possibility that the enhanced adaptations associated with the train-low model are more pronounced once a certain level of glycogen depletion is induced. In this regard, in order to induce comparable adaptations when exercise is commenced with muscle glycogen concentrations outside of such a range, significant increases in glycolytic flux, achieved via high intensity or prolonged exercise at lower intensities are likely required in order to drive sufficient glycogen depletion. For example, Impey *et al,* (2016) recently demonstrated comparable signalling and gene expression responses when exercise was commenced with high muscle glycogen (600 mmol·kg^{-1} dw). However, such responses were achieved with significantly less work (~60 min) when performed with low muscle glycogen (100 mmol·kg^{-1} dw) suggesting train-low exercise induces work-efficient signalling responses.

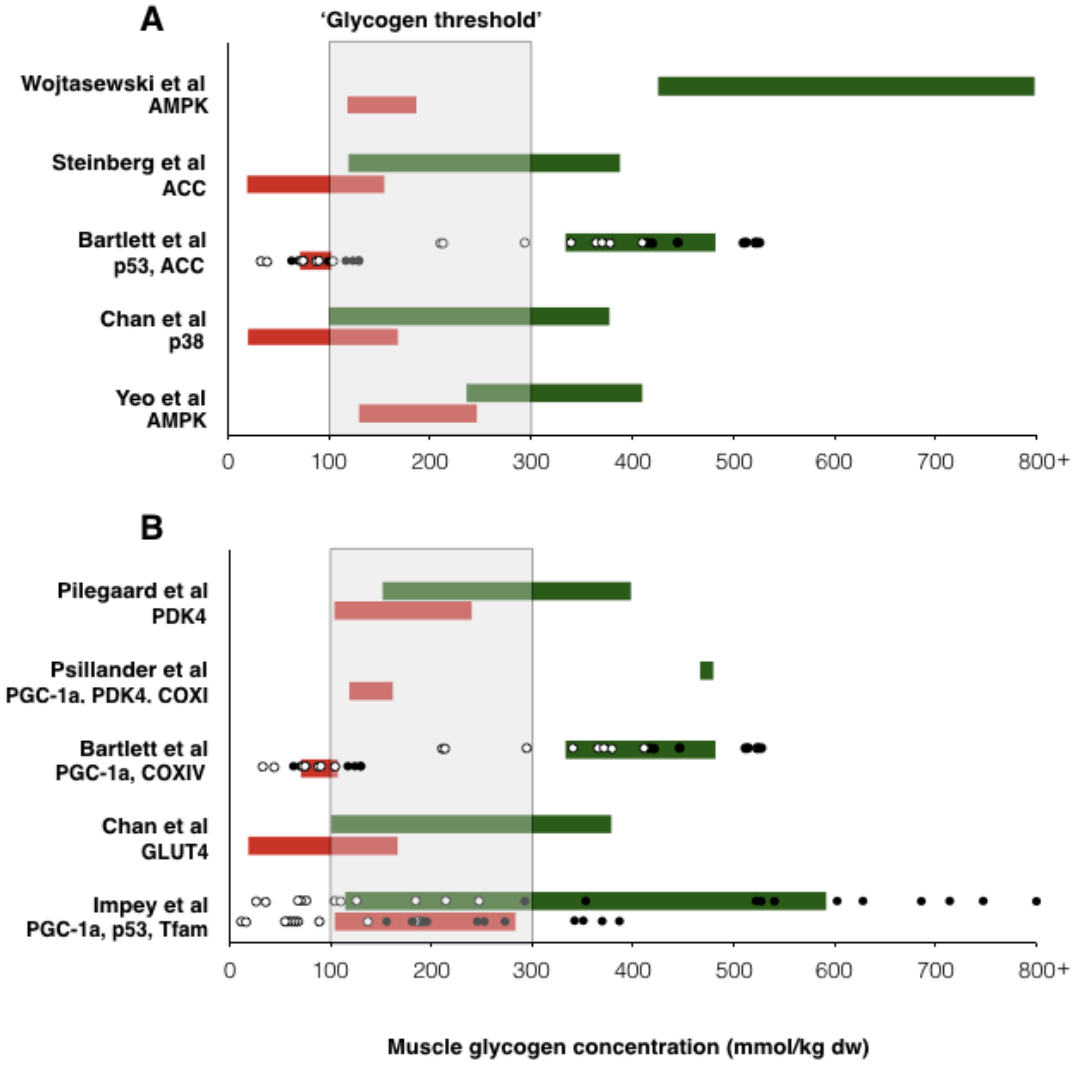

Figure 2. 10. Overview of studies supporting the glycogen threshold hypothesis. Studies are categorised into those examining **A** cell signalling and **B** gene expression responses to exercise, with the specific protein or mRNA target that is augmented in response to low muscle glycogen highlighted on the y-axis. The green bars represent the trial within the specific study that has been completed with high muscle glycogen whilst the red bars represent the trial completed with low muscle glycogen. The length of the bar corresponds to the magnitude of glycogen utilisation during the specific exercise bout. Where individual muscle glycogen data is available, black circles represent pre- and white circles represent post- exercise muscle glycogen concentrations. Adapted from Impey *et al* (2018).

2.6 Summary

From its early recognition as a primary energy source during exercise, the role of carbohydrate has evolved into an important regulatory molecule with the ability to modulate acute cell signalling pathways that control many hallmark muscle adaptations associated with exercise training. Whilst such responses can be achieved using a variety of train-low paradigms, it appears that the augmented signalling and transcriptional responses associated with train-low are reliant on the absolute concentrations of muscle glycogen and suggest the potential existence of a muscle glycogen threshold. As such, the chapters in this thesis attempt to investigate

the potential existence of a muscle glycogen threshold and to provide a definition of its potential upper and lower limits. Furthermore, the interaction between endogenous and exogenous carbohydrate availability within the context of the glycogen threshold hypothesis also warrants further investigation in order to develop a more comprehensive understanding of the signalling network that regulates these interactions.

Chapter Three:

General methodology

This chapter describes general methodologies and theory of methodologies used within this thesis.

3.1 General Methodology

3.11 Location of testing and ethical approval

Exercise tests, biochemical and molecular analysis were completed within the laboratories of the Research Institute for Sport & Exercise Sciences. Ethical approval for all of the experimental protocols and related procedures was granted from the local ethics committee at Liverpool John Moores University. Analysis of skeletal muscle cell signalling for study 1 was performed at the University of Birmingham.

3.12 Subject characteristics

All of the subjects who participated in each study were young, healthy and either recreationally active (study 1) or trained amateur cyclists (study 2 and 3). A comparison of subjects' physical and physiological characteristics are shown in Table 3.1. All subjects gave written, informed consent to participate in the study after details and procedures of the study had been fully explained. None of the subjects had any history of neurological disease, skeletal muscle abnormality or were under any pharmacological interventions during any study. All subjects were instructed to refrain from exercise, caffeine and alcohol intake at least 24 h prior to any lab visit.

Table 3. 1. Physical & physiological characteristics of subjects (mean ± SD)

	Study 1	Study 2	Study 3
Age (years)	22 ± 3	30 ± 10	31 ± 7
Height (m)	1.77 ± 0.05	1.77 ± 0.09	1.77 ± 0.08
Body mass (kg)	76.0 ± 12.7	72.6 ± 9.4	73.6 ± 6.1
$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	48.9 ± 7.0	60.4 ± 7.7	60.5 ± 5.4
Peak power output (W)	272 ± 21.1	338 ± 44.6	383± 21

3.2 Cardio-Respiratory Measures

3.21 Heart rate

Subjects were fitted with a Bluetooth heart rate sensor for the measurement of heart rate (Polar H7, Kempele, Finland) during all exercise bouts.

3.22 Assessment of respiratory gasses during exercisef

Subjects were fitted with a facemask (for the assessment of $\dot{V}O_{2peak}$) or mouthpiece (for the measurement of respiratory gases during all other exercise testing sessions). Expired oxygen and carbon dioxide breath-by-breath measurements were obtained throughout exercise using

a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, USA and Moxus Modular Metabolic System, AEI Technologies, Illinois, USA) after calibration with known reference gases.

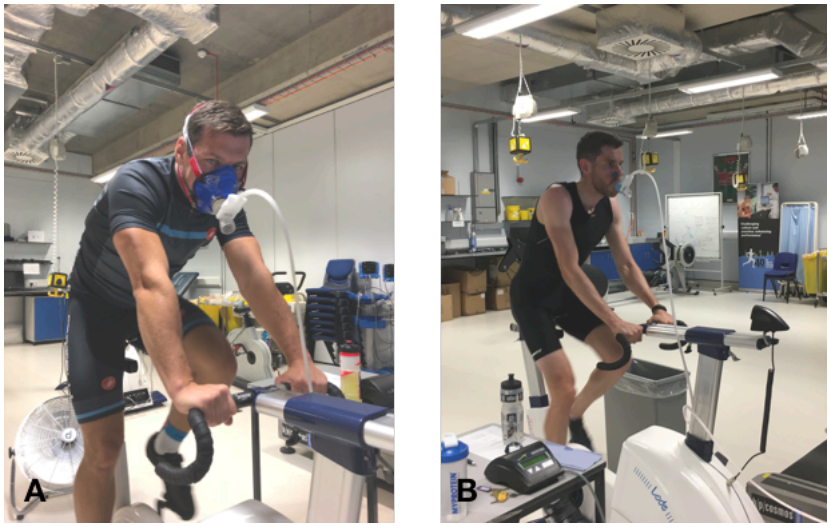

Figure 3. 1. (A) Example of face mask and (B) mouthpiece used for the collection of respiratory gases during exercise

3.23 Assessment of substrate utilisation during exercise

Rates of carbohydrate and lipid oxidation during exercise were estimated via indirect calorimetry (CPX Ultima, Medgraphics, Minnesota, USA in study 1 and 2, and Moxus Modular Metabolic System, AEI Technologies, Illinois, USA in study 3) using the equations of Jeukendrup & Wallis (2005) which assumes a negligible contribution from protein oxidation.

$$\textbf{Carbohydrate oxidation (g.min}^{-1}\textbf{):}\ (4.21 \times \dot{V}CO_2) - (2.962 \times \dot{V}O_2)$$

$$\textbf{Fat oxidation (g.min}^{-1}\textbf{):}\ (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2)$$

Oxidation of 1 gram of carbohydrate was assumed to be energetically equivalent to 17 KJ whilst oxidation of 1 gram of fat was assumed equivalent to 37.7 KJ. Volumes of oxygen and carbon dioxide are expressed in litres.min^{-1} throughout the calculation.

Indirect calorimetry principle

The method of indirect calorimetry uses measurements of gas exchange at the lungs (whole body O_2 consumption and CO_2 production) as an approximate measure of substrate oxidation during exercise given that carbohydrates, fats and proteins differ in their chemical composition and thus, the amounts of O_2 needed and CO_2 produced when oxidised (Table 3.2). Therefore, rates of substrate utilisation for energy production can be estimated through the measurement of O_2 consumed and CO_2 produced during exercise.

Table 3. 2. Energy and volumes of O_2 needed and CO_2 produced in the oxidation of carbohydrate, fat and amino acids. Taken from Jeukendrup & Wallis (2005).

	Energy yield (kcal.g)	O_2 required (L.g)	CO_2 produced (L.g)	RQ	Energy equivalent of O_2 (kcal.L)
Glucose	3.74	0.7455	0.7426	0.996	5.02
Glycogen	4.15	0.8283	0.8251	0.996	5.02
Fatty acid	9.75	2.0092	1.4136	0.704	4.85
Amino acid	4.09	0.9842	0.7931	0.807	4.16

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

Peak oxygen consumption ($\dot{V}O_{2peak}$) and peak aerobic power output (PPO) were determined during an incremental cycle test, performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Lode, Netherlands). Following the completion of a 10-minute warm up at 75 W, the test began at 100 W and consisted of 2-minute stages with 30 W increments in resistance until volitional exhaustion. Subjects received strong verbal encouragement throughout the test and exhaustion was defined as an inability to maintain a cadence of 60 $rev.min^{-1}$ for 10 consecutive seconds. Breath-by-breath measurements of expired gases were obtained throughout the duration of the test via a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US) calibrated with known internal reference gases. The highest 30 s average of O_2 uptake was considered to represent $\dot{V}O_{2peak}$ which was stated as being achieved by the following end point criteria: 1) heart rate within 10 $beats.min^{-1}$ of age-predicted maximum, 2) respiratory exchange ratio (RER) > 1.1, and 3) a plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the power during the final completed stage of the incremental test.

3.3 Measurements of Psycho-Physiological Variables

3.31 Ratings of perceived exertion

Subjects' reported ratings of perceived exertion during exercise were recorded using a 15-point scale (Borg, 1982) (Figure 3.2). Subjects were familiarised to the scale during preliminary laboratory visits to ensure comprehension of the subjective nature of this measure.

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

Figure 3. 2. Borg scale used for the assessment of ratings of perceived exertion (RPE)

3.4 Procurement, Storage and Analysis of Muscle & Blood Samples

Blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using a standard venepuncture technique. Samples were collected into serum separator tubes (SST), K_2EDTA and lithium heparin vacutainers (BD Biosciences, UK). K_2EDTA and lithium heparin vacutainers were stored on ice whilst SST vacutainers were stored at room temperature for a minimum of 30 minutes until centrifugation at 1500 g for 15-minutes at 4°C. Following centrifugation, aliquots of plasma and serum were stored at -80°C for later analysis.

3.4.1 Circulating metabolite analysis

Samples were analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol concentrations using commercially available kits and a Randox Daytona spectrophotometer (Randox Laboratories, Ireland). The coefficient of variation for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol between duplicate samples was <5%.

Spectrophotometry principle

The spectrophotometry method works by measuring the amount of light that each sample absorbs which is then used for the determination of plasma metabolite concentrations by plotting the absorbance value against the absorbance of standards of known concentrations.

Within the spectrometer, the lens transmits a straight beam of light (photons) that passes through the prism in order to be split into several component wavelengths. The desired wavelength is then selected by the silt and is transmitted through the sample in cuvette. The number

of photons that is absorbed by the sample is then detected by the photometer (Figure 3.3) which allows for determination of the plasma metabolite concentration of each sample.

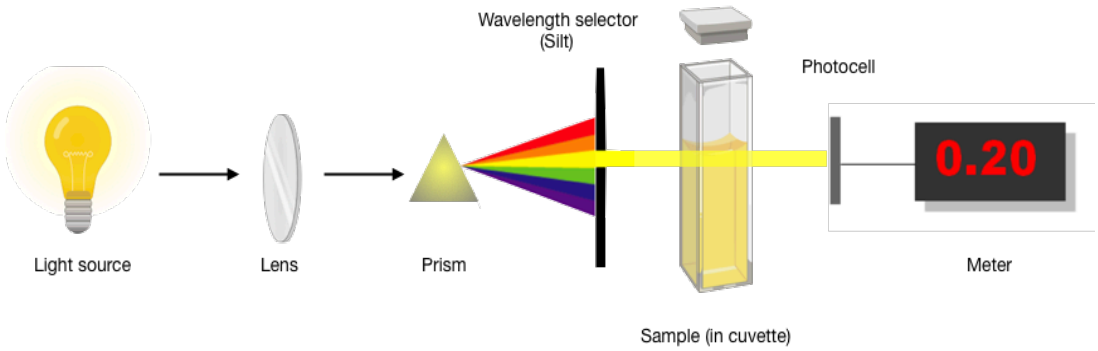

Figure 3. 3. An overview of the general principle of spectrophotometry

3.4.2 Muscle biopsies

Muscle biopsies were obtained from separate incision sites on the vastus lateralis muscle. Each incision site was located 2 cm apart and the biopsied leg was alternated for each trial to reduce the inflammatory response associated with repeated biopsies (Ekblom, 2017). Muscle samples for studies 1 and 2 were obtained using a Bard monopty disposable core biopsy instrument (12 gauge x 10 cm length, Bard Biopsy Systems, Tempe, USA) (Figure 3.4) and using the Well-Blakely conchotome technique for study 3, all under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen to minimise tissue degradation and stored at -80°C for later analysis.

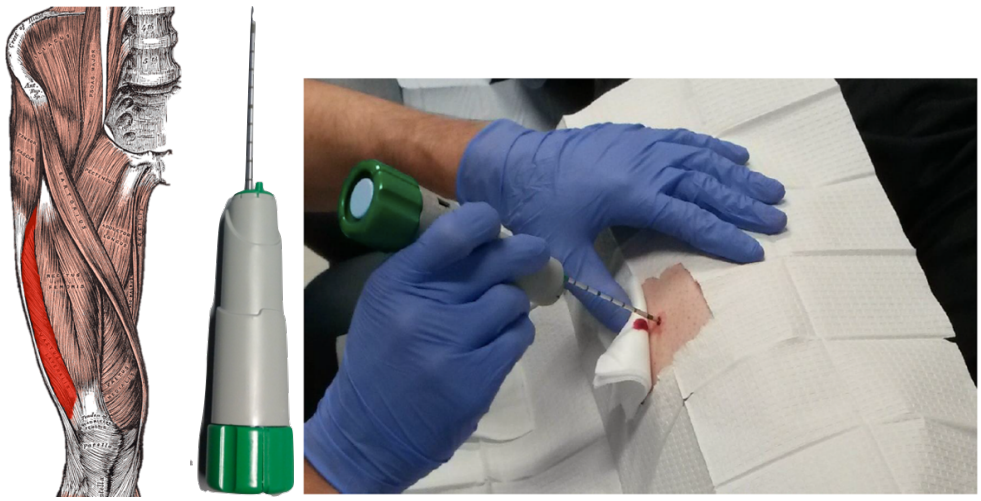

Figure 3. 4. Anatomical location of the muscle biopsy site (*Vastus Lateralis*), the Bard biopsy needle instrument used and a muscle biopsy procedure from an investigation within this thesis

3.4.3 Gene expression analysis

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was employed for the analysis of mRNA quantification in studies 1 & 2.

Polymerase chain reaction (PCR) principle

Once mRNA has been isolated from a biological sample (i.e. human skeletal muscle), the isolated mRNA is synthesised into complimentary DNA (cDNA) by reverse transcription using reverse transcriptase enzymes and nucleoside triphosphates (dNTPs). The complimentary DNA is then denatured into single stranded DNA via heating allowing for the annealing of sequence specific primers and the subsequent transcription of the target sequence via RNA polymerase (Figure 3.5). The amplified product can be detected by labelling the cDNA with SYBR green fluorescent dye that emit fluorescence, which increases proportionally with the amplification of the cDNA molecules and can be detected in real-time following each cycle of the reaction, allowing for precise semi-quantitative analysis of mRNA content.

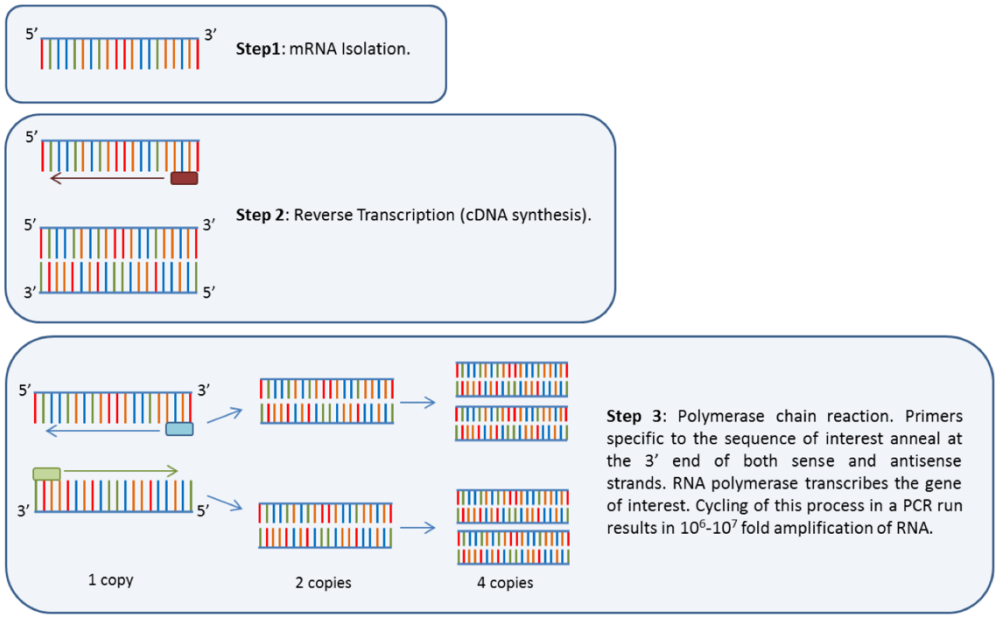

Figure 3. 5. Schematic representation of the key steps in real time quantitative polymerase chain reaction

Homogenisation of skeletal muscle samples

Skeletal muscle samples (~30-50 mg) were transferred to 2 ml lysing tubes containing ceramic beads (Roche Diagnostics, UK) and 500 µl of TRI reagent (Sigma Aldrich, UK) and homogenised through physical disruption (MagNA lyser, Roche Diagnostics, UK) at 6,000 rpm for 3 x 40 seconds, separated by 5 minutes of cooling on ice. TRI reagent contains phenol and guanidine thiocyanate that are capable of lysing cells and dissolving all other cell components

other than RNA and DNA. Furthermore, TRI reagent inhibits the activity of both DNAse and RNAse enzymes and subsequently helps to maintain RNA integrity. Following homogenisation, the tissue lysate was transferred from the lying tube into a pre-labelled RNAse-free Eppendorf.

RNA isolation method

One hundred µl of chloroform was subsequently added to the tissue-lysate sample and shaken vigorously by hand and left to stand at room temperature for 10 minutes before centrifugation at 12,000 x g for 15 minutes at 4°C. Centrifugation allowed for the sample to be separated into its red phenol, middle interphase and aqueous upper phase (Figure 3.5) The upper aqueous phase was carefully removed, transferred into a new pre-labelled RNAse-free Eppendorf and mixed with 250 µl of isopropanol. The sample was mixed by tube inversion and left to stand at room temperature for 10 minutes before further centrifugation at 12,000 x g for 10 minutes at 4°C. The resultant supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol. The sample was then vortexed on a low speed setting to wash the RNA pellet and centrifuged for 5 minutes at 7,600 x g at 4°C. The ethanol was removed and the wash process repeated. Following the second centrifugation, the ethanol was discarded and the pellet was left to air dry before re-suspension in RNAse and DNAse free, DEPC water (Thermo Fischer Scientific, UK). Samples were subsequently heated in a block heater at 35°C for 10 minutes prior to measurement of RNA quality and quantity.

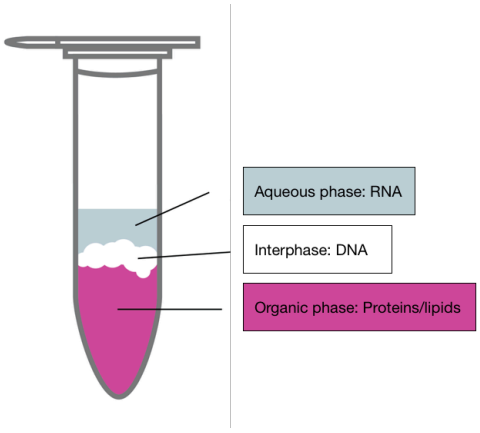

Figure 3. 6. Visual representation of phase separation that occurs with TRI reagent

Assessment of RNA quality

Both RNA purity and concentration were assessed by UV spectroscopy, using a Nandodrop spectrophotometer (Thermo Fisher Scientific). The absorbance of the diluted RNA sample was measured at known purity ratios of 260/280 and 260/230, where absorbance at 260 nm (A_{260}) provides a specific measurement of nucleic acid concentration and absorbance at 280

nm (A_{280}) and 230 nm (A_{230}) measures protein and background absorption, respectively, as an indication of possible contaminants. In general, an A_{260}/A_{280} ratio of 1.8 to 2.1 indicates very pure RNA with the A_{260}/A_{230} ratio being higher than that of A_{260}/A_{280} with common values ranging from 1.8-2.2. Despite this, there appears to be no acceptable lower limit for this ratio with previous research demonstrating no correlation between A_{260}/A_{230} and qPCR amplification efficiency (Kuang *et al.*, 2018). Both A_{260}/A_{230} and A_{260}/A_{280} ratios for each experimental study are reported in their respective experimental chapters.

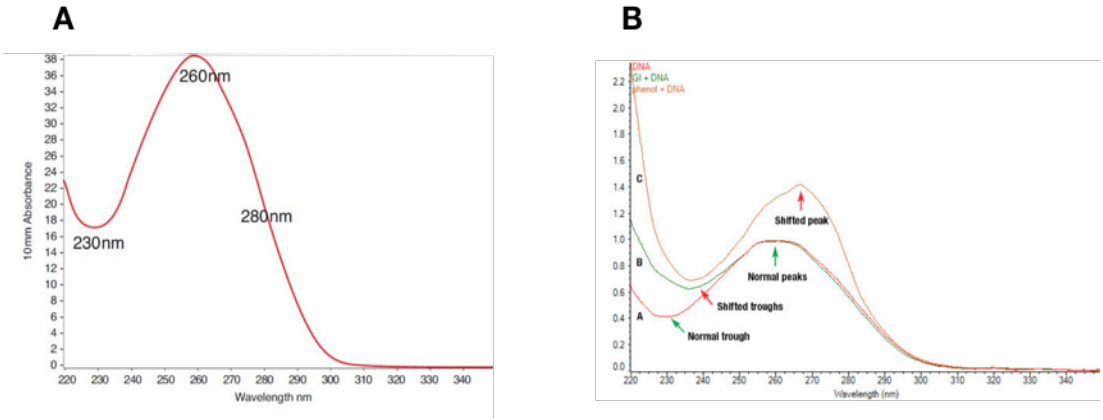

Figure 3. 7. (A) Example nucleic acid spectral image used to assess RNA purity and (B) Spectral image of samples contaminated with either guanidine or phenol

Upon measuring a sample for RNA purity and concentration, a blank sample measurement was performed by pipetting 1 µl of RNAse and DNAse free DEPC water onto the spectrophotometer for analysis. 1 µl of diluted sample was then pipetted directly onto the measurement pedestal for RNA assessment.

Method of RNA quantification

Absorbance at 260 nm was used to determine the RNA concentration given that an A_{260} reading of 1.0 is equivalent to 40 µg.ml^{-1} of RNA. The Nanodrop software used for the quantification of nucleic acid uses a modified version of the Beer-Lambert equation:

$$C = \frac{(A\ X\ \varepsilon)}{b}$$

Where:

- C is the nucleic acid concentration (ng.µl^{-1})
- A is the absorbance in AU
- ε is the wavelength-dependent extinction coefficient (ng-cm.µl^{-1}). For RNA, the wavelength-dependent extinction constant is 40nm-cm.µl^{-1}
- b is the path length in cm

Equation 3.1. The modified Beer-Lambert equation used in the current thesis to determine RNA concentration via spectrophotometric analysis

Polymerase chain reaction

For all gene expression data presented within this thesis purified RNA was diluted to 7.3 ng.µl^{-1} in 9.5 µl volume, making 70 ng total reactions (study 1) or 5.2 ng.µl^{-1} in 9.5 µl volume, making 50 ng total reactions (study 2 – in an attempt to conserve total RNA in 3/48 samples and enable the running of all genes of interest across all samples) in RNAse and DNAse free DEPC water (Thermo Fischer Scientific, UK) and amplified using specific primer sequences in a rotor-gene Q PCR machine using a one-step Quantifast SYBR Green kit (Qiagen, UK). The use of the polymerase chain reaction method allows DNA to be synthesised from a single stranded RNA template (Step 2 of Figure 3.5). This complimentary DNA is then denatured to a single stranded DNA template which is subsequently used to create two new strands of DNA. When this process is repealed for 40 cycles more than one billion copies of the original DNA segment are produced. Briefly, double stranded cDNA was first synthesised from the RNA template using dNTP nucleotides and reverse transcriptase enzymes at 50°C for 10 minutes followed by transcriptase inactivation and initial denaturation (95°C for 5 minutes). Following the initial denaturation step, complimentary DNA was then denatured to single stranded DNA at 95°C for 10 seconds allowing for subsequent annealing of primers and extension of DNA strand at 60°C for 30 seconds. The cycle of denaturation and annealing/extension was repeated for 40 cycles (Figure 3.8A). The SYBR Green mixture contains a fluorescent dye that binds to all double stranded DNA and emits fluorescence upon binding. With the use of the rotor gene software, the fluorescence signal after each cycle is quantified in real-time and the data are plotted against the cycle number to produce an amplification profile (Figure 3.8B).

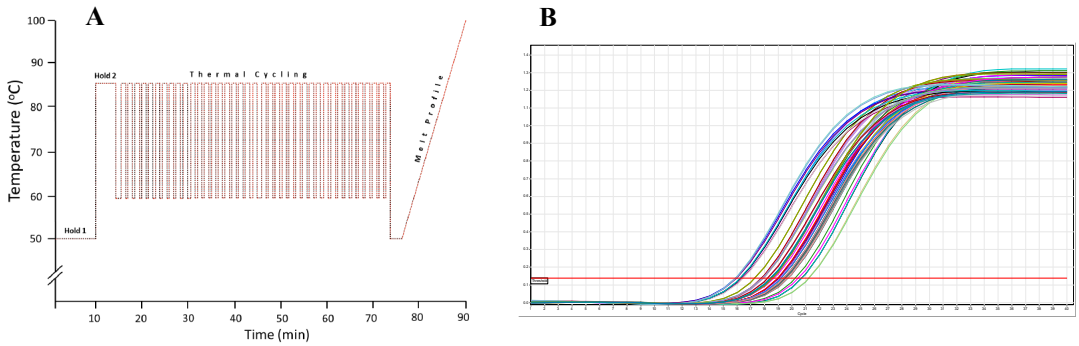

Figure 3. 8. (A) Visual representation of the polymerase chain reaction protocol used within the experiments of this thesis and (B) example of real time fluorescent detection curves.

PCR Analysis

PCR efficiency was calculated to determine the increase in the amplicon per cycle, with 100% efficiency demonstrating a perfect doubling of the amplicon at each cycle. Mean PCR efficiency of all target genes for study 1 and study 2 were 90.3 ± 3.0% and 90.1 ± 7.7% with a mean variation of 3.3% and 7.8% between samples, respectively. The mean PCR efficiency of the selected housekeeper gene for study 1 and 2 were 89.7 ± 2.7% and 93.5 ± 5.5%, respectively, with a mean variation of 3.0 and 5.9% between samples. Melt curve analysis was also performed to assess whether a single, specific product had been amplified and to allow exclusion of non-specific amplification or primer-dimer issues. In this regard, the temperature at which base-base hydrogen bonding between two DNA strands is broken depends on their length, guanine-cytosine content and their complementarity, thus, a unique melt curve will be produced for each specific DNA fragment. All melt curve analysis presented single peaks for each target gene indicating amplification of a single product (Figure 3.9).

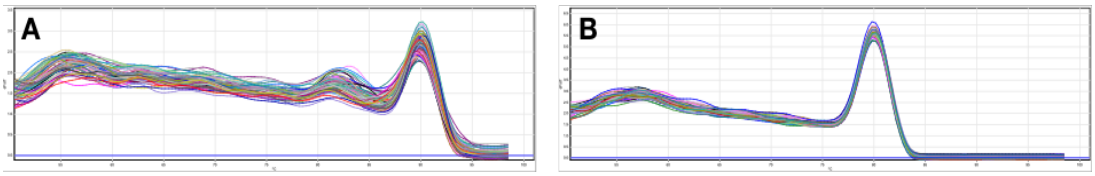

Figure 3. 9. Melt curve analysis for (A) reference gene and (B) gene of interest following RT-qPCR

Changes in mRNA content were calculated using the comparative C_t ($^{\Delta\Delta}$Ct) equation (Equation 3.2) (Schmittgen & Livak, 2008). GAPDH (study 1) or B2M (study 2) was used as the reference gene and did not change significantly between groups or time points. Mean CT values for each reference gene for each experimental study are reported in their respective experimental chapters. mRNA expression for all target genes was calculated relative to the reference

gene for the corresponding time point and normalised to a single pre-exercise time point for each individual subject which acted as the calibration condition. The specific reference sample is detailed within each respective chapter.

Equation A: $^{\Delta}$Ct = Mean Ct (target gene) - Mean Ct (housekeeper gene)
Equation B: $^{\Delta}$Ct = Mean Ct (target gene calibrator) - Mean Ct (reference gene calibtator)
Equation C: $^{\Delta\Delta}$Ct = $^{\Delta}$Ct of equation 1 - $^{\Delta}$Ct of equation 2
Equation D: $2^{\Delta\Delta}$Ct = normalised expression ratio (fold change)

Equation 3.2. Delta Delta Ct equation ($^{\Delta\Delta}$Ct) used in the current thesis to determine gene expression fold change

Primer Design

Primer sequences were designed through the use of Primer-BLAST software. Where possible, primers were designed to yield products spanning exon-exon boundaries to prevent non-specific amplification of genomic DNA containing introns which will be removed during RNA splicing. All primers were between 18 and 25 base pairs (bp) and amplified a product between 67 and 201 bp. All primers were purchased from Sigma Aldrich (Suffolk UK) with sequences for each gene presented in each respective chapter.

3.4.4 Muscle glycogen concentrations

Muscle glycogen concentration was determined using the acid hydrolysis method described by Van Loon et al (van Loon *et al.*, 2000). Approximately 1-5 mg of freeze dried tissue was powdered and dissected free of all visible non-muscle tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3 hours in a water bath set at 100°C. Samples were removed from the water bath every 45 minutes and briefly vortexed before being returned to boil. After cooling for 20 minutes at room temperature, samples were neutralised by the addition of 250 µl of 0.12 mol.L^{-1} / 2.1 mol.L^{-1} KOH, saturated with KCl and centrifuged at 500 x g for 10 minutes at 4°C. Following centrifugation, 200 µl of supernatant was frozen at -80°C and subsequently later analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, UK) as described in section 3.4.1. Glycogen concentration is expressed as mmol·kg^{-1} dw and the intra-assay coefficient of variation between duplicates was <5%.

Acid hydrolysis principle

The acid hydrolysis method allows for the cleavage of glyosidic bonds which are necessary for the attachment of glucose molecules to one another, allowing for the breakdown of di- and

polysaccharide chains into single sugar monosaccharides. Once boiled, an acid-base neutralisation reaction is performed with the addition of KOH, saturated with KCl in order to neutralise the pH of the sample.

3.4.5 Western blot analysis

Approximately 20 mg of frozen tissue was homogenised in 200 µl of ice-cold RIPA buffer (50 mM HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.1% SDS) containing protease and phosphatase inhibitors (Pierce, Thermo Scientific) and agitated end-over-end for 1 h at 4°C before centrifugation at 14,000 x g for 10 min. Following protein quantification, each sample was diluted with equal volumes of 4X Laemmli buffer and distilled water (dH^2O). to a concentration of 2 $\mu g.\mu l^{-1}$ and boiled for 5 min at 95°C.

Subcellular fractionation of skeletal muscle tissue

Isolation of nuclear and cytosolic fractions was performed on skeletal muscle samples obtained from study 2 based on the method described by Dimauro *et al.* (2012). Approximately 20 mg of frozen tissue was homogenised in 300 µl of STM buffer (comprised of 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ and 10X protease and phosphatase inhibitor cocktail). The homogenate was then centrifuged at 800 x g for 15 minutes at 4°C. The resulting supernatant was removed into a separate Eppendorf tube for subsequent isolation of the cytosolic fraction whilst the pellet was resuspended in 300 µl of STM buffer and centrifuged at 500 x g for 15 minutes at 4°C. The nuclear pellet was again resuspended in 300 µl of STM buffer and centrifuged at 1,000 x g for 15 minutes at 4°C with the supernatant discarded. The nuclear pellet was subsequently resuspended in 200 µl of NET buffer (comprised of 20 mM HEPES pH 7.9, 1.5 mM $MgCl^2$, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% 100X Triton and 10X protease and phosphatase inhibitor cocktail), kept on ice for 30 min before being sonicated for 1 min. Finally, lysates were centrifuged at 9,000 x g for 30 min at 4°C with the supernatant removed to a new tube, labelled as the nuclear fraction and stored at -80°C prior to protein quantification. Cytosolic fractions were isolated by centrifuging the supernatant, achieved from the first centrifugation step, at 800 x g for 10 minutes at 4°C. The supernatant was again centrifuged at 11,000 x g for 10 minutes at 4°C, with the resultant supernatant precipitated in 1.2 ml ice-cold 100% acetone at -20°C for 1 h followed by centrifugation at 12,000 x g for 5 min at 4°C. The resultant pellet was then resuspended in 100 µl STM buffer, labelled as the cytosolic fraction and stored at -80°C prior to protein quantification.

Validation of the purity of the nuclear and cytosolic fractions was determined by the presence of either histone 3 (H3) (nuclear) and lactate dehydrogenase (LDH) (cytosolic) 'house-keeping' protein markers, respectively, via SDS-PAGE and western blotting.

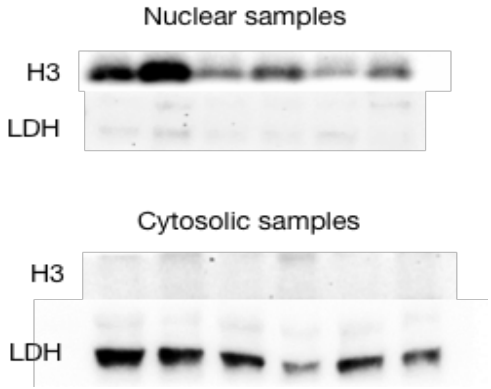

Figure 3. 10. Representative western blot images for H3 and LDH in both nuclear and cytosolic fractionated samples.

Protein quantification

Total protein concentrations for each sample were determined using the bicinchoninic acid (BCA) assay method.

BCA assay principle

The BCA assay is a colorimetric technique used for the quantification of total protein within a sample. The method works via the reduction of a Cu^{2+} ion to Cu^{1+} which is then chelated by BCA and results in the formation of a purple coloured reaction product. This reaction product demonstrates strong absorbance at 520 nm which demonstrates a near linear relationship with increasing protein concentrations.

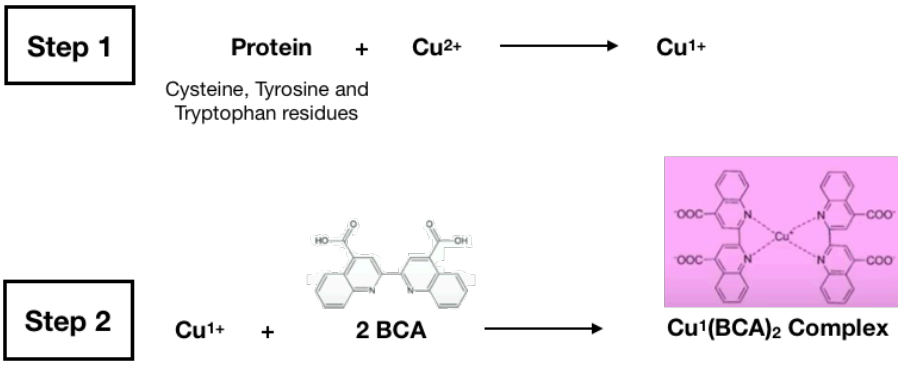

Figure 3. 11. An overview of the Biuret reaction

Preparation of standards and reagents for BCA assay

Bovine serum albumin (BSA) standards were prepared at concentrations of 2, 1.5, 1, 0.75, 0.5, 0.25 and 0.125 $mg.ml^{-1}$ through the serial dilatation of 2 mg BSA with dH^2O.

The BCA reagents were purchased as part of a commercially available BCA protein assay kit (Pierce, Rockford, USA) which contains two reagents; A & B. Reagent B (4% cupric sulphate) was thoroughly mixed with reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M of sodium hydroxide) in a trough at a ratio of 1:50.

BCA assay method

Ten µl of standard, samples and a blank (dH^2O) were pipetted in triplicate into a 96-well plate. Two hundred µl of the working agent was subsequently added to all wells using a multichannel pipette (excluding the blank) and the plate was incubated at 37°C for 30 min. Following incubation, the absorbance was recorded at 570 nM using a Clariostar plate reader (BMG Lab Tech, Germany). A standard curve was generated by plotting the average blank-corrected 570 nM measurement of each standard against its known concentration in $mg.ml^{-1}$. Sample concentrations were subsequently calculated from the standard curve.

SDS-PAGE & Western Blotting principle

SDS polyacrylamide gel electrophoresis (PAGE) allows for the separation of proteins according to their molecular weight via the application of an electrical charge running between the anode (+) and cathode (-) chambers whilst proteins are subsequently transferred from the gel to a nitrocellulose membrane. Following protein transfer, the membrane is 'blocked' with blocking agents (either BSA or non-fat milk) in order to block all areas of the membrane that do not contain any protein in order to reduce any non-specific binding of primary antibodies and reduce overall background signal. Primary antibodies bind to specific portions of the protein of interest or epitopes whereas secondary antibodies bind to the fragment crystallizable (Fc) region of the primary antibody to allow for subsequent detection (Bass *et al.*, 2017). Secondary antibodies are required for the detection of primary antibodies bound to the target antigen as primary antibodies are not typically conjugated to a label (i.e. conjugated to a reported function such as horseradish peroxidase) for detection. Furthermore, multiple secondary antibodies can bind to a single primary antibody, thus amplifying the detectable signal (Bass *et al.*, 2017). Upon detection, the luminol based chemiluminescence substrate is applied to the membrane and, in the presence of HRP (from the secondary antibody) and peroxide, results in the oxidation of luminol and forms a product that emits light at 425 nm. This light is then captured by specialist digital imaging cameras to produce western blot bands.

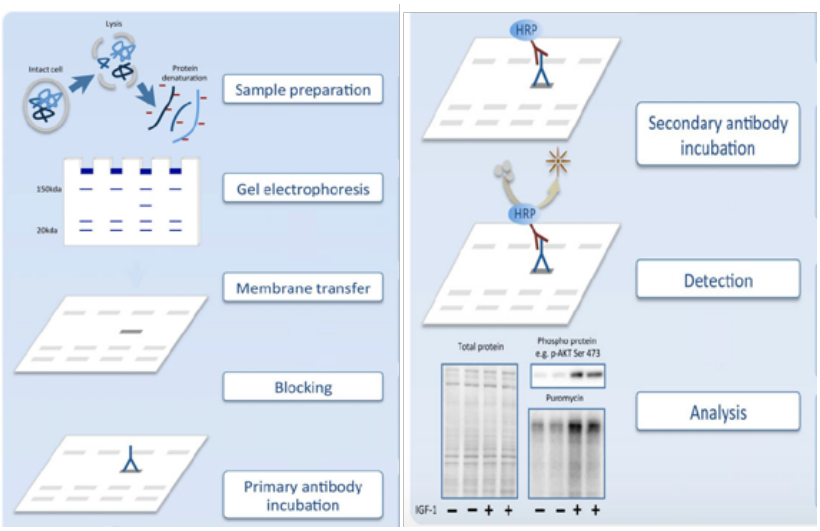

Figure 3. 12. An overview of the sequential stages of the Western blot process

SDS-PAGE and western blotting

Equivalent amounts of protein (20-30 µg) from each sample was separated by gel electrophoresis is 12% TGX Precast Midi Protein gels (Bio-Rad Laboratories, Germany) by SDS-page (15 minutes at 100V, followed by 60 minutes at 150V). Proteins were subsequently semi-dry transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Germany) for 30 minutes at 30V in transfer buffers. Following transfer, membranes were imaged using stain-free technology (Bio-Rad Laboratories, Germany) to check transfer efficiency and subsequently blocked for 1 hour at room temperature in Tris-buffered saline (TBST 0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1% Tween-20) with 5% milk. Membranes were then incubated overnight at 4°C in primary antibodies (Cell Signalling, UK) at concentrations of 1:1000 in 1X TBST. The subsequent morning, membranes were washed for 3 x 5 min in TBST and incubated in anti-species horseradish peroxidase-conjugated secondary antibody at concentrations of 1:1000-10000 (Polyclonal Goat Anti-Rabbit HRP, Cell Signalling, UK) in TBST with 2.5% milk for 1 h at room temperature. Membranes were again washed in TBST for a further 3 x 5 min and finally exposed in a chemiluminescence solution (Pierce ECL Western Blotting Substrate, Thermo Scientific, UK) and visualised using a Bio-Rad Chemi-doc system whilst band densities were determined semi-quantitatively using ImageLab software (Bio-Rad Laboratories, Germany). The volume density of each target band was normalised to the total amount of protein loaded into each lane using Stain-Free technology (Bio-Rad Laboratories, Germany) with each phosphoprotein normalised to its respective total protein (Figure 3.13).

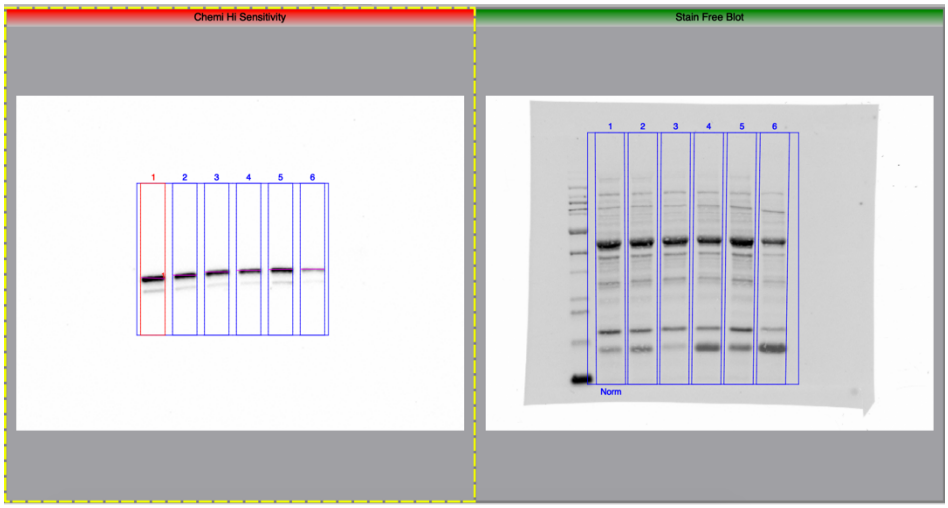

Figure 3. 13. An overview of the quantification method used for band densitometry analysis.

3.4.6 Statistical analysis

All statistical analyses were performed using the statistical package for social sciences (SPSS, Version 24, IBM, USA). For the comparison of multiple groups means, an analysis of variance (ANOVA) was used. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post-hoc tests in order to locate specific differences. All data in text, figures and tables are presented as means ± standard deviation (SD) with *P* values ≤ 0.05 indicating statistical significance. All figures were designed in Prism (Version 8, Graphpad, USA).

Chapter Four:

Graded reductions in pre-exercise muscle glycogen impair exercise capacity but do not augment skeletal muscle cell signalling

This work was presented at the International Sport, Exercise & Nutrition (ISENC) conference, Newcastle, 2017 and is published in The Journal of Applied Physiology (2019).

Hearris MA, Hammond KM, Seaborne RA, Stocks B, Shepherd SO, Philp A, Sharples AP, Morton JP & Louis JB (2019). Graded reductions in pre-exercise muscle glycogen impair exercise capacity but do not augment skeletal muscle cell signalling: implications for CHO periodisation. J Appl Physiol 126, 1587-1597.

4.1 Abstract

We examined the effects of graded muscle glycogen on exercise capacity and the modulation of skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis. In a repeated measures design, eight males completed a sleep-low, train-low model comprising an evening glycogen depleting cycling protocol followed by an exhaustive exercise capacity test (8 x 3 min at 80% PPO, followed by 1 min efforts at 80% PPO until exhaustion) the subsequent morning. Following glycogen depleting exercise, subjects ingested a total of 0 $g.kg^{-1}$ (L-CHO), 3.6 $g.kg^{-1}$ (M-CHO) or 7.6 $g.kg^{-1}$ (H-CHO) of carbohydrate during a 6 h period prior to sleeping, such that exercise was commenced the next morning with graded ($P < 0.05$) muscle glycogen concentrations (Mean ± SD) (L-CHO: 88 ± 43, M-CHO: 185 ± 62, H-CHO: 278 ± 47 $mmol \cdot kg^{-1}$ dw). Despite differences ($P < 0.05$) in exercise capacity at 80% PPO between trials (L-CHO: 18 ± 7, M-CHO: 36 ± 3, H-CHO: 44 ± 9 min) exercise induced comparable $AMPK^{Thr172}$ phosphorylation (~4 fold) and PGC-1α mRNA expression (~5 fold) post- and 3 h post-exercise, respectively. In contrast, exercise nor CHO availability affected the phosphorylation of $p38MAPK^{Thr180/Tyr182}$, $CaMKII^{Thr268}$ or mRNA expression of p53, Tfam, CPT-1, CD36 or PDK4. Data demonstrate that when exercise is commenced with muscle glycogen below 300 $mmol \cdot kg^{-1}$ dw, further graded reductions of 100 $mmol \cdot kg^{-1}$ dw impairs exercise capacity but does not augment skeletal muscle cell signalling.

4.2 Introduction

Skeletal muscle glycogen is recognised as the predominant energy substrate used during intense aerobic exercise (Hawley & Leckey, 2015) and plays an important role in regulating the capacity to sustain exercise at a given workload above 60% $\dot{V}O_{2max}$ (Bergström *et al.*, 1967; Hermansen *et al.*, 1967; Hawley *et al.*, 1997). Additionally, muscle glycogen acts as a regulatory molecule (Philp *et al.*, 2012) that is able to modulate cell signalling and transcriptional responses to exercise and subsequently augment selected skeletal muscle markers of training adaptation (e.g succinate dehydrogenase, citrate synthase and β-hydroxyacyl-CoA-dehydrogenase enzyme activity and cytochrome c oxidase subunit IV content). Most notably, exercise commenced with reduced muscle glycogen (as defined as a 'train-low' session (Stellingwerff *et al.*, 2019)) augments the AMPK-PGC-1α signaling axis (Steinberg *et al.*, 2006; Yeo *et al.*, 2010; Bartlett *et al.*, 2013; Psilander *et al.*, 2013) and results in the augmented expression of target genes with putative roles in the regulation of mitochondrial biogenesis and substrate utilisation (Pilegaard *et al.*, 2002; Bartlett *et al.*, 2013; Lane *et al.*, 2015). Whilst a multitude of research designs have been used to study the physiological and molecular responses to 'train-low' exercise, the recently developed 'sleep-low, train-low' model (which requires athletes to perform an evening training session, restrict carbohydrate (CHO) during overnight recovery, and then complete a fasted training session the subsequent morning) provides a potent strategy to augment mitochondrial related cell signalling (Bartlett *et al.*, 2013; Lane *et al.*, 2015; Burke *et al.*, 2018). Furthermore, repeated bouts of sleep-low, train-low is the only train-low model shown to enhance performance in trained endurance athletes (Marquet *et al.*, 2016*a*, 2016*b*).

Given the enhanced training response associated with the sleep-low, train-low model is potentially regulated by muscle glycogen availability, it is prudent to consider the absolute glycogen concentrations required to facilitate this response. In this regard, examination of available data demonstrate that the augmented signalling and transcriptional responses associated with train-low models are particularly apparent when absolute pre-exercise muscle glycogen concentrations are ≤300 mmol·kg^{-1} dw (Impey *et al.*, 2018). 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 accordance with data derived from acute exercise protocols, the notion of a glycogen threshold is also apparent when investigating selected skeletal muscle markers of training adaptation (Impey *et al.*, 2018). For example, train-low sessions commenced with glycogen concentrations < 300 mmol·kg^{-1} dw (Hansen *et al.*, 2005; Yeo *et al.*, 2008; Morton *et al.*, 2009) result in augmented oxidative enzyme activity and/or content following 3-10 weeks of training. 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). Nonetheless, whist training with low muscle glycogen augments selected signalling events, absolute training volume (Impey *et al.*, 2016) and/or intensity (Yeo *et al.*, 2008; Hulston *et al.*, 2010; Lane *et al.*, 2013) may be reduced due to a lack of muscle substrate and/or an impairment in the contractile apparatus of skeletal muscle (Ørtenblad *et al.*, 2011; Gejl *et al.*, 2014). When taken together, the challenge that exists is to therefore facilitate the pro-signalling environment whilst simultaneously maintaining the ability to complete the desired workload and intensity in order to promote training adaptation.

As such, the aim of the present study was to examine the effects of graded pre-exercise glycogen concentrations on both exercise capacity and the modulation of selected skeletal muscle signalling pathways with putative roles in the regulation of mitochondrial biogenesis. This model of graded pre-exercise muscle glycogen was achieved through a sleep-low, train-low model that adopted CHO intakes considered practically viable (within the time-course of sleep-low designs) and representative of real-world refeeding strategies. Whilst the use of such sleep-low, train-low models are primarily designed for athletic populations, the use of recreational populations allows for a greater understanding of the molecular events that occur in response to such 'train-low' designs, given the difficulties of collecting muscle biopsies from elite athletes. It was hypothesised that the activation of skeletal muscle signalling pathways would be proportionally dependent on pre-exercise muscle glycogen concentrations.

4.3 Methods

4.3.1 Participants

Eight recreationally active males (mean ± SD: age, 22 ± 3 years; body mass 76.0 ± 12.7 kg; height, 177.9 ± 5.7 cm) took part in this study. Mean $\dot{V}O_{2peak}$ and peak power output (PPO) for the cohort were 48.9 ± 7.0 mL kg^{-1} min^{-1} and 273 ± 21 W, respectively. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All subjects provided written informed consent and all procedures conformed to the standards set by the Declaration of Helsinki (2008). The study was approved by the local Research Ethics Committee of Liverpool John Moores University.

4.3.2 Experimental Design

Using a sleep-low, train-low model and a repeated measures design, with each experimental trial separated by a minimum of 7 days, subjects undertook an evening bout of glycogen depletion exercise followed by the consumption of graded quantities of CHO (L-CHO: 0 g, M-CHO: 3.6 g kg^{-1}, H-CHO: 7.6g kg^{-1}) across a 6 h period, so that exhaustive exercise was commenced the next morning with three different levels of pre-exercise muscle glycogen concentrations. Skeletal muscle biopsies were obtained from the vastus lateralis immediately before, post- and 3 h post-exercise. During the H-CHO and M-CHO trials, an additional muscle biopsy was obtained at a matched time point corresponding to the point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials. Consequently, all subjects completed the L-CHO trial first, whilst the subsequent M-CHO and H-CHO trials were completed in a randomised and counterbalanced order. An overview of the experimental protocol is shown in Figure 4.1.

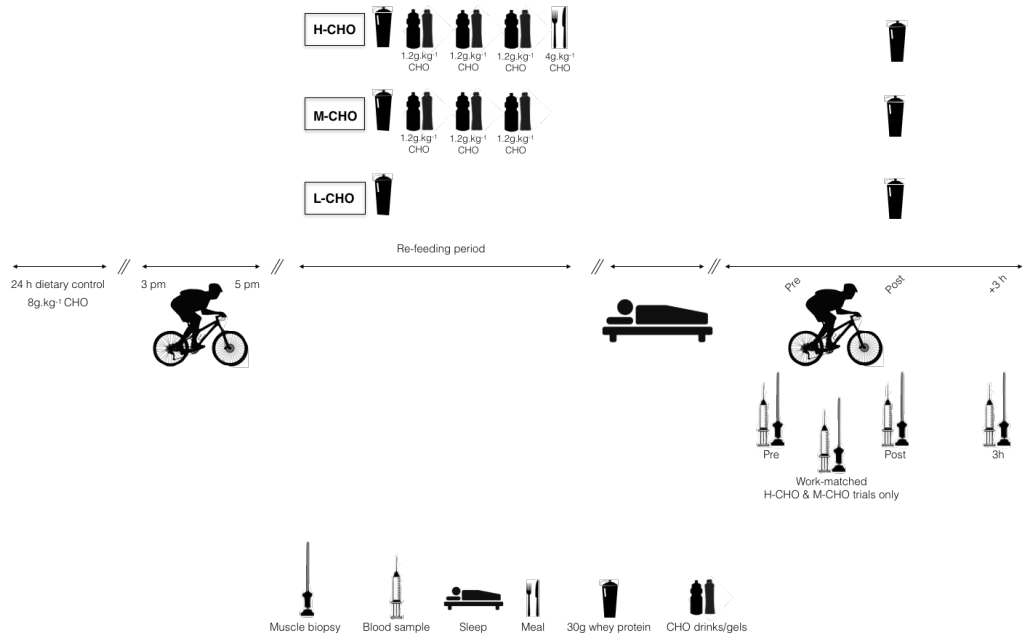

Figure 4. 1. Schematic overview of the experimental sleep-low, train-low protocol. Following 24 h of standardised dietary conditions, subjects completed an evening bout of glycogen depleting cycling exercise. Upon completion, subjects received three graded levels of CHO in order to manipulate pre-exercise muscle glycogen the subsequent morning. Following an overnight fast, subjects completed an exhaustive bout of cycling exercise. Muscle biopsies were obtained pre-exercise, at the point of exhaustion (post exercise) and 3 h post exercise. During H-CHO and M-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials.

4.3.3 Assessment of peak oxygen uptake

At least 7 days prior to experimental trials, all subjects were assessed for peak oxygen consumption ($\dot{V}O_{2peak}$) and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Following the completion of a 10 min warm-up at 75 W, the test began at 100 W and consisted of 2 minute stages with 30 W increments in resistance until volitional exhaustion. $\dot{V}O_{2peak}$ was stated as being achieved by the following end-point criteria: (1) heart rate within 10 beats min^{-1} of age-predicted maximum, (2) respiratory exchange ratio > 1.1 and (3) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

4.3.4 Overview of sleep-low, train-low model

Phase 1: Glycogen depletion exercise

In the 24 h preceding glycogen depleting exercise (i.e. from 12 pm the day prior), subjects received a standardised high CHO diet (8 g kg^{-1} CHO, 2 g kg^{-1} protein and 1 g kg^{-1} fat) and refrained from alcohol and vigorous physical exercise for the previous 48 h. The standardised diet was provided to subjects and consisted of 3 main meals and 3 CHO rich snacks, with subjects required to stop eating 3 h prior to commencing glycogen depleting exercise. On the day of glycogen depleting exercise, subjects reported to the laboratory at approximately 3 pm to perform a bout of intermittent glycogen depleting cycling, as previously completed in our laboratory (Taylor *et al.*, 2013; Impey *et al.*, 2016). Following a self-selected warm-up, subjects cycled for 2 min at 90% PPO, followed immediately by a 2 min recovery period at 50% PPO. This work to rest ratio was repeated until subjects could do longer sustain the workload at 90% PPO for 2 min at which point, exercise intensity was reduced to 80% PPO with the same work to rest ratio. When subjects were unable to maintain this intensity, it was reduced to 70% PPO and finally 60% PPO, maintaining the same work to rest ratio throughout. When subjects were unable to maintain the workload at 60% PPO for 2 min the exercise protocol was terminated. The pattern of exercise and total time to exhaustion in the subject's initial trial was recorded and replicated in all subsequent trials. Subjects were permitted to consume water *ad libitum* during exercise, with the pattern of ingestion replicated during subsequent trials.

Phase 2: Carbohydrate re-feeding strategy

To facilitate the overnight sleep-low model, subjects were fed 30 g of whey protein isolate (Science in Sport, Nelson, UK) mixed with 500 ml of water immediately following the cessation of glycogen depleting exercise to reflect real-world practice as per current nutritional guidelines (Thomas *et al.*, 2018). Subjects in the L-CHO trial then refrained from eating for the remainder of the evening whereas subjects within the M-CHO and H-CHO trials were

provided with a mixture of CHO drinks (Maltodextrin, Science in Sport, Nelson UK) and gels (GO isotonic energy gel, Science in Sport, Nelson UK) to be consumed at hourly intervals. In the M-CHO trial, subjects were provided with CHO at a rate of 1.2 g kg^{-1} h^{-1} for 3 h whereas subjects in the H-CHO trial were provided with 1.2 g kg^{-1} h^{-1} for 3 h followed by a high carbohydrate meal (4 g kg^{-1} CHO, 51 ± 1 g protein and 17 ± 1 g fat) consisting of bread, soup, rice, fresh juice, rice pudding and jam after 4 h of recovery. In this way, total CHO intakes in the L-CHO, M-CHO and H-CHO trials equated to 0, 3.6 and 7.6 g kg^{-1}, respectively, with fluid intake allowed *ad libitum.*

Phase 3: High intensity interval cycling and exercise capacity test

To facilitate the train-low exercise session, subjects arrived the subsequent morning between 8-9 am, in a fasted state, where a venous blood sample was collected from the antecubital vein and a muscle biopsy taken from the vastus lateralis. Subjects then completed the high-intensity interval (HIIT) cycling protocol, consisting of 8 x 3 min intervals at 80% PPO, interspersed with 1 min rest. During exercise, heart rate (HR) was continuously measured and the final HR for each 3 min interval was recorded, whilst ratings of perceived exertion (RPE) were recorded upon completion of each interval. Expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 1.5 min of each interval and substrate utilisation was assessed using the equations of Jeukendrup & Wallis (Jeukendrup & Wallis, 2005) given the validity of indirect calorimetry for the assessment of substrate utilisation at exercise intensities up to 80-85% $\dot{V}O_{2max}$ (Romijn *et al.*, 1992). Upon completion of the high-intensity cycling protocol, subjects were provided with 5 min of active recovery prior to commencing an exercise capacity test consisting of intermittent "1 min efforts" corresponding to 80% PPO interspersed with 1 min recovery periods at 40% PPO. This intermittent protocol was followed until the subjects reached volitional exhaustion and has been previously utilised in our laboratory (Impey *et al.*, 2016).

4.3.5 Blood analysis

Venous blood samples were collected in vacutainers containing K_2EDTA, lithium heparin or serum separation tubes and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4°C. Samples were collected immediately prior to exercise, at the point of exhaustion (post exercise) and 3 h post exercise, whilst an additional sample was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial during the M-CHO and H-CHO trials. Plasma was aliquoted and stored at -80°C until analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol using

commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, UK) as per manufacturer instructions.

4.3.6 Muscle biopsies

Skeletal muscle biopsies (~60 mg) were obtained from the vastus lateralis immediately prior to exercise, at the point of exhaustion (post exercise) and 3 h post exercise. During the M-CHO and H-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial, thereby allowing for 'work-matched' comparison between trials. For the work-matched biopsy, subjects dismounted the cycle ergometer and were moved to the adjacent biopsy suite. Following collection of the biopsy sample (~5 min), subjects recommenced cycling exercise. Muscle biopsies were obtained from separate incision sites 2-3 cm apart using a Bard Monopty Disposable Core Biopsy Instrument (12 gauge x 10 cm length, Bard Biopsy Systems, Tempe, AZ, USA) under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80ºC for later analysis.

4.3.7 Muscle glycogen concentration

Muscle glycogen concentrations were determined according to the acid hydrolysis method described by Van Loon et al (van Loon *et al.*, 2000). Approximately 2-5 mg of freeze-dried tissue was powdered, dissected of all visible blood and connective tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3 h at 95ºC. After cooling to room temperature, samples were neutralised by the addition of 250 µl 0.12 mol L^{-1} Tris/2.1 mol L^{-1} KOH saturated with KCl. Following centrifugation, 200 µl of 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 and intra-assay coefficients of variation were <5%.

4.3.8 RNA isolation and analysis

Muscle samples (~20 mg) were homogenised in 1 ml TRIzol reagent (Thermo Fisher Scientific, UK) and total RNA isolated according to the manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at optical densities (OD's) of 260 and 280 nm, using a Nanodrop 3000 (Fisher, Rosklide, Denmark) with an average 260/280 ratio of 1.9 ± 0.1. A quantity of 70 ng RNA was used for each 20 µl PCR reaction.

4.3.9 Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR)

RT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one-step kit on a Rotogene 300Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA). RT-qTR-PCR was performed as follows: hold 50ºC for 10 min (reverse transcription/cDNA synthesis), 95ºC for 5 min (transcriptase inactivation and initial denaturation step), and PCR steps of 40 cycles; 95ºC for 10 sec (denaturation), 60ºC for 30 sec (annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). Following initial screening of suitable reference genes, GAPDH showed the most stable C_t values across all RT-PCR runs and subjects, regardless of experimental condition (25.3 ± 1.0) and was therefore selected as the reference gene in all RT-PCR assays. The average PCR efficiency for all RT-PCR runs (90 ± 2%) was similar for all genes across all time points and experimental conditions. As such, the relative gene expression levels were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (Schmittgen & Livak, 2008) where the relative expression was calculated as $2^{-\Delta\Delta ct}$ where C_t represents the threshold cycle. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH) within the same subject and condition and relative to the pre-exercise value in the H-CHO condition.

Table 4. 1. Primers used for real-time RT-PCR

Gene	Forward primer	Reverse primer
PGC-1	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCCTCTCTGCT
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
Tfam	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA
CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGGTTCTTATTCACA
PDK4	TGGTCCAAGATGCCTTTGAGT	GTTGCCCGCATTGCATTCTT
CPT1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG
GAPDH	AAGACCTTGGGCTGGGACTG	TGGCTCGGCTGGCGAC

4.3.10 SDS page & Western blotting

Muscle samples were prepared, separated by SDS-PAGE and transferred to nitrocellulose membranes according to the method outlined in section 3.4.5. Following transfer, membranes were stained for protein with Ponceau S (Sigma-Aldrich, Gillingham, UK), blocked in TBS-Tween containing 3% non-fat milk for 1 h and incubated overnight in primary antibodies (1:1000) (AMPKα (2603), p-AMPK^{Thr172} (2531), ACC (3676), p-ACC^{Ser79} (3661), p38MAPK (9212), p-p38MAPK$^{Thr180/Tyr182}$ (4511), CaMKII (3362) and p-CaMKII^{Thr268} (12716) from Cell Signaling Technologies), before incubation in relevant secondary antibodies (1:2000) (anti-

rabbit (7074) from Cell Signaling Technologies) for 1 h at room temperature. Proteins were detected via chemiluminescence (Millipore, Watford, UK) and quantified by densitometry as outlined in section 3.4.5.

4.3.11 Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS Version 24). Comparison of average physiological responses and exercise capacity were analysed using a one-way repeated-measures general linear model whereas changes in physiological and molecular responses between conditions (i.e. muscle glycogen, mRNA expression and activity of signalling molecules) were analysed using a two-way repeated measures general linear model, where the within factors were time and condition. Here, the post-exercise sampling point in the L-CHO trial was also used as the work-matched sampling point as this corresponded to the same sampling point and allowed for comparison between trials. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post-hoc tests in order to locate specific differences. All data in text, figures and tables are presented as means ± SD with P values ≤ 0.05 indicating statistical significance.

4.4 Results

4.4.1 Skeletal muscle glycogen and exercise capacity

The exercise and nutritional strategy employed was successful in achieving graded levels of pre-exercise muscle glycogen (H-CHO; 278 ± 47, M-CHO; 185 ± 62, L-CHO; 88 ± 43 mmol·kg^{-1} dw) such that exercise was commenced with three distinct levels of muscle glycogen (P = 0.016) (Figure 2A). Exhaustive exercise significantly reduced (P < 0.001) muscle glycogen concentration to comparable levels (<100 mmol·kg^{-1} dw) with no difference between conditions (P = 0.11). In accordance with the observed differences in pre-exercise muscle glycogen concentration, total exercise time spent at 80% PPO in the H-CHO trial (44 ± 9 min) was significantly greater than both M-CHO (36 ± 3 min) (P = 0.037) and L-CHO (18 ± 6 min) trials (P < 0.001) whilst the M-CHO trial was significantly greater than the L-CHO trial (P < 0.001) (Figure 2B). Given the low pre-exercise muscle glycogen concentration of subjects in the L-CHO trial, 6 of the 8 subjects were unable to complete the prescribed HIIT protocol. As such, exercise capacity data is presented as the total amount of time spent at 80% PPO and is inclusive of the exercise performed during the prescribed HIIT protocol and subsequent capacity test and excludes all time spent at rest/recovery.

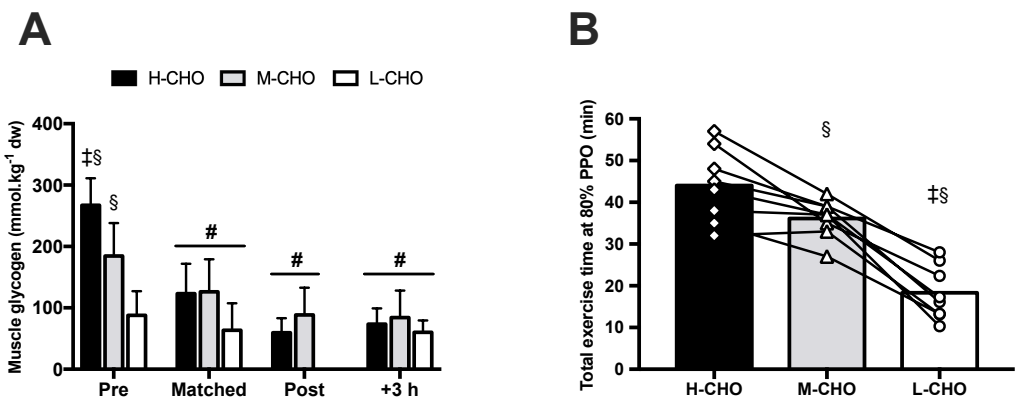

Figure 4. 2. (A) Skeletal muscle glycogen concentration and (B) Exercise capacity at 80% PPO (reflective of set work protocol plus time to exhaustion). $^{\#}P < 0.05$, significantly different from pre-exercise, §$P < 0.05$, significantly different from H-CHO, ‡$P < 0.05$, significantly different from M-CHO. Data is presented as means and individual data points represent individual subjects.

4.4.2 Physiological and metabolic responses to exercise

Subject's average heart rate (Figure 3A) across the HIIT session, when matched for work done (H-CHO; 163 ± 16, M-CHO; 167 ± 15, L-CHO; 171 ± 17 beats.min^{-1}) was significantly higher in the L-CHO trial compared with H-CHO ($P = 0.031$) only. Similarly, subject's average RPE (Figure 3B) across the HIIT session (H-CHO; 13 ± 1, M-CHO; 14 ± 1, L-CHO; 16 ± 1 arbitrary units) was significantly higher in the L-CHO trial compared with both M-CHO ($P = 0.041$) and H-CHO ($P = 0.012$) trials, respectively. Exhaustive exercise resulted in a significant reduction in plasma glucose ($P = 0.036$), where plasma glucose was significantly lower in the L-CHO trial when compared with the H-CHO trial only ($P = 0.015$) (Figure 3C). Exhaustive exercise resulted in a significant increase in plasma lactate ($P = 0.001$), NEFA ($P < 0.001$) and glycerol ($P = 0.012$) but did not display any significant differences between trials (Figures 3D, E and F, respectively). However, when matched for work done, plasma NEFA ($P = 0.01$) and plasma glycerol ($P = 0.017$) was increased to a significantly greater extent in the L-CHO trial when compared with the H-CHO trial only. In addition, subjects in the L-CHO trial oxidised significantly less CHO ($P = 0.048$) and greater amounts of lipid ($P = 0.004$) during the HIT exercise when compared with the H-CHO trial only (Figure 3G and H, respectively).

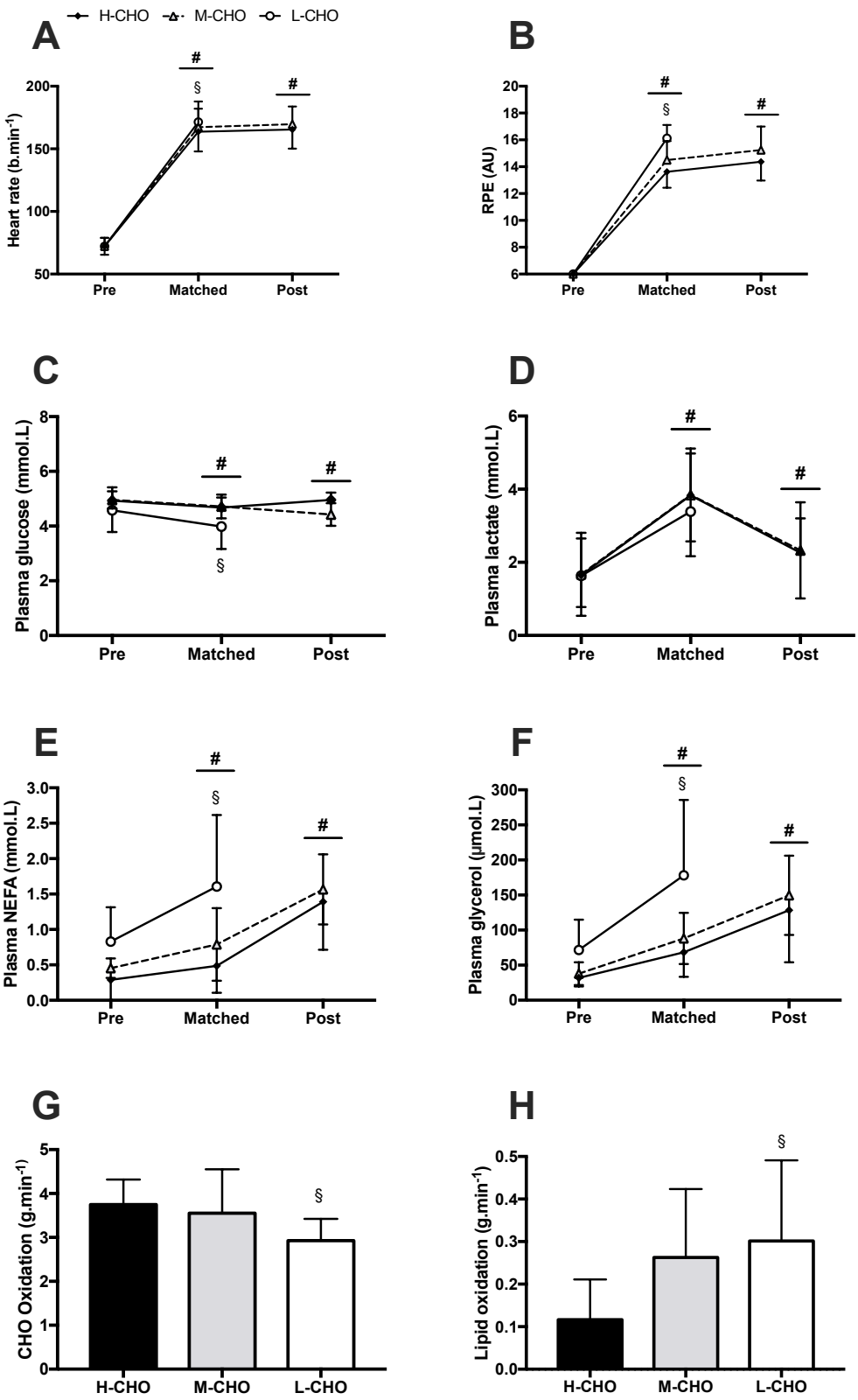

Figure 4. 3. (A) Heart rate, (B) RPE and plasma (C) Glucose, (D) Lactate, (E) NEFA (F) glycerol pre-exercise, at work-matched time points and post-exercise, (E) Average rate of CHO and (F) lipid oxidation during exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, §$P < 0.05$, significantly different from H-CHO. Data is presented as mean ± SD (A-D) and individual data points represent individual subjects (E & F).

4.4.3 Regulation of mitochondrial biogenesis related cell signalling

Exhaustive exercise induced significant increases in $AMPK^{Thr172}$ phosphorylation ($P = 0.017$) but did not display any significant differences between trials ($P = 0.548$) (Figure 4A). Similarly, exhaustive exercise induced significant increases in ACC^{Ser79} phosphorylation ($P = 0.005$), although phosphorylation was higher in the M-CHO trial when compared with the L-CHO trial only ($P = 0.021$) (Figure 4B). When exercise duration was matched to the post-exercise sampling point in the L-CHO group, the increase in $AMPK^{Thr172}$ phosphorylation remained comparable between groups ($P = 0.269$) and the increase in ACC^{Ser79} phosphorylation still remained higher in the M-CHO trial when compared with the L-CHO trial ($P = 0.021$). In contrast, exhaustive exercise did not induce phosphorylation of $p38MAPK^{Thr180/Tyr182}$ ($P = 0.656$) (Figure 4C) or $CaMKII^{Thr286}$ ($P = 0.707$) (Figure 4D) ($P < 0.05$). Representative Western blots are shown in Figure 4E. With regard to exercise induced gene expression, exhaustive exercise induced a significant increase in PGC-1α mRNA expression at 3 h post-exercise ($P = 0.001$) but did not display any significant differences between trials (Figure 5A). In contrast, p53, Tfam, CPT-1, CD36 and PDK4 mRNA expression (Figures 5B, C, D, E, F, respectively) was unaffected by either glycogen availability or the exhaustive exercise protocol ($P > 0.05$).

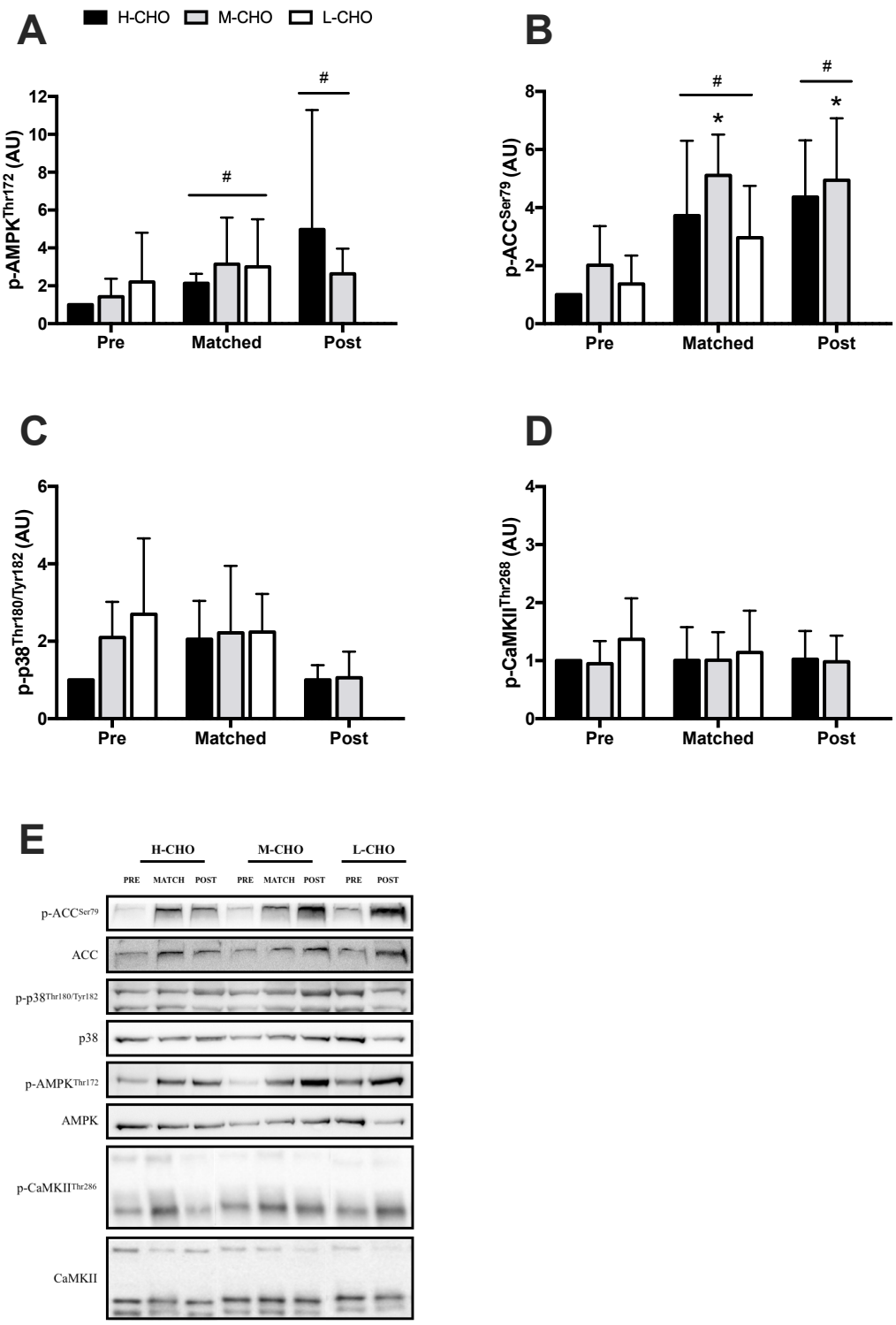

Figure 4. 4. (A) AMPK^{Thr172} phosphorylation, (B) ACC^{Ser79} phosphorylation, (C) p38$^{Thr180/Tyr182}$ phosphorylation, (D) CaMKII^{Thr286} phosphorylation and (E) representative Western blot images at pre-exercise, work-matched time point and post-exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, $^{*}P < 0.05$, significantly different from L-CHO. Data is presented as means and individual data points represent individual subjects.

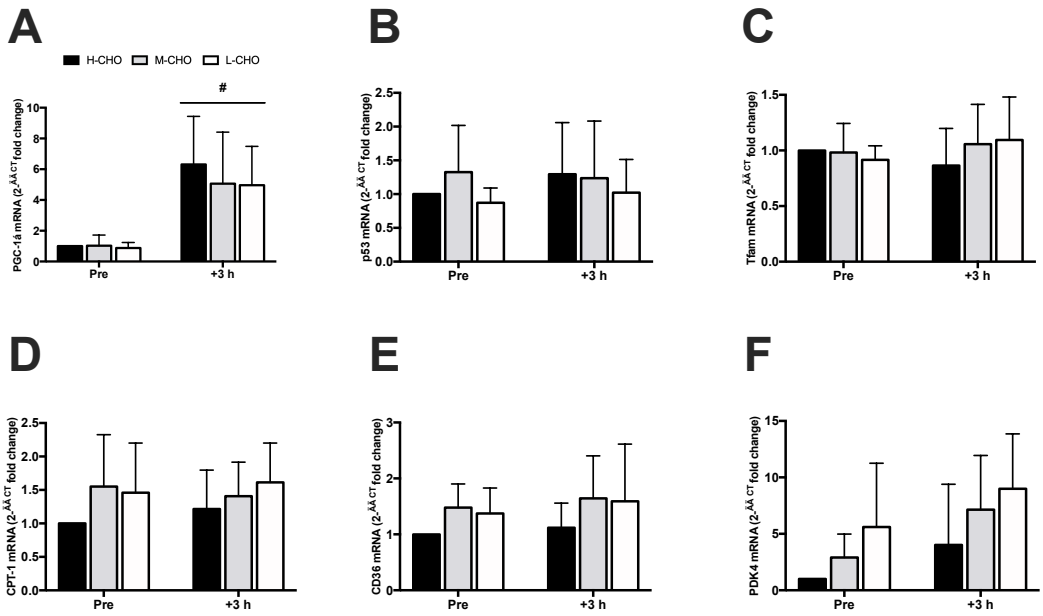

Figure 4. 5. (A) PGC-1α, (B) p53, (C) Tfam, (D) CPT-1, (E) CD36 and (F) PDK4 mRNA expression pre- and 3 h post-exercise. $^{\#}P < 0.05$, significantly different from pre-exercise. Data is presented as means and individual data points represent individual subjects.

4.5 Discussion

Using a sleep-low, train-low model, we examined the effects of three distinct levels of pre-exercise muscle glycogen on exercise capacity and the modulation of selected skeletal muscle signalling pathways with putative roles in mitochondrial biogenesis. We provide novel data by demonstrating that 1) graded reductions in pre-exercise muscle glycogen of 100 mmol·kg^{-1} dw reduce exercise capacity at 80% PPO by ~20-50% and 2) despite significant differences in pre-exercise muscle glycogen availability, we observed comparable increases in AMPK^{Thr172} phosphorylation and PGC-1α mRNA. In contrast to our hypothesis, these data suggest that graded levels of muscle glycogen below 300 mmol·kg^{-1} dw do not augment skeletal muscle cell signalling, a finding that may be related to the fact that commencing exercise with <300 mmol·kg^{-1} dw is already a critical level of absolute glycogen (as suggested by Impey et al. (Impey *et al.*, 2018)) that is required to induce a metabolic milieu conducive to cell signalling. In relation to the goal of promoting cell signalling, our data therefore suggest that reducing pre-exercise glycogen concentrations below 300 mmol·kg^{-1} dw in recreationally trained individuals does not confer any additional benefit within the context of the sleep-low, train-low model.

To achieve the intended model of graded glycogen concentrations, we adopted a sleep-low, train-low design whereby subjects performed an evening bout of glycogen depleting exercise and subsequently ingested three graded quantities of CHO that were practically viable within

the time-course of the sleep-low model. This strategy was effective in achieving graded differences in pre-exercise muscle glycogen concentration (278 vs. 185 vs. 88 mmol·kg^{-1} dw in H-CHO, M-CHO and L-CHO, respectively) and represent muscle glycogen resynthesis rates (approximately 30 mmol·kg^{-1} h^{-1}) commonly observed with CHO feeding rates of 1-1.2 g kg^{-1} h^{-1} (Jentjens & Jeukendrup, 2003). A novel aspect of the chosen study design was that we employed a sampling point in both the H-CHO and M-CHO trials that was matched to the point of exhaustion in the L-CHO trial, thus allowing for the assessment of mitochondrial related signalling events at both work-matched and exhaustive exercise time points, whilst also allowing for the assessment of exercise capacity. In accordance with differences in muscle glycogen, both NEFA availability and lipid oxidation were greater in the L-CHO trial when compared with the H-CHO trial at the work-matched sampling point. However, at the point of exhaustion, plasma NEFA and glycerol were comparable between all conditions which is likely reflective of the post-exercise muscle glycogen concentrations in all three conditions given the well documented effects of muscle glycogen (Arkinstall *et al.*, 2004) on substrate utilisation during exercise.

Consistent with the well documented effects of muscle glycogen on exercise capacity (Bergström *et al.*, 1967; Hermansen *et al.*, 1967) we observed that even small differences in pre-exercise muscle glycogen concentrations (~100 mmol·kg^{-1} dw) can induce changes in exercise capacity at 80% PPO of between ~20-50% (8-18 minutes). Whilst we acknowledge that the lack of blinding to each experimental condition may have influenced exercise capacity (despite subjects receiving no feedback during exercise), it is unclear whether prior knowledge of CHO intake alone would enhance exercise performance (Hulston & Jeukendrup, 2009). Nonetheless, these data are consistent with previous data (Casey *et al.*, 2000; Areta & Hopkins, 2018) that suggest differences in muscle glycogen of 100-120 mmol·kg^{-1} dw enhance exercise capacity at 70% $\dot{V}O_{2max}$ by 5-12 minutes. As such, the 8 minute difference in exercise capacity between M-CHO and H-CHO trials is likely more representative of changes in muscle glycogen concentration. Whilst the present data may help to characterise what is considered a worthwhile change in absolute muscle glycogen concentration in determining exercise capacity, it should be acknowledged that these changes should be considered in the context of each individual given the interindividual variability between subjects in the present study. Furthermore, as the capacity for glycogen storage is enhanced and its utilisation during exercise reduced amongst well-trained populations (Karlsson *et al.*, 1974; Henriksson, 1977; Areta & Hopkins, 2018) such small differences in muscle glycogen (as observed within the present study) may allow for extended exercise times amongst well-trained individuals.

In relation to post-exercise mitochondrial related signalling, it is widely accepted that commencing work-matched exercise protocols with reduced muscle glycogen induces greater skeletal muscle signalling (Impey *et al.*, 2018). For example, AMPK^{Thr172} phosphorylation (Yeo *et al.*, 2010), AMPK-α2 activity (Wojtaszewski *et al.*, 2003) and nuclear abundance (Steinberg *et al.*, 2006) are all augmented when acute exercise is commenced with reduced pre-exercise muscle glycogen. In contrast, no enhancement in AMPK^{Thr172} or ACC^{Ser79} phosphorylation was observed at our work-matched time point (i.e. following the completion of ~20 min high-intensity cycling) despite graded reductions in pre-exercise muscle glycogen concentrations. This apparent lack of augmented cell signalling may be explained by subjects already commencing exercise with pre-exercise glycogen concentrations below 300 mmol·kg^{-1} dw, an absolute concentration that was previously suggested to facilitate the enhanced cell signalling responses associated with low glycogen availability (Impey *et al.*, 2018). Indeed, the range of pre-exercise muscle glycogen concentrations are distinctly lower than previous work that report greater skeletal muscle signalling following work-matched exercise protocols. For example, high glycogen trials are commonly commenced with muscle glycogen concentrations between 400 and 600 mmol·kg^{-1} dw (Roepstorff *et al.*, 2005; Bartlett *et al.*, 2013) and remain above 300 mmol·kg^{-1} dw post-exercise (Wojtaszewski *et al.*, 2003; Roepstorff *et al.*, 2005; Bartlett *et al.*, 2013). In such instances, these researchers observed attenuated (Wojtaszewski *et al.*, 2003) or abolished (Roepstorff *et al.*, 2005; Bartlett *et al.*, 2013) activation of cell signalling pathways. Interestingly, despite the completion of significantly more work in both the M-CHO and H-CHO trials, no further increases in AMPK^{Thr172} phosphorylation were observed following exhaustive exercise. Whilst both AMPK activity and ACC phosphorylation are known to be regulated by exercise duration (Stephens *et al.*, 2002) these responses appear to be closely linked to changes in muscle glycogen concentrations (Stephens *et al.*, 2002; Fiorenza *et al.*, 2018). In this way, the lack of augmented signalling in response to further exercise in the present study may be explained by the relatively small changes in muscle glycogen from the work-matched time point to exhaustion.

In contrast to AMPK and ACC, we did not observe any change in the phosphorylation status of p38MAPK$^{Thr180/Tyr182}$ or CAMKII ^{Thr286} either in response to exercise or muscle glycogen concentration, though we note the large inter-individual variability and recommend the use of larger sample sizes in future. These data are in agreement with previous work that demonstrate no change in p38MAPK or CAMKII phosphorylation using a variety of train-low methodologies, including sleep-low, train-low (Bartlett *et al.*, 2013; Lane *et al.*, 2015), twice-per day training (Yeo *et al.*, 2010) and fasted training (Stocks *et al.*, 2019). Whilst augmented p38MAPK phosphorylation has been observed when pre-exercise muscle glycogen is reduced (163 vs. 375 mmol·kg^{-1} dw), this is only apparent within the nucleus and not the cytoplasm

(Chan *et al.*, 2004). As such, further work should utilise cellular fractionation methodologies in order to investigate the cellular localisation of such exercise-inducible kinases.

Despite the observed augmented mRNA expression of PGC-1α within the post-exercise recovery period, exhaustive exercise did not augment the mRNA expression of other mitochondrial (p53 or Tfam) or substrate utilization related genes (PDK4, CPT1 or CD36). Although the time-course of mRNA expression for these genes is not well understood, the lack of change in mRNA expression in the present study may be explained by the present chosen sampling points in accordance with the sleep-low, train-low exercise model. Indeed, given that the pre-exercise biopsy was sampled within ~14 h of glycogen depleting exercise, it is difficult to determine whether mRNA expression was already elevated at pre-exercise. For instance, time-course studies have revealed that the mRNA expression of Tfam (Perry *et al.*, 2010), PDK4 and CPT1 (Pilegaard *et al.*, 2005) is enhanced for up to 24 h post-exercise which coincides with the present pre-exercise sampling time point (~14 h between the two exercise bouts). However, given the time-course of phosphorylation of our chosen protein targets (1-3 h) (Cochran *et al.*, 2010) it is highly unlikely that any of these proteins would be phosphorylated at pre-exercise as a result of the previous evenings glycogen depletion exercise.

Practically, these data suggest that in the context of the sleep-low, train-low model, where muscle glycogen is depleted to very low levels (~100 mmol·kg^{-1} dw), insufficient time is available to restore muscle glycogen to normal levels. As such, individuals undertaking sleep-low, train-low models, that reduces muscle glycogen to very low levels, should consume CHO in accordance with the energetic requirements of the subsequent morning session, given that withholding CHO intake overnight appears to confer no additional benefit in relation to cell signalling but impairs exercise capacity. In contrast, it appears that when muscle glycogen is not depleted to such low levels (> 300 mmol·kg^{-1} dw), withholding CHO intake in the post-exercise period may prolong the acute cell signalling and gene expression responses (Pilegaard *et al.*, 2005; Lane *et al.*, 2015). With this in mind, it should be noted that driving glycogen depletion below 300 mmol·kg^{-1} dw would likely be more difficult and require considerably more work in well-trained individuals (Gejl *et al.*, 2017) given they display an enhanced capacity for glycogen storage and reduced utilisation during exercise (Karlsson *et al.*, 1974; Henriksson, 1977; Coyle *et al.*, 1988; Areta & Hopkins, 2018). In practice, it appears that careful consideration of the individuals training status and the metabolic demands of each training session is required to ensure appropriate day-to-day periodisation of CHO in order to ensure absolute training intensity is not compromised whilst also creating a metabolic milieu conducive to facilitating the metabolic adaptations associated with ‘train low’.

In summary, we provide novel data by demonstrating that graded reductions in pre-exercise muscle glycogen below 300 mmol·kg^{-1} dw (as achieved using a sleep-low, train-low model) impairs exercise capacity but does not augment skeletal muscle cell signalling responses. Practically, these data suggest that, within the context of the sleep-low, train-low model (when muscle glycogen is depleted to very low levels) overnight CHO restriction is not required to augment skeletal muscle cell signalling, and thus, CHO should be consumed in accordance with the metabolic demands of the subsequent morning session. Future studies should investigate step-wise reductions in pre-exercise muscle glycogen, within a wider range (i.e. 100-600 mmol·kg^{-1} dw), in order to investigate the existence of a potential glycogen threshold and allow for a better definition of its potential upper and lower limits.

Chapter Five:

Graded reductions in pre-exercise skeletal muscle glycogen concentration does not augment exercise-induced nuclear AMPK and PGC-1α protein content

This work was presented at the European College of Sport Science (ECSS) conference, Dublin, 2018

5.1 Abstract

We examined the effects of graded muscle glycogen on the subcellular location of AMPK and PGC-1α protein content and mRNA expression of genes associated with the regulation of mitochondrial biogenesis and substrate utilisation in human skeletal muscle. In a repeated measures design, eight trained male cyclists completed acute high-intensity interval (HIT) cycling (8 x 5 min at 80% peak power output) with graded concentrations of pre-exercise muscle glycogen. Following initial glycogen depleting exercise, subjects ingested 2 g kg^{-1} (L-CHO), 6 g kg^{-1} (M-CHO) or 14 g kg^{-1} (H-CHO) of carbohydrate during a 36 h period, such that exercise was commenced with graded ($P < 0.05$) muscle glycogen concentrations (H-CHO; 531 ± 83, M-CHO; 332 ± 88, L-CHO; 208 ± 79 mmol·kg^{-1} dw). Exercise depleted muscle glycogen to < 300 mmol·kg^{-1} dw in all trials (H-CHO; 270 ± 88, M-CHO; 173 ± 74, L-CHO; 100 ± 42 mmol·kg^{-1} dw) and induced comparable increases in nuclear AMPK protein content (~2 fold) and PGC-1α (~5 fold), p53 (~1.5 fold) and CPT-1 (~2 fold) mRNA between trials (all $P < 0.05$). The magnitude of increase in PGC-1α mRNA was also positively correlated with post-exercise glycogen concentration ($P < 0.05$). In contrast, exercise nor carbohydrate availability affected the subcellular location of PGC-1α protein or PPAR, SCO2, SIRT1, DRP1, MFN2 or CD36 mRNA. Using a sleep-low, train-low model with a high-intensity endurance exercise stimulus, we conclude that pre-exercise muscle glycogen does not modulate skeletal muscle cell signalling.

5.2 Introduction

The concept of deliberately commencing endurance exercise with reduced muscle glycogen (i.e. the train-low paradigm (Burke *et al.*, 2018)) is now recognised as a potent nutritional strategy that is able to modulate cell signalling (Wojtaszewski *et al.*, 2003; Yeo *et al.*, 2010; Bartlett *et al.*, 2013) and transcriptional responses (Pilegaard *et al.*, 2002; Bartlett *et al.*, 2013; Psilander *et al.*, 2013) to acute exercise. Furthermore, repeated bouts of train-low exercise can subsequently augment many hallmark muscle adaptations inherent to the endurance phenotype. Indeed, the strategic periodisation of dietary CHO intake to facilitate the commencement of exercise with low muscle glycogen during 3-10 weeks of training enhances mitochondrial enzyme activity and protein content (Hansen *et al.*, 2005; Yeo *et al.*, 2008; Morton *et al.*, 2009), whole body and intra-muscular lipid metabolism (Hulston *et al.*, 2010) and, in some instances, improves exercise capacity (Hansen *et al.*, 2005) and performance (Marquet *et al.*, 2016*a*, 2016*b*). Whilst not all studies demonstrate clear positive effects of such train-low models on skeletal muscle adaptation and performance (Burke *et al.*, 2017; Gejl *et al.*, 2017; Riis *et al.*, 2019) the train-low paradigm has nonetheless gained increased recognition amongst athletic populations (Stellingwerff, 2012).

Most notably, skeletal muscle glycogen appears to exert its regulatory effects primarily through the AMPK-PGC-1α signalling axis, whereby AMPKα2 activity (Wojtaszewski *et al.*, 2003), phosphorylation (Yeo *et al.*, 2010) and nuclear abundance (Steinberg *et al.*, 2006) are all augmented under conditions of low muscle glycogen. These effects may partly be mediated through the glycogen binding domain present on the β sub-unit of AMPK (McBride *et al.*, 2009) which allows for the physical tethering of AMPK to the glycogen granule and may subsequently inhibit its translocation to the nucleus and its activation of transcriptional regulatory proteins such as PGC-1α. In this regard, the subcellular localisation of AMPK may play an important role in the signal transduction pathway regulating train-low responses, whereby its translocation to the nucleus could allow for interaction with transcriptional regulatory proteins, such as PGC-1α, to control gene expression (McGee *et al.*, 2003). Indeed, endurance exercise also appears to increase the nuclear abundance of PGC-1α (Little *et al.*, 2011), via AMPK phosphorylation (Little *et al.*, 2010), and may constitute the initial phase of exercise-induced adaptive responses.

Despite the potential regulatory role of muscle glycogen availability on exercise-induced cell signalling, the absolute concentrations of muscle glycogen required to facilitate such responses is currently unknown. Recent suggestions propose the potential existence of a muscle glycogen threshold, whereby the augmented signalling and transcriptional responses associated with train-low models are particularly apparent when absolute pre-exercise muscle

glycogen concentrations are ≤300 mmol·kg^{-1} dw (Impey *et al.*, 2018). However, using a sleep-low, train-low model, chapter 4 of this thesis demonstrated no additional benefit of stepwise reductions in pre-exercise muscle glycogen concentrations below 300 mmol·kg^{-1} dw (100-300 mmol·kg^{-1} dw) on exercise-induced cell signalling or gene expression following exhaustive exercise. This apparent lack of augmented response may be explained by subjects commencing exercise with pre-exercise glycogen concentrations below 300 mmol·kg^{-1} dw and the use of exhaustive exercise which reduced post-exercise muscle glycogen concentrations below 100 mmol·kg^{-1} dw in all conditions. Nonetheless, whilst reducing pre-exercise muscle glycogen concentrations below 300 mmol·kg^{-1} dw appears to confer no additional benefit to mitochondrial related signalling, it still remains unclear whether concentrations of 300 mmol·kg^{-1} dw are indicative of a train-low session. As such, in order to elucidate the potential existence of a muscle glycogen threshold and allow for a better definition of its potential upper and lower limits, investigations using a wider range of pre-exercise muscle glycogen concentrations (i.e. 100-600 mmol·kg^{-1} dw) and work-matched, non-exhaustive exercise are now required.

With this in mind, the aim of the present study was to examine the effects of wide-ranging graded glycogen concentrations on the modulation of AMPK-PGC-1α signalling and the transcription of genes with putative roles in the regulation of mitochondrial biogenesis. Our model of graded pre-exercise muscle glycogen concentrations was achieved through a modified sleep-low, train-low model previously used within our laboratory (Impey *et al.*, 2016), providing an additional day between exercise sessions in order to allow sufficient time for the restoration of muscle glycogen between exercise bouts and to subsequently achieve our intended pre-exercise muscle glycogen concentrations. It was hypothesised that stepwise reductions in muscle glycogen would potentiate early molecular responses to exercise.

5.3 Methods

5.3.1 Participants

Eight endurance-trained amateur male cyclists (mean ± SD: age, 30 ± 10 years; body mass 72.6 ± 9.4 kg; height, 177.0 ± 8.9 cm) took part in this study. Mean $\dot{V}O_{2peak}$ and peak power output (PPO) for the cohort were 60.4 ± 7.7 mL kg^{-1} min^{-1} and 338 ± 45 W, respectively. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All subjects provided written informed consent and all procedures conformed to the standards set by the Declaration

of Helsinki (2008). The study was approved by the local Research Ethics Committee of Liverpool John Moores University.

5.3.2 Experimental Design

In a repeated-measures design, with each experimental trial separated by a minimum of 7 days, subjects undertook an evening bout of glycogen depletion exercise followed by the consumption of graded quantities of CHO (L-CHO: 2 g kg^{-1}, M-CHO: 6 g kg^{-1}, H-CHO: 14g kg^{-1}) across a ~36 h period so as to manipulate pre-exercise muscle glycogen prior to a bout of high-intensity interval exercise (8 x 5 min at 80% PPO) with all trials performed in a randomised and counterbalanced order. Skeletal muscle biopsies were obtained from the vastus lateralis immediately before, post- and 3 h post-exercise. An overview of the experimental protocol is shown in Figure 5.1.

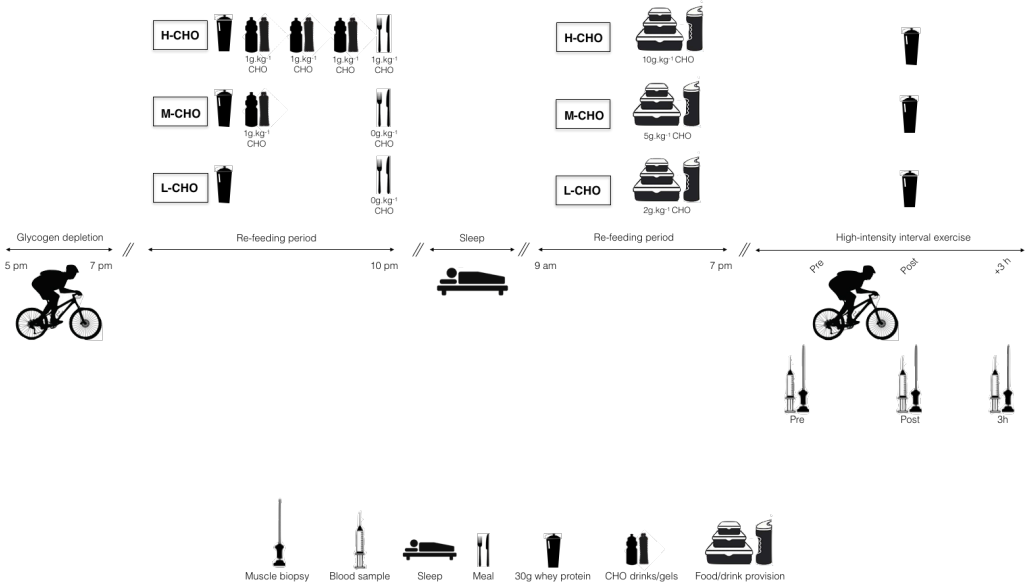

Figure 5. 1. Schematic overview of the experimental protocol. Subjects completed an evening bout of glycogen depleting cycling exercise and, upon completion, received three graded quantities of CHO in order to manipulate pre-exercise muscle glycogen across 36 h. Following an overnight fast, subjects completed a bout of high-intensity interval cycling. Muscle biopsies were obtained pre-exercise, post-exercise and 3 h post-exercise.

5.3.3 Assessment of peak oxygen uptake

At least 7 days prior to experimental trials, all subjects were assessed for peak oxygen consumption ($\dot{V}O_{2peak}$) and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Following the completion of a 10 min warm-up at 75 W, the test began at 100 W and consisted of 2 min stages with 30 W increments in resistance until volitional exhaustion. $\dot{V}O_{2peak}$ was stated as being achieved by the following end-point criteria: (1) heart rate within 10 beats min^{-1} of age-predicted maximum, (2)

respiratory exchange ratio > 1.1 and (3) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

5.3.4 Overview of sleep-low, train-low model

Phase 1: Glycogen depletion exercise

In the 24 h preceding glycogen depleting exercise, subjects recorded all food and drink consumed and were asked to replicate this for all subsequent trials and also refrained from alcohol and vigorous physical exercise for the previous 48 h. On the day of glycogen depleting exercise, subjects reported to the laboratory at approximately 5 pm to perform a bout of intermittent glycogen depleting cycling, as previously completed in our laboratory (Taylor *et al.*, 2013; Impey *et al.*, 2016) and detailed within section 4.3.4. The pattern of exercise and total time to exhaustion in the subject's initial trial was recorded and replicated in all subsequent trials. Subjects were permitted to consume water *ad libitum* during exercise*,* with the pattern of ingestion replicated during subsequent trials.

Phase 2: Carbohydrate re-feeding strategy

To facilitate the goal of achieving graded differences in muscle glycogen concentrations between trials, subjects were provided with varying amounts of carbohydrate during the ~36 h recovery period prior to the subsequent bout of high-intensity interval exercise. Within the immediate ~4 h recovery period following glycogen depleting exercise, subjects in the H-CHO trial were provided with CHO at a rate of 1 g kg^{-1} h^{-1} for 3 h from a mixture of CHO drinks and gels (Science in Sport, Nelson UK) followed by a high carbohydrate meal providing a further 1 g kg^{-1} CHO. Subjects in the M-CHO trial consumed 1 g kg^{-1} CHO immediately following exercise whilst subjects in the L-CHO trial refrained from CHO intake throughout the remainder of the evening. Across all trials, subjects also consumed 30 g of whey protein isolate (Science in Sport, Nelson, UK) mixed with 500 ml of water immediately following the cessation of glycogen depleting exercise to reflect real-world practice as per current nutritional guidelines (Thomas *et al.*, 2018). Over the course of the following day, subjects consumed either 10 g kg^{-1} (H-CHO), 5 g kg^{-1} (M-CHO) or 2 g kg^{-1} (L-CHO) carbohydrate (i.e. between 9 am and 9 pm). In this way, total CHO intakes in the H-CHO, M-CHO and L-CHO trials equated to 14, 6 and 2 g kg^{-1} CHO, respectively, over the course of the ~ 36 h following glycogen depletion. In all trials, subjects also consumed 2 g kg^{-1} protein and 1 g kg^{-1} fat with fluid intake allowed *ad libitum.*

Phase 3: High intensity interval cycling

Subjects arrived at the laboratory between 8-9 am, in a fasted state, where a venous blood sample was collected from the antecubital vein and a muscle biopsy taken from the vastus lateralis. Subjects then completed the high-intensity interval (HIIT) cycling protocol, consisting of 8 x 5 min intervals at 80% PPO, interspersed with 1 min rest. During exercise, heart rate (HR) was continuously measured and the final HR for each 5 min interval was recorded, whilst ratings of perceived exertion (RPE) were recorded upon completion of each interval. Expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2.5 min of every alternative interval (i.e. intervals 2, 4, 6 and 8) and substrate utilisation was assessed using the equations of Jeukendrup & Wallis (Jeukendrup & Wallis, 2005) given the validity of indirect calorimetry for the assessment of substrate utilisation at exercise intensities up to 80-85% $\dot{V}O_{2max}$ (Romijn *et al.*, 1992). Following the completion of the exercise capacity test and collection of the post-exercise biopsy, subjects were fed 30 g whey protein (Science in Sport, Nelson, UK) mixed with 500 ml of water.

5.3.5 Blood analysis

Venous blood samples were collected in vacutainers containing K_2EDTA, lithium heparin or serum separation tubes and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4ºC. Samples were collected immediately prior to and after exercise. Plasma was aliquoted and stored at -80ºC until analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, UK) as per manufacturer instructions.

5.3.6 Muscle biopsies

Skeletal muscle biopsies (~60 mg) were obtained from the vastus lateralis immediately prior to exercise, following completion of the exercise bout and 3 h post exercise. Muscle biopsies were obtained from separate incision sites 2-3 cm apart using a Bard Monopty Disposable Core Biopsy Instrument (12 gauge x 10 cm length, Bard Biopsy Systems, Tempe, AZ, USA) under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80ºC for later analysis.

5.3.7 Muscle glycogen concentration

Muscle glycogen concentrations were determined according to the acid hydrolysis method described by Van Loon et al (van Loon *et al.*, 2000). Approximately 2-5 mg of freeze-dried

tissue was powdered, dissected of all visible blood and connective tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3 h at 95ºC. After cooling to room temperature, samples were neutralised by the addition of 250 µl 0.12 mol L^{-1} Tris/2.1 mol L^{-1} KOH saturated with KCl. Following centrifugation, 200 µl of 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 and intra-assay coefficients of variation were <5%.

5.3.8 RNA isolation and analysis

Muscle samples (~20 mg) were homogenised in 1 ml TRIzol reagent (Thermo Fisher Scientific, UK) and total RNA isolated according to the manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at optical densities (OD's) of 260 and 280 nm, using a Nanodrop 3000 (Fisher, Rosklide, Denmark) with an average 260/280 ratio of 1.93 ± 0.08. A quantity of 50 ng RNA was used for each 20 µl PCR reaction.

5.3.9 Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR)

RT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one-step kit on a Rotogene 300Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA). RT-qTR-PCR was performed as follows: hold 50ºC for 10 min (reverse transcription/cDNA synthesis), 95ºC for 5 min (transcriptase inactivation and initial denaturation step), and PCR steps of 40 cycles; 95ºC for 10 sec (denaturation), 60ºC for 30 sec (annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). Following initial screening of suitable reference genes, β-2 microglobulin (B2M) showed the most stable C_t values across all RT-PCR runs and subjects, regardless of experimental condition (25.3 ± 1.0) and was therefore selected as the reference gene in all RT-PCR assays. The average PCR efficiency for all RT-PCR runs (90 ± 2%) was similar for all genes across all time points and experimental conditions. As such, the relative gene expression levels were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (Schmittgen & Livak, 2008) where the relative expression was calculated as $2^{-\Delta\Delta ct}$ where C_t represents the threshold cycle. mRNA expression for all target genes was calculated relative to the reference gene (B2M) within the same subject and condition and relative to the pre-exercise value in the H-CHO condition.

Table 5. 1. Primers used for real-time RT-PCR

Gene	Forward Primer	Reverse Primer
PGC-1	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCCTCTCTGCT
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
PPAR	ATGGAGCAGCCACAGGAGGAAGCC	GCATGAGGCCCCGTCACAGC
SCO2	CTTCACTCACTGCCCTGACA	CGGTCAGACCCAACAGCTT
SIRT1	GAGCCATGAAGTATGACAAAGATGA	GGCACTTCATGGGGTATGGAA
DRP1	CACCCGGAGACCTCTCATTC	CCCCATTCTTCTGCTTCCAC
MFN2	CCCCCTTGTCTTTATGCTGATGTT	TTTTGGGAGAGGTGTTGCTTATTTC
CPT-1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG
CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGGTTCTTATTCACA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT

5.3.10 Subcellular fractionation

Isolation of nuclear and cytosolic fractions was performed using the method described by Di-mauro *et al* (2012) as outlined in section 3.4.5. Protein concentrations were determined for both fractions via BCA assay (Thermo Fisher Scientific, UK) and validation of the enrichment of nuclear and cytosolic fractions was determined by examination of histone 3 (CST: 9715) and lactate dehydrogenase (CST: 2012) 'house-keeper' protein markers, respectively, via SDS-PAGE and western blotting (Figure 5.4B)

5.3.11 SDS page & Western blotting

Following the determination of protein concentration within each fraction, samples were re-suspended in 4X Laemlli buffer, boiled for 5 min at 95°C and separated by SDS-PAGE using 12% Mini-Protean TGX Stain-Free Gels (Bio-Rad). After electrophoresis, gels were activated according to manufacturer instructions and semi-dry transferred to nitrocellulose membranes. Following transfer, a stain-free image was obtained for total protein loading normalisation. Membranes were subsequently blocked in TBS-Tween containing 5% non-fat milk for 1 h and incubated overnight in primary antibodies (AMPKα (CST: 2603) and PGC-1α (Calbio-chem: st-1202) before incubation in relevant secondary antibody (anti-rabbit (CST: 7074) for 1 h at room temperature the following morning. Proteins were detected via chemilumines-cence (Thermo Fisher Scientific, UK) and quantified by densitometry. The volume density of each target band was normalised to the total amount of protein loaded into each lane using Stain-Free technology (Figure 5.4A).

5.3.12 Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS Version 24). Comparison of average physiological responses (e.g. total substrate oxidation)

were analysed using a one-way repeated-measures general linear model whereas changes in physiological and molecular responses between conditions (i.e. muscle glycogen, mRNA expression and activity of signalling molecules) were analysed using a two-way repeated measures general linear model, where the within factors were time and condition. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post-hoc tests in order to locate specific differences. All data in text, figures and tables are presented as means ± SD with P values ≤ 0.05 indicating statistical significance.

5.4 Results

5.4.1 Skeletal muscle glycogen

The exercise and nutritional strategy employed was successful in achieving graded levels of pre-exercise muscle glycogen (H-CHO; 531 ± 83, M-CHO; 332 ± 88, L-CHO; 208 ± 79 mmol·kg^{-1} dw) such that exercise was commenced with three distinct levels of muscle glycogen (P < 0.001) (Figure 5.2A). Exercise significantly reduced (P < 0.001) muscle glycogen concentrations, with post-exercise muscle glycogen concentrations (H-CHO; 270 ± 88, M-CHO; 173 ± 74, L-CHO; 100 ± 42 mmol·kg^{-1} dw) remaining higher in the H-CHO trial when compared with the L-CHO trial only (P = 0.013). Furthermore, muscle glycogen utilisation during exercise was significantly higher in the H-CHO trial (260 ± 90 mmol·kg^{-1} dw) when compared with the L-CHO trial (107 ± 55 mmol·kg^{-1} dw) only (P = 0.011) with no significant differences observed between M-CHO and either H-CHO or L-CHO trials (P > 0.05).

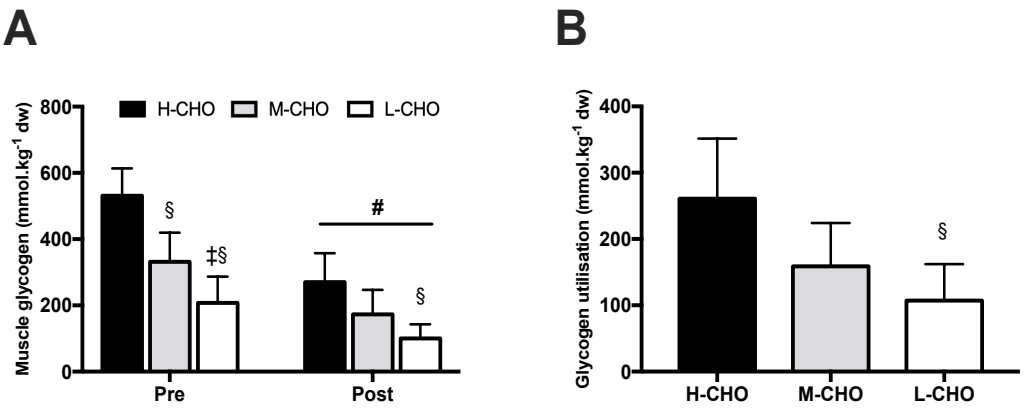

Figure 5. 2. (A) Skeletal muscle glycogen concentration and (B) utilisation during exercise. $^{\#}P$ < 0.05, significantly different from pre-exercise, §P < 0.05, significantly different from H-CHO, ‡P < 0.05, significantly different from M-CHO. Data is presented as mean ± SD

5.4.2 Physiological and metabolic responses to exercise

Subject's average heart rate (Figure 5.3A) (H-CHO; 173 ± 9, M-CHO; 174 ± 8, L-CHO; 176 ± 7 beats.min^{-1}) and RPE (Figure 5.3B) (H-CHO; 15 ± 1, M-CHO; 16 ± 2, L-CHO; 16 ± 2 arbitrary units) across the HIIT session was similar between trials ($P > 0.05$). Despite remaining unchanged following HIIT exercise, plasma glucose was significantly lower in the L-CHO when compared with the H-CHO trial only ($P = 0.039$). In contrast, the HIIT session resulted in a significant increase in plasma lactate ($P = 0.012$), NEFA ($P = 0.002$) and glycerol ($P < 0.001$). Furthermore, plasma NEFA was significantly elevated in the L-CHO trial when compared with the H-CHO trial only ($P = 0.003$) whilst plasma glycerol was significantly elevated when compared with both H-CHO ($P = 0.003$) and M-CHO trials ($P = 0.002$) (Figures 5.3 C-F respectively). Similarly, subjects in the L-CHO trial achieved significantly lower rates of CHO oxidation and greater rates of lipid oxidation when compared with both M-CHO and H-CHO trials ($P < 0.005$) although no significant differences were observed between M-CHO and H-CHO trials for either CHO ($P = 0.427$) or lipid oxidation ($P = 0.687$) (Figures 5.3 G & H respectively).

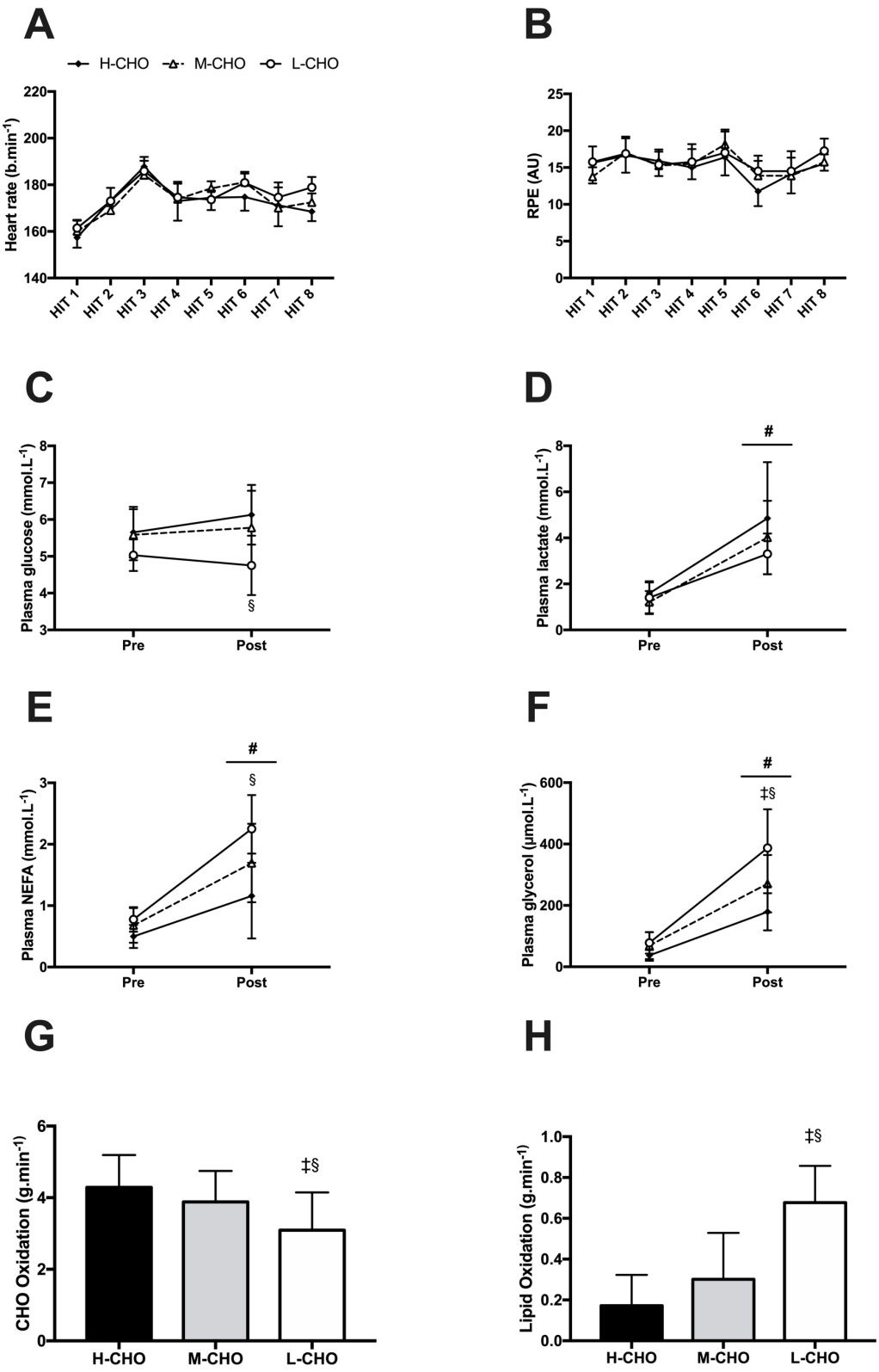

Figure 5. 3. (A) Heart rate, (B) RPE and plasma (C) Glucose, (D) Lactate, (E) NEFA (F) glycerol pre- and post-exercise, (E) Average rate of CHO and (F) lipid oxidation during exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, §$P < 0.05$, significantly different from H-CHO, ‡$P < 0.05$, significantly different from M-CHO. Data is presented as mean ± SD

5.4.3 Regulation of mitochondrial biogenesis related cell signalling

HIIT exercise induced an increase in the content of AMPK protein (P = 0.025) within the nucleus with no significant change within the cytosol (P = 0.20). Despite this, there was no significant effect of pre-exercise muscle glycogen concentrations on AMPK localisation (P = 0.207). In contrast, HIIT exercise did not induce any change in the content of PGC-1α protein in either nuclear (P = 0.31) or cytosolic (P = 0.36) fractions (Figure 5.5 B-E). With regard to exercise induced gene expression, HIIT exercise induced a significant increase in PGC-1α, p53 and CPT-1 mRNA expression at 3 h post-exercise ($P < 0.05$) but did not display any significant differences between trials (Figure 5.5 A, B, I). However, PPAR, SCO2, SIRT1, DRP1, MFN2 and CD36 mRNA expression (Figures 5.5C, D, E, F, G, H respectively) were unaffected by either glycogen availability or the HIIT exercise protocol ($P > 0.05$).

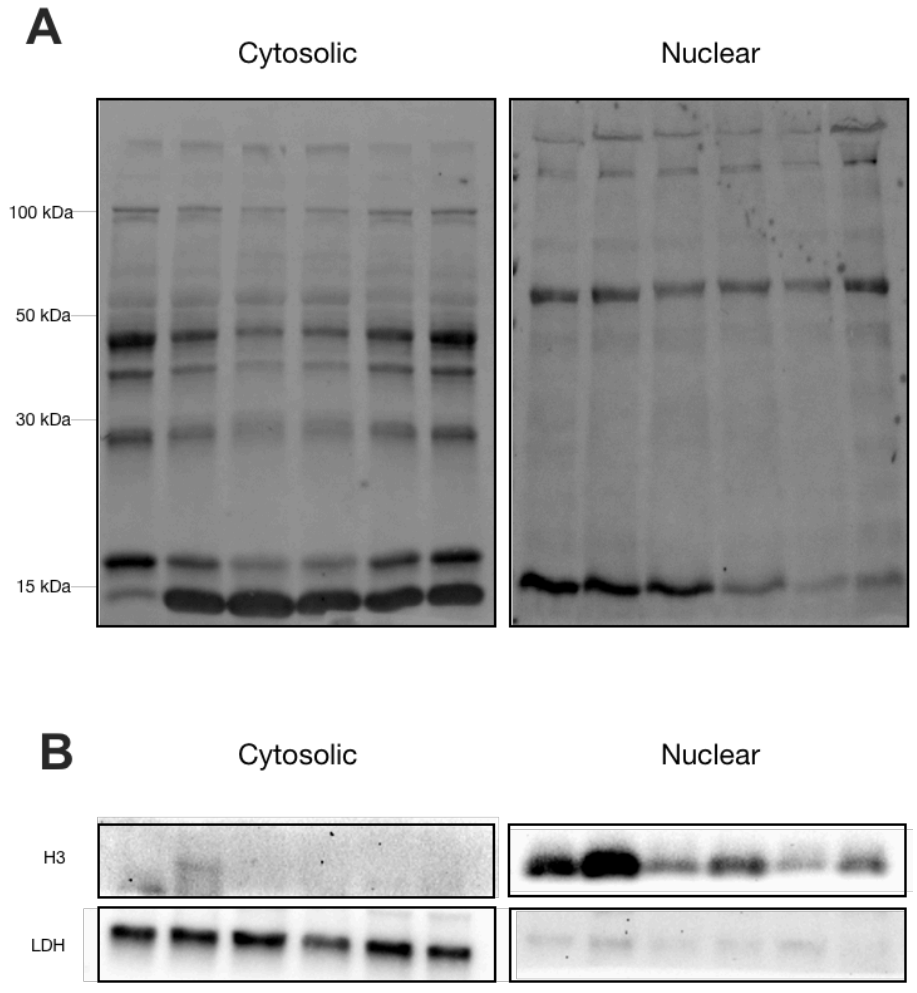

Figure 5. 4. (A) Whole lane stain-free imaging for both nuclear and cytosolic fractions and (B) histone H3 and LDHA used as indicators of nuclear and cytosolic enrichment, respectively.

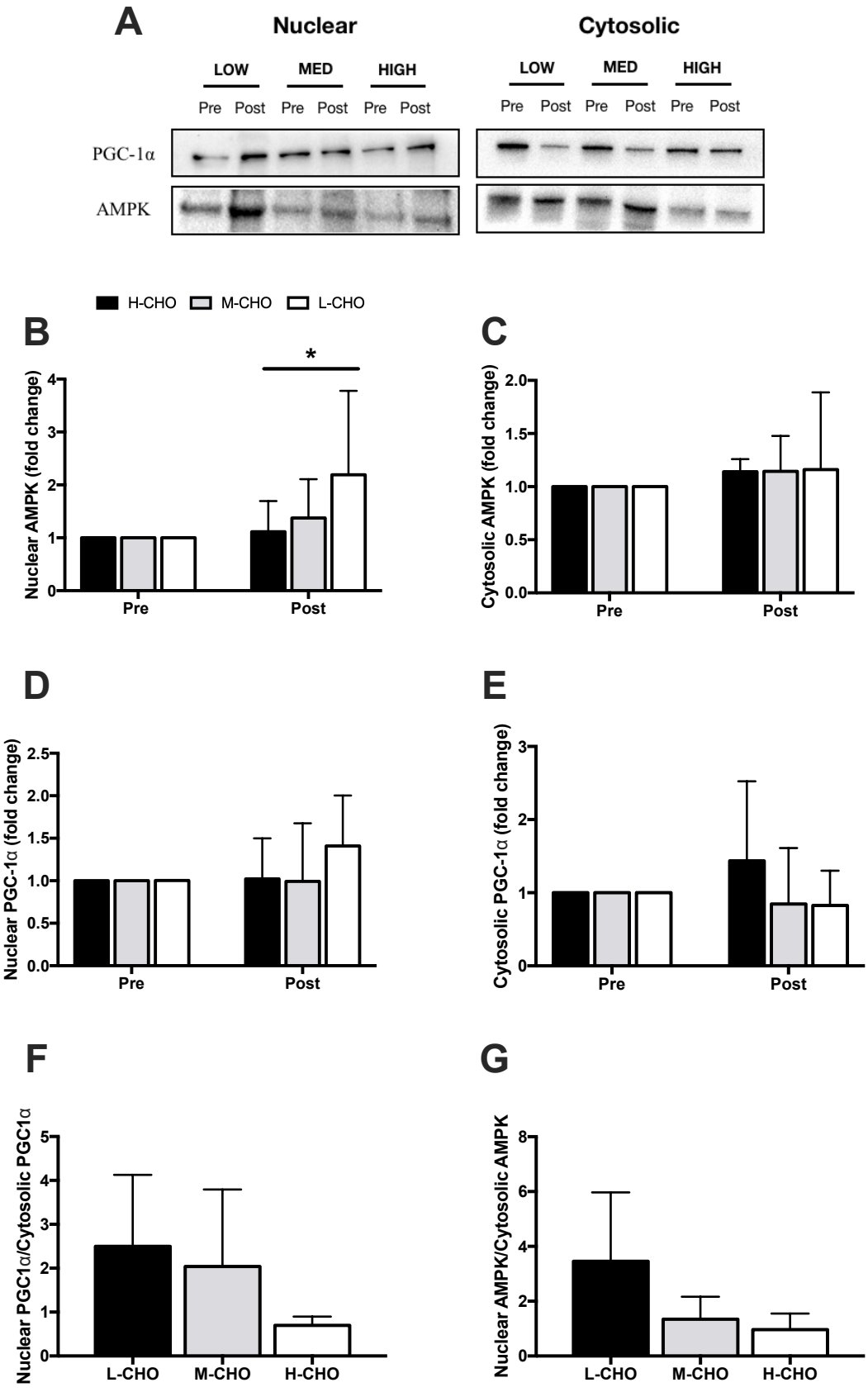

Figure 5. 5. (A) Representative Western blot images in nuclear and cytosolic fractions (B) nuclear AMPK, (C) cytosolic AMPK, (D) nuclear PGC-1α, (E) cytosolic PGC-1α, (F) nuclear/cytosolic AMPK ratio and (G) nuclear/cytosolic PGC-1α ratio pre- and post-exercise, Data is presented as means ± SD.

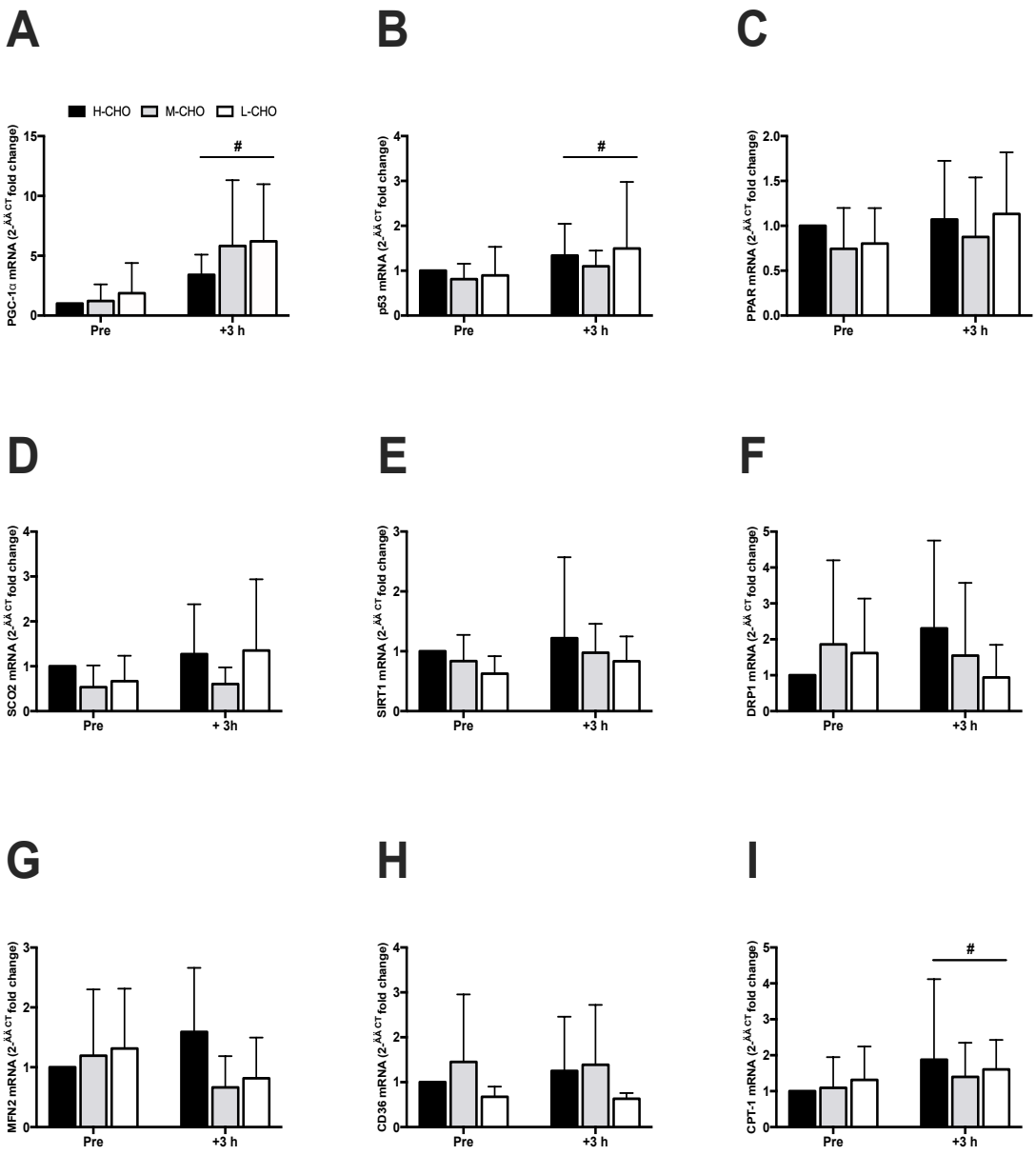

Figure 5. 6. (A) PGC-1α, (B) p53, (C) PPAR, (D) SCO2, (E) SIRT1, (F) DRP1, (G) MFN2, (H) CD36 and (I) CPT-1 mRNA expression pre- and 3 h post-exercise. $^{\#}P < 0.05$, significantly different from pre-exercise. Data is presented as means ± SD

5.5 Discussion

The aim of the present study was to test the hypothesis that graded pre-exercise muscle glycogen concentrations modulate the exercise-induced nuclear abundance of AMPK and PGC-1α protein content as well as the transcription of genes with putative roles in the regulation of mitochondrial biogenesis. Using trained male cyclists, we demonstrate that commencing an acute bout of work-matched and non-exhaustive HIT cycling with graded pre-exercise muscle glycogen (within a range of 600-200 mmol·kg^{-1} dw) does not modulate such early signalling responses. In the context of manipulating CHO availability around training, our data suggest

that the metabolic stress of HIT exercise may override any potential effect of pre-exercise muscle glycogen and induce negligible modulatory effects on skeletal muscle that is already subjected to the local metabolic challenge of high-intensity exercise.

Based on the data obtained in chapter 4 of this thesis, we adopted a modified sleep-low, train-low design, creating an extended recovery period following glycogen depleting exercise, in which larger amounts of CHO could be consumed, allowing for the investigation of a wider range of pre-exercise muscle glycogen concentrations (i.e. > 300 mmol·kg^{-1} dw). This strategy was effective in achieving graded differences in pre-exercise muscle glycogen concentration (H-CHO; 531 ± 83, M-CHO; 332 ± 88, L-CHO; 208 ± 79 mmol·kg^{-1} dw) and represent high, normal and low resting muscle glycogen concentrations of trained individuals (Areta & Hopkins, 2018). Whilst we acknowledge that the manipulation of CHO intake also results in considerable differences in total energy intake, such short-term differences in energy intake do not appear to influence the molecular response to exercise (Hammond *et al.*, 2019). In contrast to chapter 4, we also employed a work-matched, non-exhaustive exercise protocol to allow for a matched comparison of metabolic and signalling responses and to prevent the depletion of muscle glycogen to similarly low levels across all trials. Interestingly, despite differences in pre-exercise muscle glycogen, subjects RPE was similar between trials, thus suggesting that the previously reported reductions in self-selected power output with low muscle glycogen may not be associated with changes in perception of effort (Yeo *et al.*, 2008).

In relation to post-exercise signalling, the exercise-induced increase in nuclear AMPK protein content was not augmented in response to stepwise reductions in pre-exercise muscle glycogen concentrations and is in contrast to previous reports of enhanced nuclear AMPK content when exercise is commenced with low pre-exercise muscle glycogen (Steinberg *et al.*, 2006). Given that nuclear AMPK translocation in response to exercise may be partly regulated by absolute glycogen concentrations (due to the physical tethering of AMPK to the glycogen granule; Steinberg *et al.*, 2006) the magnitude of muscle glycogen utilisation during exercise may also be important for AMPK's disassociation from the granule and subsequent translocation to other subcellular regions. With this in mind, the similar rates of glycogen utilisation (~50%) between trials, leading to absolute post-exercise glycogen concentrations < 300 mmol·kg^{-1} dw, may explain the comparable increases in nuclear AMPK content across all trials and subsequent discrepancies with previous work. Whilst the absolute concentrations of muscle glycogen required to facilitate the subcellular shuttling of AMPK are relatively unknown, post-exercise glycogen concentrations in the present study are markedly higher (H-CHO; 270 ± 88, M-CHO; 173 ± 74, L-CHO; 100 ± 42 mmol·kg^{-1} dw) when compared with that of the low glycogen trial in previous work (17 ± 6 mmol·kg^{-1} dw) (Steinberg *et al.*, 2006).

In fact, post-exercise muscle glycogen concentrations in the present L-CHO trial are comparable to the control condition (111 ± 35 mmol·kg^{-1} dw) of previous work (Steinberg *et al.*, 2006) and may suggest that the augmentation of nuclear AMPK may only occur at extremely low absolute glycogen concentrations normally associated with exhaustion.

Irrespective of muscle glycogen, the use of high-intensity endurance exercise within the present study is known to induce significantly greater metabolic stress and augments the phosphorylation and activation of AMPK when compared with low-intensity exercise models (Wojtaszewski *et al.*, 2000; Egan *et al.*, 2010; Combes *et al.*, 2015; Fiorenza *et al.*, 2018). Given that such metabolic fluctuations ultimately regulate signalling kinase activity, the completion of high-intensity endurance exercise may provide a sufficient local metabolic challenge to skeletal muscle whereby reducing pre-exercise muscle glycogen induces negligible modulatory effects on skeletal muscle. Whilst augmented signalling in response to high-intensity exercise commenced with low muscle glycogen has been previously observed (Yeo *et al.*, 2010), recent data suggest this may be explained by the metabolic stress of training twice-per-day, as opposed to low pre-exercise glycogen concentrations *per se* (Andrade-Souza *et al.*, 2019). Furthermore, as the exercise-induced activation of AMPK is reduced in trained individuals (McConell *et al.*, 2020), the use of trained cyclists within the present study may offer further explanation for the discrepancy in results when compared with those of Steinberg *et al.* (2006).

Despite exercise-induced increases in nuclear AMPK we failed to observe concomitant increases in nuclear PGC-1α. These data are in contrast to most (Little *et al.*, 2010, 2011; Andrade-Souza *et al.*, 2019) but not all (Tachtsis *et al.*, 2016) reports of enhanced nuclear PGC-1α in response to acute endurance exercise. Our data are, however, in support of previous findings that demonstrate exercise-induced increases in nuclear PGC-1α is unaffected by muscle glycogen availability (Andrade-Souza *et al.*, 2019). The lack of change in nuclear PGC-1α in the present study may be explained by our chosen sampling time point, given that, in some cases, increases in nuclear PGC-1α have only been observed 3 h post-exercise (Little *et al.*, 2011). Thus, it is plausible that the immediate post-exercise increase in nuclear AMPK may result in a temporal and coordinated increase in nuclear PGC-1α later in the recovery period, given the requirement for AMPK in order to achieve full activation of PGC-1a and thus, its nuclear translocation (Safdar *et al.*, 2011). Alternatively, it is possible that post-exercise increases in nuclear PGC-1α may represent increased protein stability, and not translocation from the cytoplasm *per se*, a response that appears to be linked with increased p38 MAPK activity (Puigserver *et al.*, 2001). Although not measured in the present study, p38 MAPK phosphorylation does not appear to be affected by either glycogen availability or a similar

bout of high-intensity interval exercise (Yeo *et al.*, 2010) and may explain the lack of nuclear PGC-1α observed post-exercise. Nonetheless, as PGC-1α has been shown to activate its own promoter through a feed-forward loop (Handschin *et al.*, 2003), the lack of nuclear PGC-1α following exercise is somewhat surprising given the exercise-induced increase in PGC-1α mRNA. When taken together, these data suggest a potential disconnect between exercise-induced nuclear PGC-1α protein and PGC-1α mRNA expression (Granata *et al.*, 2017) and point towards the involvement of other proteins involved in the signal transduction pathway.

In relation to exercise-induced gene expression, we demonstrate that graded reductions in pre-exercise muscle glycogen across a wide range of concentrations (100 - 600 mmol·kg^{-1} dw) do not augment the expression of genes with putative roles in mitochondrial biogenesis and substrate utilisation, providing further support to the findings from Chapter 4. Although in contrast to the previously reported effects of muscle glycogen concentrations on the regulation of gene expression (Pilegaard *et al.*, 2002, 2005; Bartlett *et al.*, 2013; Psilander *et al.*, 2013), these data may be explained by the attainment of post-exercise muscle glycogen concentrations (i.e. < 300 mmol·kg^{-1} dw) associated with augmented gene expression responses to exercise (Impey *et al.*, 2018) and raise the possibility that absolute post-exercise muscle glycogen concentrations may also be an important factor in regulating mitochondrial-related skeletal muscle signalling responses to exercise. In line with this rationale, our laboratory has previously demonstrated comparable increases in AMPK activity and mRNA expression of its downstream targets with the depletion of muscle glycogen to similar concentrations (~100 mmol·kg^{-1} dw), despite marked differences in pre-exercise concentrations (600 vs. 300 mmol·kg^{-1} dw) (Impey *et al.*, 2016). Furthermore, when taken together with previous data that demonstrate post-exercise muscle glycogen concentrations regulate exercise-induced PGC-1α mRNA expression (Pilegaard *et al.*, 2005; Psilander, 2014; Fiorenza *et al.*, 2018) and total protein abundance (Mathai *et al.*, 2008) these findings raise the possibility that the regulation of gene expression may occur through changes in the absolute concentrations of glycogen obtained post-exercise.

In summary, we provide novel data by demonstrating that graded pre-exercise muscle glycogen (within a range of 600-200 mmol.kg^{-1} dw) does not modulate the exercise-induced nuclear abundance of AMPK or PGC-1α nor does it affect the expression of genes with regulatory roles in mitochondrial biogenesis and substrate utilization. Practically, these data suggest that the additional stress of low pre-exercise muscle glycogen may not be required when performing high-intensity exercise that already subjects skeletal muscle to a sufficient metabolic challenge and may be better suited during conditions that do not elicit such cellular perturbations (e.g. prolonged low-intensity exercise completed below lactate threshold). Restriction of CHO

availability for the latter training sessions would also circumvent the impairment in self-selected training intensity observed when high-intensity exercise is performed with reduced muscle glycogen (Yeo *et al.*, 2008; Lane *et al.*, 2013). Given that post-exercise muscle glycogen concentrations were reduced to low levels across all trials (i.e. 100-250 $mmol \cdot kg^{-1}$ dw), our data raise the possibility that the absolute post-exercise muscle glycogen concentrations may also be an important factor in regulating exercise-induced skeletal muscle signalling responses associated with mitochondrial biogenesis and further work is now required to test this hypothesis directly.

Chapter Six:

Exogenous CHO feeding during exercise does not blunt post-exercise skeletal muscle signalling responses to steady-state cycling

6.1 Abstract

We examined the effects of ingesting graded quantities of exogenous CHO during steady state exercise on the modulation of skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis. In a repeated measures design, eight endurance trained males ingested graded quantities of exogenous carbohydrate (0, 45 or 90 g CHO.h^{-1}) during 3 h of steady-state cycling at 100% of lactate threshold (208 ± 11 W) commenced with high muscle glycogen concentrations (0g; 767 ± 87, 45g; 742 ± 109, 90g; 698 ± 98 mmol·kg^{-1} dw) in the fed state. Exogenous CHO feeding during exercise did not alter the pattern of muscle glycogen utilisation during exercise (0g; 515 ± 93, 45g; 473 ± 133, 90g; 405 ± 132 mmol·kg^{-1} dw, $P > 0.05$) and thus post-exercise muscle glycogen concentrations (0g; 252 ± 85, 45g; 269 ± 88, 90g; 293 ± 109 mmol·kg^{-1} dw, $P > 0.05$) were similar between trials. Exercise-induced comparable increases in AMPK^{Thr172} (~4 fold), CaMKII^{Thr268} (~2 fold), and p53^{Ser15} (~2 fold), ($P < 0.05$), independent of CHO availability whilst neither exercise nor CHO availability affected the phosphorylation of p38MAPK$^{Thr180/Tyr182}$ or CREB^{Ser133}. When taken together, these data demonstrate that exogenous CHO feeding during prolonged endurance exercise does not spare muscle glycogen or modulate exercise-induced signalling responses controlling mitochondrial biogenesis.

6.2 Introduction

During exercise, transient perturbations to cellular homeostasis (e.g. the rise in cytoplasmic free Ca^{2+}, increased free AMP, increased ADP/ATP ratio, reductions in muscle glycogen, increased fatty acid availability and lactate accumulation) are able to activate specific signalling cascades regulating skeletal muscle plasticity (Hawley *et al.*, 2014). Specifically, exercise induced activation of AMP-activated protein kinase (AMPK), p38 mitogen activated protein kinase (p38 MAPK) and calmodulin dependent kinase II (CaMKII) appear to be central to the signal transduction network given their ability to converge upon and activate downstream transcription factors and co-activators to augment the transcription of genes that dictate the synthesis of new, functional proteins (Coffey & Hawley, 2007; Egan *et al.*, 2010)

Undertaking endurance exercise with low endogenous CHO availability (defined as a train-low session; (Burke *et al.*, 2018) induces significant metabolic shifts compared with when exercise is undertaken with high endogenous CHO availability. Indeed, at comparable submaximal work intensities, exercise with low endogenous CHO availability substantially reduces glycogen utilisation (Arkinstall *et al.*, 2004) whilst concomitantly increasing circulatory free fatty acid (FFA) availability (Wojtaszewski *et al.*, 2003) and circulatory catecholamines (Hansen *et al.*, 2005). Consequently, the altered metabolic milieu created through exercising with low endogenous CHO availability has a direct impact on molecular signalling events controlling training adaptation. This response appears largely mediated through AMPK-dependent mechanisms given that $AMPK^{Thr172}$ phosphorylation (Yeo *et al.*, 2010), AMPK-α2 activity (Wojtaszewski *et al.*, 2003) and nuclear abundance (Steinberg *et al.*, 2006) are all augmented when acute exercise is commenced with low endogenous CHO availability. In contrast, data from chapter 4 and 5 suggest reducing pre-exercise muscle glycogen concentrations do not alter exercise-induced signalling responses associated with mitochondrial biogenesis when concentrations are depleted to sufficiently low levels post-exercise. These data therefore raise the possibility that the absolute concentration of muscle glycogen post-exercise (opposed to that obtained pre-exercise) may also be an important factor in regulating such responses.

Consuming exogenous CHO intake during exercise may provide one strategy to manipulate post-exercise muscle glycogen concentrations, given its potential ability to spare muscle glycogen utilisation during exercise (Bjorkman *et al.*, 1984; Erickson *et al.*, 1987), especially in specific fibre types (Stellingwerff *et al.*, 2007). In line with this rationale, exogenous CHO feeding during exercise appears to attenuate AMPK-α2 activity only when the pattern of muscle glycogen utilisation is altered, and thus absolute post-exercise concentrations are elevated

compared with when CHO is not fed (Akerstrom *et al.*, 2006; Lee-Young *et al.*, 2006). Taken together, these data suggest that, as exogenous CHO feeding may exert its effects through changes in endogenous glycogen concentrations, absolute muscle glycogen concentrations may be the main substrate regulating exercise-induced AMPK signalling. Nonetheless, the effect of exogenous CHO feeding during exercise on alternative signalling pathways within human skeletal muscle remains incompletely explored and, given that exogenous CHO feeding during exercise may blunt training-induced adaptations (Morton *et al.*, 2009) provides an area of research that warrants further investigation.

With this in mind, the aim of the present study was to examine the effects of graded exogenous CHO feeding during steady-state exercise on the modulation of selected skeletal muscle signalling pathways with putative roles in the regulation of mitochondrial biogenesis. As such, it was hypothesised that exogenous CHO feeding would only modulate cell signalling responses in concert with differences in the pattern of muscle glycogen utilisation during exercise and, ultimately differences in post-exercise muscle glycogen concentrations.

6.3 Methods

6.3.1 Participants

Eight endurance-trained amateur male cyclists (mean ± SD: age, 31 ± 7 years; body mass 73.6 ± 6.1 kg; height, 177.0 ± 8.0 cm) took part in this study. Mean $\dot{V}O_{2peak,}$ peak power output (PPO) and power output at lactate threshold (LT) for the cohort were 60.5 ± 5.4 mL kg^{-1} min^{-1}, 383 ± 21 W and 208 ± 11 W, respectively. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All subjects provided written informed consent and all procedures conformed to the standards set by the Declaration of Helsinki (2008). The study was approved by the local Research Ethics Committee of Liverpool John Moores University.

6.3.2 Experimental Design

In a repeated-measures, randomised, cross-over design separated by a minimum of 7 days, subjects undertook 180 min of steady state cycling at 100% lactate threshold (208 ± 11 W) whilst consuming exogenous carbohydrate at a rate of either 0, 45 or 90 grams per hour. To promote high CHO availability prior to the main experimental trial, subjects undertook 90 min of glycogen depleting exercise followed by the consumption of a high CHO diet (11.6 g kg^{-1}) and a high CHO meal (2 g kg^{-1}) 3 h prior to exercise. Muscle biopsies were obtained from the vastus lateralis muscle immediately before and after the completion of the submaximal cycling protocol. An overview of the experimental protocol is shown in Figure 6.1.

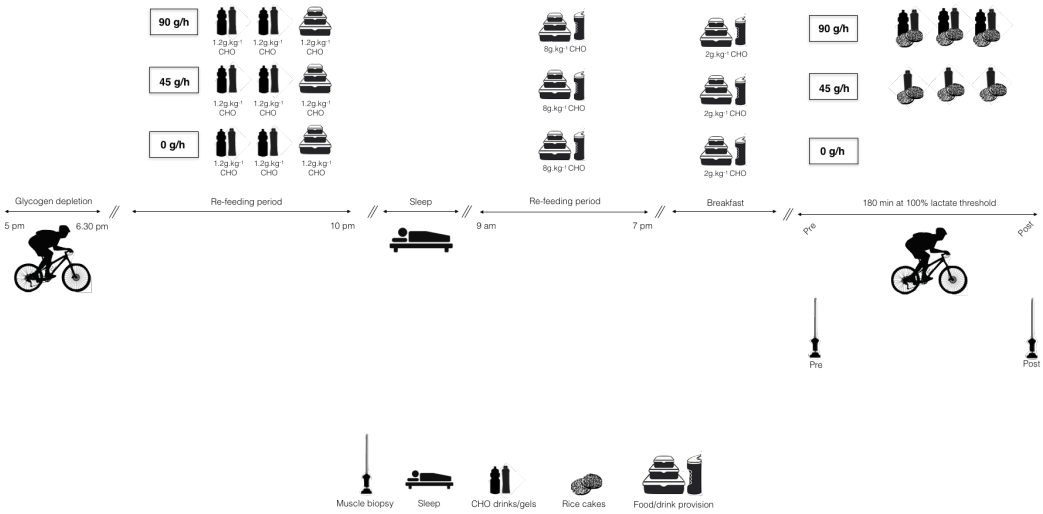

Figure 6. 1. Schematic overview of the experimental protocol. Subjects completed an evening bout of glycogen depleting cycling exercise followed by the consumption of a high CHO diet for 24 h prior to the main experimental trial. Following a high CHO pre-exercise breakfast, subjects completed a bout steady state cycling, during which graded quantities of exogenous CHO were ingested. Muscle biopsies were obtained pre- and post-exercise.

6.3.3 Preliminary testing

At least 10 days prior to experimental trials, all subjects performed a two-part incremental cycle test to determine peak oxygen consumption ($\dot{V}O_{2peak}$), lactate threshold (LT) and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Following the completion of a 10 min warm-up at 75 W, the LT test began at 125 W and consisted of 4 min stages with 25 W increments in resistance until blood lactate concentrations increased above 4 mmol·L^{-1}, with LT defined as the first deflection point in the blood lactate accumulation curve . On completion of the LT test, subjects were provided with a 15 min rest period, prior to the completion of the assessment for $\dot{V}O_{2peak}$. The test recommenced at a power output corresponding to that of the penultimate stage of the LT test and continued with 60 second stages with 25 W increments in resistance until volitional exhaustion. $\dot{V}O_{2peak}$ was stated as being achieved by the following end-point criteria: (1) heart rate within 10 beats min^{-1} of age-predicted maximum, (2) respiratory exchange ratio > 1.1 and (3) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

Subjects also undertook full familiarisation of the experimental trial, at least 7 days prior to the main experimental trials, and all refrained from CHO intake during the steady state cycling protocol, thus replicating the same nutritional conditions as per the 0g trials.

6.3.4 Glycogen depletion exercise

On the day of glycogen depleting exercise, subjects reported to the laboratory at approximately 5 pm to perform 90 min of intermittent glycogen depleting cycling, as previously completed in our laboratory (Taylor *et al.*, 2013; Impey *et al.*, 2016) and detailed within section 4.3.4. The pattern of exercise and total time to exhaustion in the subject's initial trial was recorded and replicated in all subsequent trials. Subjects were permitted to consume water *ad libitum* during exercise*,* with the pattern of ingestion replicated during subsequent trials.

6.3.5 Carbohydrate re-feeding strategy

Immediately following glycogen depleting exercise, subjects were provided with CHO at a rate of 1.2 g kg^{-1} h^{-1} for 3 h through a mixture of drinks and bars (Go Energy, Science in Sport, Nelson, UK) in accordance with nutritional guidelines to optimise muscle glycogen resynthesis (Thomas *et al.*, 2018). In this way, subjects consumed 3.6 g kg^{-1} CHO, 0.6 g kg^{-1} protein and 0 g kg^{-1} fat across the first 3 h of recovery. For the following 24 h, subjects consumed a standardised high CHO diet (8 g kg^{-1} CHO, 2 g kg^{-1} protein and 1 g kg^{-1} fat) with fluid intake allowed *ad libitum.*

6.3.6 Steady-state cycling

On the morning of each experimental trial subjects arrived at the laboratory at 8 am and were provided with a standardised high CHO breakfast (2 g kg^{-1} CHO, 40 g protein and 35 g fat; comprising porridge oats, semi-skimmed milk, honey, orange juice, bread, jam, banana and eggs) which was consumed 3 h prior to commencing exercise. Immediately prior to exercise, an indwelling cannula (Safety lock 22G, BD Biosciences, UK) was inserted into the antecubital vein in the anterior crease of the forearm and a resting blood sample was taken, followed by the collection of a muscle biopsy sample from the vastus lateralis. The steady-state cycling protocol consisted of 180 min of cycling at 100% LT (208 ± 11 W) at a self-selected cadence, during which carbohydrate was ingested at a rate of either 0 g h^{-1} (0g), 45 g h^{-1} (45g) or 90 g h^{-1} (90g). In both 45g and 90g trials, carbohydrate was provided in the form of gels (GO Energy, Science in Sport, Nelson, UK) and rice cakes (21 g CHO per rice cake) whilst subjects in the 90g trial were also provided with a specially formulated maltodextrin based drink containing 22 g CHO per 500 ml. Fluid intake during exercise was matched at 500 ml h^{-1} across all three trials, whereby subjects in the 0g and 45g trials consumed water flavoured with electrolyte tablets (Go Hydro, Science in Sport, Nelson, UK) and subjects in the 90g trial consumed the aforementioned maltodextrin drink. During exercise, heart rate (HR) and ratings of perceived exertion (RPE) were recorded every 15 min whilst expired air was collected via a mouthpiece connected to an online gas analysis system (MOXUS modular uptake system, AEI

technologies, Pittsburgh, PA) for a total of 5 min at regular 30 min intervals and substrate utilisation was assessed using the equations of Jeukendrup & Wallis (Jeukendrup & Wallis, 2005).

6.3.7 Muscle biopsies

Skeletal muscle biopsies (~100 mg) were obtained from the vastus lateralis immediately pre- and post-exercise. Muscle biopsies were obtained from separate incision sites 2-3 cm apart using the Well-Blakesey conchotome technique under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80ºC for later analysis.

6.3.8 Muscle glycogen concentration

Muscle glycogen concentrations were determined according to the acid hydrolysis method described by Van Loon et al (van Loon *et al.*, 2000). Approximately 2-5 mg of freeze-dried tissue was powdered, dissected of all visible blood and connective tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3 h at 95ºC. After cooling to room temperature, samples were neutralised by the addition of 250 µl 0.12 mol L^{-1} Tris/2.1 mol L^{-1} KOH saturated with KCl. Following centrifugation, 200 µl of 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 and intra-assay coefficients of variation were <5%.

6.3.9 SDS page & Western blotting

Muscle samples were prepared, separated by SDS-PAGE and transferred to nitrocellulose membranes according to the method outlined in section 3.4.5. Following transfer, a stain-free image was obtained for total protein loading normalisation. Membranes were subsequently blocked in TBS-Tween containing 5% non-fat milk for 1 h and incubated overnight in primary antibodies; AMPKα (CST: 2532), p-AMPK^{Thr172} (CST: 2531), p38MAPK (CST: 9212), p-p38MAPK$^{Thr180/Tyr182}$ (CST: 9211), CaMKII (CST: 3362), p-CaMKII^{Thr268} (CST: 3361), p53 (CST: 9282), p-p53 9284 (CST: 9281), CREB (CST: 9197), p-CREB^{Ser133} (CST: 9198), before incubation in relevant secondary antibody (anti-rabbit (CST: 7074) for 1 h at room temperature the following morning. Proteins were detected via chemiluminescence (Millipore, Watford, UK) and quantified by densitometry as outlined in section 3.4.5.

6.3.10 Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS Version 24). Comparison of average physiological responses across the exercise bout (i.e.

total substrate oxidation) were analysed using a one-way repeated-measures general linear model whereas changes in physiological and molecular responses between conditions (i.e. muscle glycogen, and activity of signalling molecules) were analysed using a two-way repeated measures general linear model, where the within factors were time and condition. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post-hoc tests in order to locate specific differences. All data in text, figures and tables are presented as means ± SD with *P* values ≤ 0.05 indicating statistical significance.

6.4 Results

6.4.1 Skeletal muscle glycogen

Subjects commenced exercise with similar muscle glycogen concentrations ($P = 0.137$) across all three trials (0g; 767 ± 87, 45g; 742 ± 109, 90g; 698 ± 98 mmol·kg^{-1} dw). Muscle glycogen was significantly reduced following 180 min of steady state cycling (0g; 252 ± 85, 45g; 269 ± 88, 90g; 293 ± 109 mmol·kg^{-1} dw) to similar concentrations in all trials ($P = 0.502$), demonstrating no difference in absolute muscle glycogen utilisation (0g; 515 ± 93, 45g; 473 ± 133, 90g; 405 ± 132 mmol·kg^{-1} dw) between the trials ($P = 0.155$) (Figure 6.2 A-B).

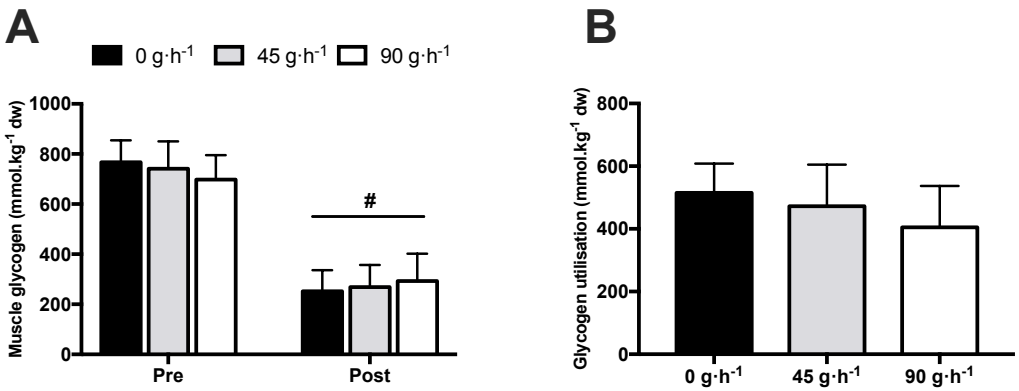

Figure 6. 2. (A) Skeletal muscle glycogen concentration and (B) utilisation during exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, Data is presented as mean ± SD

6.4.2 Physiological responses to exercise

Heart rate (HR) and rating of perceived exertion (RPE) were significantly elevated by exercise ($P < 0.05$) but did not differ between trials ($P > 0.05$) (Figure 6.3 A-B). Average rate of CHO oxidation was significantly higher in the 90g trial (2.3 ± 1.5 g.min^{-1}) when compared with the 0g (1.8 ± 1.6 g) trial ($P = 0.01$) with no significant differences between either 45g (2.1 ± 1.4 g.min^{-1}) and 90g or 45g and 0g trials ($P > 0.05$) despite large differences in total CHO intake.

Similarly, average rate of lipid oxidation was significantly lower in the 90g trial (0.58 ± 0.05 g.min^{-1}) when compared with the 0g (0.78 ± 0.05 g.min^{-1}) trial ($P < 0.001$) with no significant differences between either 45g (0.63 ± 0.04 g.min^{-1}) and 90g or 45g and 0g trials ($P > 0.05$) (Figure 6.2 C-D).

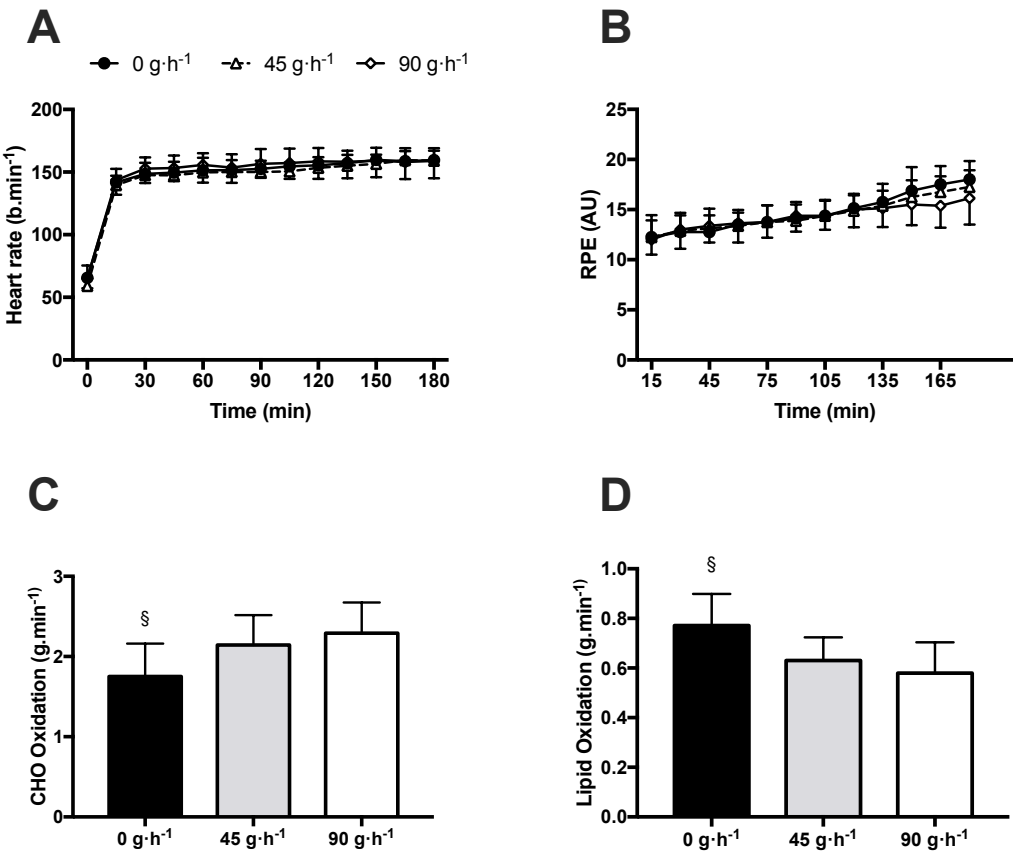

Figure 6. 3. (A) Heart rate and (B) during exercise. (C) Average rate of CHO and (D) lipid oxidation during exercise. §$P < 0.05$, significantly different from 90 g.h^{-1}, Data is presented as mean ± SD.

6.4.3 Skeletal muscle signalling

Exercise significantly increased the phosphorylation of AMPK^{Thr172} ($P < 0.001$) although increases (~ 4-fold) were similar between conditions ($P = 0.851$). Similarly, exercise increased the phosphorylation of both CaMKII^{Thr286} (2-fold; $P = 0.003$) and p53^{Ser15} (2-fold; $P. = 0.025$) independent of CHO availability ($P = 0.360$ and $P = 0.784$, respectively). In contrast, neither p38 MAPK$^{Thr180/Tyr182}$ (main effect of condition; $P = 0.645$, time; $P = 0.064$) or CREB^{Ser133} (main effect of condition; $P = 0.601$, time; $P = 0.733$) phosphorylation were significantly altered by either exercise or CHO availability, which both displayed large inter-individual variance in their responses. Western blots are shown in Figure 6.5.

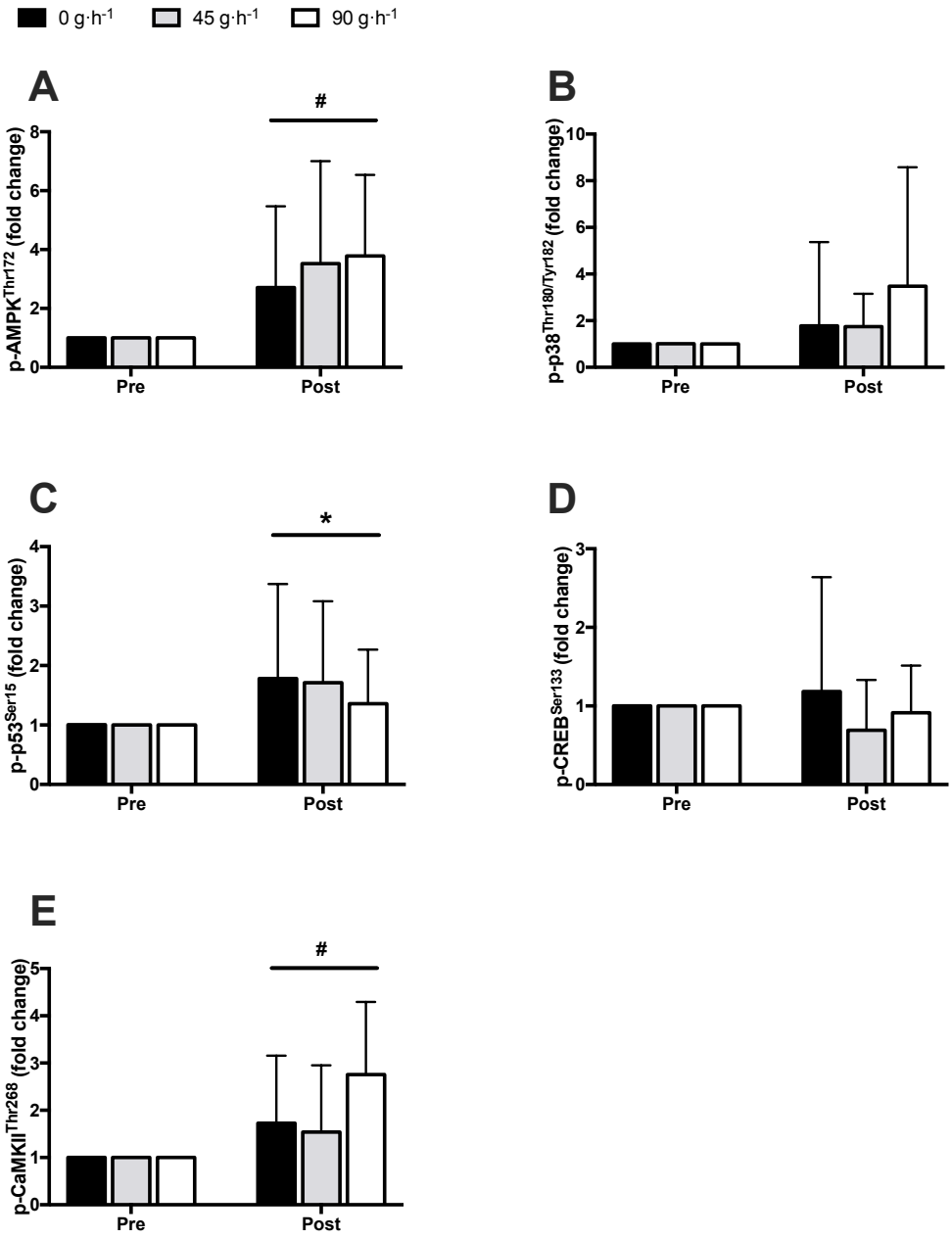

Figure 6. 4. (A) AMPK^{Thr172} phosphorylation, (B) p38$^{Thr180/Tyr182}$ phosphorylation, (C) p53^{Ser15} phosphorylation, (D) CREB^{Ser133} phosphorylation and (E) CaMKII^{Thr286} phosphorylation at pre- and post-exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, Data is presented as means ± SD

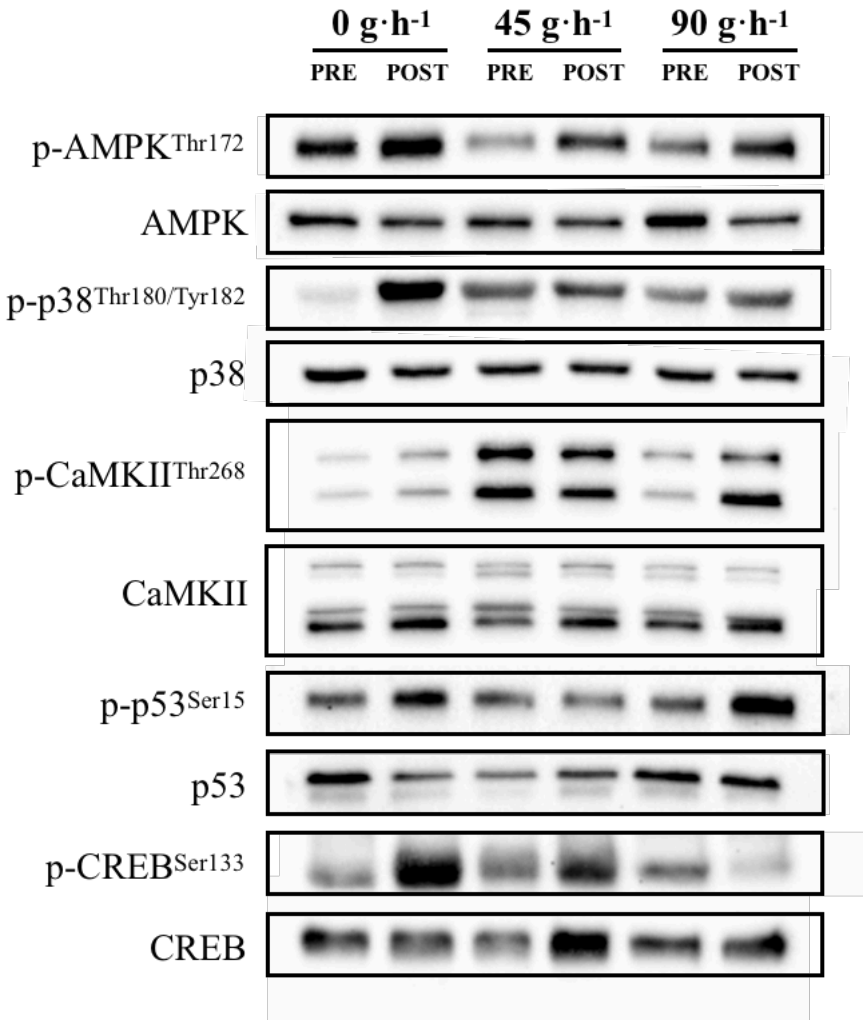

Figure 6. 5. Representative Western blot images at pre-exercise, work-matched time point and post-exercise

6.5 Discussion

The aim of the present study was to test the hypothesis that exogenous CHO feeding during prolonged endurance exercise would modulate exercise-induced skeletal muscle signalling. Using trained male cyclists, we provide novel data by demonstrating that exogenous CHO feeding during 180 min of steady state exercise does not alter muscle glycogen utilisation nor does it alter post-exercise mitochondrial-related signalling. In the context of manipulating CHO availability around training, these data suggest that CHO feeding during exercise does not provide a glycogen sparing effect within skeletal muscle but does not impair the acute regulatory signalling associated with mitochondrial biogenesis.

In order to ensure exercise was commenced with sufficient endogenous CHO availability, subjects performed a bout of evening glycogen depletion exercise followed by high CHO intake in the immediate 3 h recovery period (1.2 $g.kg^{-1}$ per hour), the following day (8 $g.kg^{-1}$)

and 3 h prior (3 $g.kg^{-1}$) to commencing the steady state cycling protocol, in accordance with current nutritional guidelines (Thomas *et al.*, 2018) to maximise both muscle and liver glycogen. Whilst our intended model was successful in achieving pre-exercise glycogen concentrations (0g; 767 ± 87, 45g; 742 ± 109, 90g; 698 ± 98 $mmol \cdot kg^{-1}$ dw) consistent with a CHO loaded state (Areta & Hopkins, 2018) the consumption of graded quantities of exogenous CHO during exercise did not significantly alter the pattern of glycogen utilisation (0g; 515 ± 93, 45g; 473 ± 133, 90g; 405 ± 132 $mmol \cdot kg^{-1}$ dw) during exercise. These results are consistent with previous findings (Stellingwerff *et al.*, 2007; Gonzalez *et al.*, 2015) that demonstrate exogenous CHO feeding at a rate of 50 and 102 $g.h^{-1}$, respectively, does not alter whole muscle glycogen utilisation during 3 h of endurance exercise. However, exogenous CHO feeding does appear to significantly reduce muscle glycogen utilisation within type I fibres across 3 h (Stellingwerff *et al.*, 2007) and type IIa fibres across 2 h of endurance exercise (De Bock *et al.*, 2006). With this in mind, future studies should aim to investigate the effects of exogenous CHO feeding on muscle fibre type specific protein signalling, given the differential regulation of AMPK and downstream targets between fibre types (Kristensen *et al.*, 2015).

In relation to post-exercise signalling, we observed comparable exercise induced increases in $AMPK^{Thr172}$ phosphorylation between all trials, independent of exogenous CHO availability (Figure 6.4A). These findings support previous suggestions that CHO ingestion, either before or during exercise (or in combination) do not alter skeletal muscle AMPK signalling (De Bock *et al.*, 2005; Akerstrom *et al.*, 2006) when the rate of glycogen utilisation is unaltered. Thus, the comparable increases in $AMPK^{Thr172}$ phosphorylation observed within the present study are likely explained by the similar pattern of glycogen utilisation (~ 50%) across all trials given the well documented effects of muscle glycogen on AMPK signalling within skeletal muscle (Wojtaszewski *et al.*, 2003; Steinberg *et al.*, 2006; Yeo *et al.*, 2010; Bartlett *et al.*, 2013). Given the physical tethering of AMPK to the glycogen granule via its ß-subunit, it is proposed that exercise-induced glycogen depletion results in the dissociation of AMPK from the glycogen granule and allows greater access for kinase phosphorylation (Steinberg *et al.*, 2006).

In contrast to AMPK, we did not observe any change in the phosphorylation status of p38 $MAPK^{Thr180/Tyr182}$ either in response to exercise or exogenous CHO feeding, though we note the large inter-individual variability between subjects. When taken together with previous data that demonstrate p38 MAPK phosphorylation is unaltered by endogenous CHO availability (Yeo *et al.*, 2010; Bartlett *et al.*, 2013) or pre-exercise CHO feeding (Stocks *et al.*, 2019), these data provide further evidence to support the notion that substrate availability does not modulate the phosphorylation of p38 MAPK in whole muscle homogenate. Although p38

MAPK phosphorylation does not appear to be affected by exercise intensity (Egan *et al.*, 2010; Bartlett *et al.*, 2012), its phosphorylation status may be more sensitive to fluctuations in specific metabolites during exercise (Combes *et al.*, 2015). Specifically, exercise-induced increases in p38 MAPK phosphorylation appear to strongly correlate with fluctuations in both plasma lactate and ATP (Fiorenza *et al.*, 2018). Given the continuous nature of the present exercise bout, when performed at 100% LT, large incremental fluctuations in plasma lactate would be unexpected (Baldwin *et al.*, 2000) and thus, it could be suggested that insufficient metabolic stress may explain the lack of p38 MAPK phosphorylation. The lack of p38 MAPK phosphorylation is, however, interesting given the observed changes in CaMKII phosphorylation, as p38 MAPK phosphorylation also appears to be sensitive to cytosolic calcium (Wright *et al.*, 2007*a*).

Similar to that of AMPK, we observed comparable exercise induced increases in CaMKII^{Thr268} phosphorylation between all trials. (Figure 6.4E). Although CaMKII phosphorylation appears to occur in an intensity dependent manner (Rose *et al.*, 2006; Egan *et al.*, 2010) the observed increases within the present data may reflect continuous calcium release from the SR with prolonged contraction (Baar, 2014) opposed to the intermittent oscillations likely observed with high-intensity exercise (Rose *et al.*, 2006). With this in mind, the lack of observed change in CaMKII phosphorylation in response to high-intensity exercise within chapter 4 suggest that exercise duration may also be an important regulator of CaMKII phosphorylation due to the continuous exposure to calcium during prolonged exercise. Nonetheless, we also demonstrate that exogenous CHO availability does not appear to modulate CaMKII phosphorylation. One mechanism by which exogenous CHO feeding may alter CaMKII signalling, would be via alterations in muscle glycogen depletion, given that low muscle glycogen (< 300 mmol·kg^{-1} dw) is associated with impaired SR Ca^{2+} release (Gejl *et al.*, 2014). However, given the comparable post-exercise muscle glycogen concentrations between trials, the present results are unsurprising.

In addition to the primary upstream signalling kinases, metabolic adaptations within skeletal muscle are regulated by downstream transcription factors and co-activators, including p53 and CREB. The tumor suppressor protein p53 has recently emerged as part of the signalling network controlling exercise-induced training adaptations and may be phosphorylated via upstream kinases AMPK and p38 MAPK (She *et al.*, 2001; Jones *et al.*, 2005). In accordance with this, the present study demonstrates comparable exercise-induced increases in p53^{Ser15} phosphorylation in concert with the observed increase in AMPK^{Thr172} phosphorylation. Although augmented p53^{Ser15} phosphorylation has been observed in response to endurance exercise with low muscle glycogen (Bartlett *et al.*, 2013), the present data suggest that p53 may

not be sensitive to exogenous carbohydrate availability. Given the ability of both AMPK and CaMKII to phosphorylate CREB (Egan *et al.*, 2010), we also examined CREB signalling in response to both exercise and exogenous CHO feeding. However, despite the observed increases in post-exercise AMPK and CaMKII phosphorylation, we observed no change in the phosphorylation status of $CREB^{Ser133}$ either in response to exercise or exogenous CHO feeding. These findings may be explained by our chosen sampling time point, given that increases in CREB phosphorylation are typically observed 3 h post-exercise and are actually reduced immediately upon the completion of exercise (Egan *et al.*, 2010; Stocks *et al.*, 2019) as seen in the present data. Unfortunately, owing to a lack of muscle tissue, we were unable to measure the transcription of genes with putative roles in mitochondrial biogenesis, namely PGC-1α given its purported role as a master regulator. Nonetheless, others have reported that PGC-1α mRNA expression is unaffected by exogenous CHO feeding during exercise (Cluberton *et al.*, 2005).

In summary, we provide novel data by demonstrating that steady state endurance exercise induces phosphorylation of signalling kinases AMPK and CaMKII and downstream transcription factor p53, independent of exogenous CHO availability. These data extend previous findings by characterising the response of alternative signalling pathway responses to carbohydrate feeding during endurance exercise and, when taken together, indicate that exogenous CHO feeding does not modulate post-exercise signalling responses regulating mitochondrial biogenesis in conditions where muscle glycogen utilisation is unaltered.

Chapter Seven:

Synthesis of findings

7.1 Achievement of aims and objectives

The primary aim of this thesis was to examine the effects of both endogenous and exogenous CHO availability on the regulation of molecular pathways associated with mitochondrial biogenesis. On this basis, a secondary aim was to investigate the existence of a potential muscle glycogen threshold and provide an understanding of its potential upper and lower limits.

Aim 1 - To examine the effects of graded reductions in pre-exercise muscle glycogen (below 300 mmol·kg^{-1} dw) on skeletal muscle cell signalling and gene expression responses associated with the regulation of mitochondrial biogenesis and substrate metabolism (Study 1, Chapter 4).

When matched for work done, commencing exercise with low muscle glycogen appears to augment many acute signalling responses that regulate training adaptation. However, the absolute concentration of muscle glycogen required to achieve such responses is unclear. Using a sleep-low, train-low model, the data presented within this chapter demonstrate that further reductions in pre-exercise muscle glycogen below 300 mmol·kg^{-1} dw do not augment early post-exercise signalling or transcriptional responses and, in fact, may impair exercise capacity. A novel aspect of the chosen study design was that we employed a sampling point in both the H-CHO and M-CHO trials that was matched to the point of exhaustion in the L-CHO trial, thus allowing for the assessment of mitochondrial related signalling events at both work-matched and exhaustive exercise time points, whilst also allowing for the assessment of exercise capacity.

Aim 2 – To examine the effects of graded reductions in pre-exercise muscle glycogen (between the range of 100-600 mmol·kg^{-1} dw) on skeletal muscle cell signalling and gene expression responses associated with the regulation of mitochondrial biogenesis and substrate metabolism (Study 2, Chapter 5).

Having observed no additional benefit of stepwise reductions in pre-exercise muscle glycogen concentrations below 300 mmol·kg^{-1} dw (100-300 mmol·kg^{-1} dw) on exercise-induced cell signalling or gene expression following exhaustive exercise in study 1, study 2 required the investigation of a wider range of muscle glycogen concentrations to be used during work-matched, non-exhaustive exercise. Using a modified version of the sleep-low, train-low model from study 1 (in order to allow for greater muscle glycogen resynthesis) the data presented in this study extend the findings of study 1 and demonstrate no benefit of commencing exercise

with reduced muscle glycogen on early signalling and gene expression responses regulating mitochondrial biogenesis.

Aim 3 – To examine the effects of graded exogenous CHO intake during exercise on skeletal muscle cell signalling responses associated with the regulation of mitochondrial biogenesis (Study 3, Chapter 6).

In addition to muscle glycogen, exogenous CHO feeding during exercise also has the ability to induce changes in metabolism and thus modulate skeletal muscle signalling regulating mitochondrial biogenesis, although the early signalling responses to CHO feeding during exercise are not well characterised. Furthermore, data derived from both study 1 and 2 raise the possibility that post-exercise muscle glycogen may also be an important factor in regulating such responses and thus, exogenous CHO feeding may provide a practical model in which post-exercise muscle glycogen can be modified whilst matching total work done. However, the data within chapter 6 demonstrate that feeding graded quantities of CHO during exercise (ranging from 0 to 90 g h^{-1}) does not alter muscle glycogen utilisation or post-exercise signalling cascades regulating mitochondrial biogenesis.

7.2 General discussion & practical implications

Since the initial discovery and description of glycogen in 1857, its role has evolved considerably, from that of a simple energy source through to a potential regulator of molecular signalling events in response to exercise. The concept of train-low is founded on the premise that undertaking endurance exercise with reduced muscle glycogen induces significant metabolic shifts compared with when exercise is undertaken with high CHO availability. For instance, at comparable work intensities, exercise with low CHO availability substantially reduces glycogen utilisation (Arkinstall *et al.*, 2004) whilst concomitantly increasing circulatory free fatty acid (FFA) availability (Wojtaszewski *et al.*, 2003) and circulatory catecholamines (Hansen *et al.*, 2005). Consequently, the altered metabolic milieu created through exercising with low CHO availability has a direct impact on molecular signalling events controlling training adaptation, which appears to occur through the AMPK/PGC-1α pathway.

Does pre-exercise muscle glycogen concentration regulate training responses?

Given that the enhanced training response associated with train-low is potentially mediated by muscle glycogen availability, examination of available train-low studies (Figure 2.10) that demonstrate augmented mitochondrial-related signalling suggest these responses are particularly evident when absolute pre- exercise muscle glycogen concentrations are < 300 $mmol \cdot kg^{-1}$ dw and elude to the potential existence of a glycogen threshold. Nonetheless, it should be

acknowledged that augmented skeletal muscle signalling is not always observed with reduced pre-exercise muscle glycogen (Lane *et al.*, 2015; Impey *et al.*, 2016; Hammond *et al.*, 2019) or that any potential threshold does not allude to the suggestion that individuals will not adapt to endurance exercise if they do not exercise with glycogen concentrations below the proposed limit. In support of this, the data generated from study 1 and 2 demonstrate that, in the context of the high-intensity endurance exercise, reducing pre-exercise muscle glycogen confers no additional benefit to molecular signalling events that govern exercise-induced adaptation. Despite marked differences in pre-exercise muscle glycogen, the lack of augmented effect may be explained by the fact that absolute glycogen concentrations were reduced to concentrations already associated with enhanced mitochondrial-related signalling (i.e. < 300 mmol·kg^{-1} dw) across all trials (Figure 7.1) likely reflective of the high-intensity nature of the exercise protocols undertaken. This rationale seems plausible given that muscle glycogen appears to modulate the activity of AMPK through its physical tethering of the glycogen binding domain located on the β sub-unit, and thus, it is the absolute concentration of glycogen (regardless of whether is achieved through pre-exercise nutritional manipulation or degradation during exercise) that is the primary driver, allowing for the de-attachment of AMPK from the glycogen granule and increased access for AMPK phosphorylation. When taken together, these data raise the possibility that obtaining low absolute muscle glycogen concentrations post-exercise may also be an important factor in augmenting mitochondrial-related signalling.

Does post-exercise muscle glycogen concentration regulate training responses?

In support of this rationale, post-exercise muscle glycogen concentrations appear to regulate exercise-induced PGC-1α mRNA expression (Psilander, 2014; Fiorenza *et al.*, 2018) and total protein abundance (Mathai *et al.*, 2008), whilst the maintenance of low post-exercise muscle glycogen prolongs exercise induced increases in PGC-1α mRNA expression. Furthermore, our lab has previously demonstrated comparable signalling responses can still be achieved despite marked differences in pre-exercise muscle glycogen (600 vs. 300 mmol·kg^{-1} dw) when muscle glycogen is depleted to similar levels (~ 100 mmol.kg^{-1} dw) post-exercise (Impey *et al.*, 2016). Upon this basis, the high rates of glycogen utilisation and subsequent achievement of low post-exercise glycogen concentrations in study 1 and 2 raise the possibility that when post-exercise muscle glycogen concentrations are depleted to concentrations associated with mitochondrial-related signalling (i.e. < 300 mmol·kg^{-1} dw; Figure 7.1) the impact of pre-exercise glycogen availability is reduced. Collectively, these data raise the possibility that absolute post-exercise glycogen concentrations (in combination with the amount of glycogen

utilised during exercise) may play an important role in regulating mitochondrial-related signalling although further research is now warranted to directly test this hypothesis.

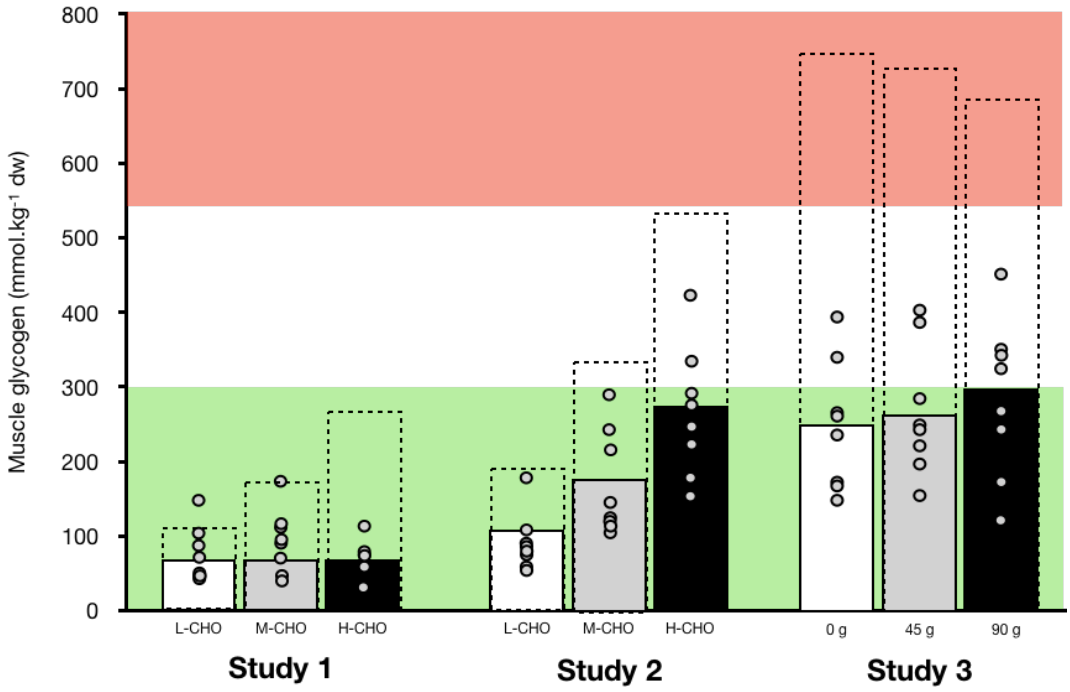

Figure 7. 1. Mean post-exercise muscle glycogen concentrations across experimental trials in study 1, 2 and 3. Individual post-exercise muscle glycogen concentrations are shown in grey circles. Mean pre-exercise muscle glycogen concentrations are represented by dashed bars to indicate the magnitude of glycogen depletion during the exercise bout. Data indicate that mean post-exercise muscle glycogen concentrations across all experimental trials reached low (i.e. < 300 mmol·kg^{-1} dw) levels.

Sending the signal: The interaction of muscle glycogen concentrations and exercise stress

The goal of endurance training is to induce an array of physiological and metabolic adaptations and is underpinned by disruptions in cellular homeostasis required to induce the activation of signalling networks that govern such responses. Whilst the manipulation of muscle glycogen concentrations presents one strategy to induce such cellular perturbations, the importance of key training variables (e.g. intensity and duration) should not be overlooked given their role in regulating mitochondrial related signalling (Stephens *et al.*, 2002; Egan *et al.*, 2010; Granata *et al.*, 2017). As such, the role of nutrient availability should be considered within the context of the specific exercise being undertaken whereby the manipulation of muscle glycogen may only induce modulatory effects on skeletal muscle that has not been subjected to sufficient local metabolic challenge via the manipulation of both exercise intensity and duration. In essence, when exercise stress is low, commencing the exercise bout with low-

pre-exercise glycogen concentrations may provide a viable strategy to induce perturbations to cellular homeostasis required to activate mitochondrial related signalling cascades. However, if exercise is of sufficient intensity and duration to induce cellular perturbations (and coincidently the attainment of low absolute muscle glycogen concentrations) commencing such exercise with low muscle glycogen appears unnecessary given the potential to impair exercise capacity.

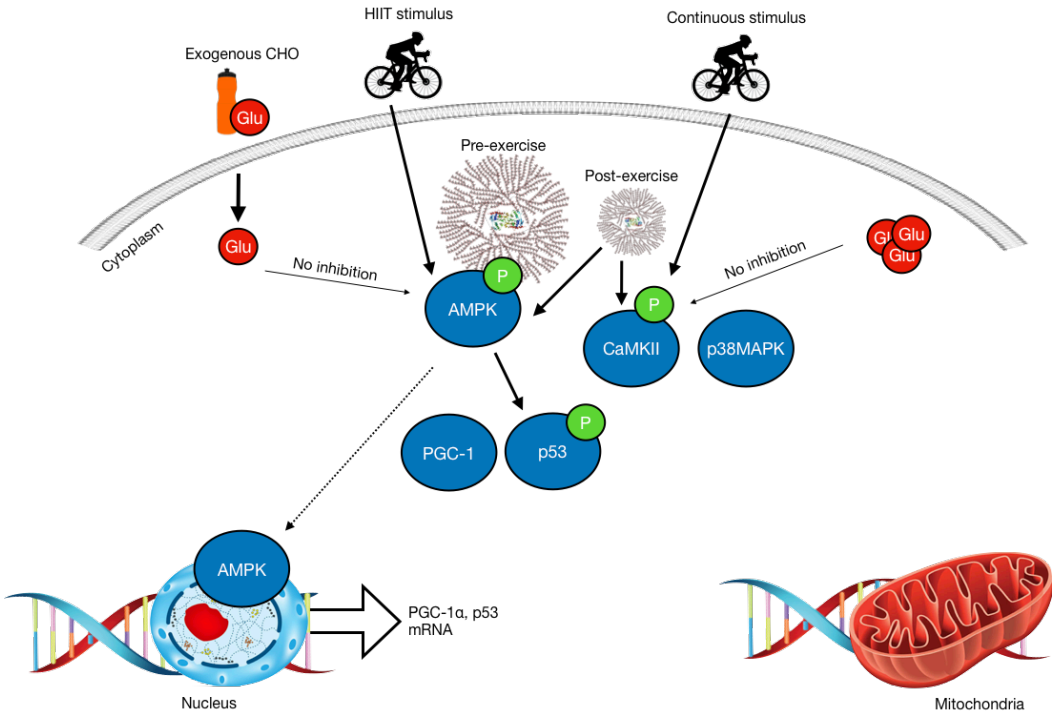

Figure 7. 2. Schematic summary of the alterations in skeletal muscle signalling originating from this thesis. Commencing high-intensity endurance exercise with low pre-exercise muscle glycogen concentrations does not appear to augment mitochondrial related-signalling events when exercise induces sufficient glycogen depletion, and results in low post-exercise glycogen concentrations. High-intensity endurance exercise results in AMPK phosphorylation and translocation to the nucleus and results in the augmented mRNA expression of both PGC-1α and p53 whilst continuous exercise may also phosphorylate CaMKII. Furthermore, feeding exogenous CHO during exercise does not attenuate AMPK, CaMKII or p53 signalling, potentially due to the lack of muscle glycogen sparing observed.

Practical application

Practically, these data ultimately lend support to the concept of 'fuelling for the work required' whereby CHO availability is adjusted in accordance with the demands of the specific training session to be completed. When considering the glycogen cost of habitual training sessions, it should be noted that driving glycogen depletion would likely be more difficult and require considerably more work in well-trained individuals (Gejl *et al.*, 2017) given they display an enhanced capacity for glycogen storage and reduced utilisation during exercise (Karlsson *et*

al., 1974; Henriksson, 1977; Coyle *et al.*, 1988; Areta & Hopkins, 2018). In practice, it appears that careful consideration of the individuals training status and the metabolic demands of each training session is required to ensure appropriate day-to-day periodisation of CHO in order to create a metabolic milieu conducive to facilitating the metabolic adaptations associated with train-low.

7.3 Limitations

Amalgamating train-low models – In both chapter 4 and 5, we adopted a sleep-low, train-low model that utilised an amalgamation of multiple train-low models (e.g. low glycogen, fasted exercise, post-exercise CHO restriction), all of which are able to independently modify acute signalling responses to exercise. With this in mind, the effect of absolute muscle glycogen concentrations may have been dampened and may confine the applicability of the present data to the sleep-low, train-low model. Furthermore, given that the pre-exercise biopsy was sampled within ~14 h of glycogen depleting exercise in study 1, it is difficult to determine whether mRNA expression was already elevated at pre-exercise which may also result in a dampened response to exercise. Although the time-course of gene expression is not well characterized, some the mRNA expression of certain genes measured within this chapter are elevated for up to 24 h post-exercise which coincides with the present pre-exercise sampling time point. Based upon this, future studies may wish to employ a longer period between exercise bouts, as can be observed within chapter 5.

Translation of acute signalling data – Whilst an understanding of the acute signal transduction network that regulates mitochondrial biogenesis provides useful insights into the early responses that regulate training adaptation, there appears to be a disconnect between the activation of such pathways and functional increases in protein abundance (Hornberger, 2016; Granata *et al.*, 2018). Furthermore, the assessment of post-translational modifications (i.e. phosphorylation) within chapter 4 and 6 can only act as a proxy marker for the activity of such proteins. Therefore, future studies will benefit from the development of specific kinase activity assays in order to provide a more detailed understanding of intracellular signalling in response to carbohydrate manipulation.

Subcellular fractionation – Although the fractionation protocol used within this thesis was successful in achieving samples enriched with either nuclear or cytosolic proteins, it should be noted that this method is unable to provide 'pure' fractions without any contamination from other cellular regions. Furthermore, our fractionation protocol was performed on frozen tissue samples which may have resulted in the fracture of nuclei and therefore selectively limit

analysis of these fractions to those that survived the freeze/thaw process. Unfortunately, due to a lack of muscle lysate generated from the fractionation protocol, it was not possible to measure the phosphorylation status of several proteins which would yield important information to their sub-fraction activation in response to exercise.

Dietary protocols – Whilst the dietary protocols used in this thesis were successful in inducing differences in muscle glycogen concentrations, it should be noted such differences also result in differences in energy availability between trials and thus, the responses observed may not solely be related to carbohydrate manipulation *per se.* Although recent data demonstrate that acute alterations in energy availability do not influence mRNA expression in response to exercise (Hammond *et al.*, 2019) its effect on other early signalling responses to exercise (i.e. protein phosphorylation) are not well understood.

7.4 Recommendations for future research

Recommendation 1 – Based upon the data obtained from chapter 5, it appears that AMPK nuclear translocation may, in part, regulate the exercise-induced increase in PGC-1α mRNA expression. However, future studies should examine the phosphorylation status of AMPK, as well as other signalling kinases and transcription factors, within the nucleus in order to further elucidate the molecular mechanisms that may underpin adaptation to exercise and the modulatory role CHO availability may have on this pathway. Furthermore, measurement of translocation and phosphorylation status of such proteins within mitochondrial fractions would provide a more comprehensive understanding of such pathways.

Recommendation 2 – Whilst the data presented within this thesis has primarily focused on the transcription of genes and post-translational modification of proteins known to be regulated by either exercise and/or substrate availability within skeletal muscle this represents a small, selective part of the signal transduction network that regulates skeletal muscle adaptation. With this in mind, the adoption of global 'omic' approaches to quantify metabolic, transcriptional and phosphorylation responses to exercise with low CHO availability would allow for a greater understanding of the signal transduction network that is regulated by CHO availability and help to identify novel regulators of train-low. Furthermore, this approach would allow for the adoption of multiple linear regression analysis to allow for a better understanding of the relationships between changes in metabolic and molecular variables in response to low CHO exercise.

Recommendation 3 – The data obtained from both chapter 4 and 5, appear to suggest that post-exercise muscle glycogen may be a critical factor regulating signalling responses to exercise. Given that muscle glycogen utilisation during exercise is intensity-dependent, future studies should address whether train-low sessions should be left to low-intensity exercise sessions (which typically do not result in substantial depletion of muscle glycogen) or whether the completion of high-intensity exercise to further drive glycogen depletion is required. In light of this, an understanding of the modulatory role of training status should also be considered given the differential use of muscle glycogen between trained and untrained individuals.

Recommendation 4 – The data obtained from chapter 6, demonstrate that exogenous CHO feeding during exercise does not modulate post-exercise signalling responses to exercise. However, given that exogenous CHO feeding may spare muscle glycogen in a fibre-type dependent manner, future studies should now investigate fibre-type specific signalling in response to CHO feeding, given that AMPK and its downstream targets are activated in a fibre-type dependent manner (Kristensen *et al.*, 2015).

7.5 Closing thoughts

In summary, the data presented within this thesis demonstrate that neither pre-exercise muscle glycogen nor exogenous CHO feeding during exercise appear to significantly modify early signalling responses to endurance exercise. These data raise the possibility that the absolute concentration of muscle glycogen post-exercise may also be an important factor in regulating the molecular responses that underpin exercise adaptation although this now warrants direct investigation. From a practical perspective, these data suggest that performing exercise with reduced CHO availability may not be required when exercise is of sufficient intensity and/or duration to induce sufficient changes to the cellular milieu and the depletion of sufficient muscle glycogen. As such, low CHO training may be best suited to situations where exercise intensity and/or duration is insufficient to elicit metabolic perturbations (e.g. prolonged low-intensity exercise completed below lactate threshold). Restriction of CHO availability for the latter training sessions would also circumvent the impairment in self-selected training intensity observed when high-intensity exercise is performed with reduced muscle glycogen (Yeo *et al.*, 2008; Lane *et al.*, 2013). Nonetheless, these data now pose several fundamental questions that are relevant across many sporting models, in order to further our understanding of the train-low paradigm (see section 7.4). When considered in this way, it is remarkable that the study of only 500 g of substrate (the approximate whole- body storage of CHO) remains as exciting as ever.

Chapter Eight:

References

Akerstrom TCA, Birk JB, Klein DK, Erikstrup C, Plomgaard P, Pedersen BK & Wojtaszewski JFP (2006). Oral glucose ingestion attenuates exercise-induced activation of 5′-AMP-activated protein kinase in human skeletal muscle. *Biochem Biophys Res Commun* **342,** 949–955.

Akimoto T, Li P & Yan Z (2008). Functional interaction of regulatory factors with the Pgc-1alpha promoter in response to exercise by in vivo imaging. *Am J Physiol - Cell Physiol* **295,** C288–C292.

Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS & Yan Z (2005). Exercise stimulates Pgc-1α transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem* **280,** 19587–19593.

American Dietetic Association (1993). Position of The American Dietetic Association and The Canadian Dietetic Association: Nutrition for physical fitness and athletic performance for adults. *J Am Diet Assoc* **93,** 691–696.

Andersen P & Henriksson J (1977). Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* **270,** 677–690.

Andrade-Souza VA, Ghiarone T, Sansonio A, Silva KAS, Tomazini F, Arcoverde L, Fyfe J, Perri E, Saner N, Kuang J, Bertuzzi R, Leandro CG, Bishop DJ & Lima-Silva AE (2019). Exercise twice-a-day potentiates markers of mitochondrial biogenesis in men. *FASEB J* **34,** 1602–1619.

Arany Z, Lebrasseur N, Morris C, Smith E, Yang W, Ma Y, Chin S & Spiegelman BM (2007). The transcriptional coactivator PGC-1β drives the formation of oxidative type IIX fibers in skeletal muscle. *Cell Metab* **5,** 35–46.

Areta JL & Hopkins WG (2018). Skeletal muscle glycogen content at rest and during endurance exercise in humans: A meta-analysis. *Sport Med* **48,** 2091–2102.

Arkinstall M, Bruce C, Clark SA, Rickards C, Burke LM & Hawley JA (2004). Regulation of fuel metabolism by preexercise muscle glycogen content and exercise intensity. *J Appl Physiol* **97,** 2275–2283.

Baar K (2014). Nutrition and the adaptation to endurance training. *Sport Med*; DOI: 10.1007/s40279-014-0146-1.

Baar K & McGee SL (2008). Optimising training adaptations by manipulating glycogen. *Eur J Sport Sci* **8,** 97–106.

Baldwin J, Snow RJ & Febbraio MA (2000). Effect of training status and relative exercise intensity on physiological responses in men. *Med Sci Sports Exerc* **32,** 1648–1654.

Baldwin KM, Fitts RH, Booth FW, Winder WW & Holloszy JO (1975). Depletion of muscle and liver glycogen during exercise - Protective effect of training. *Pflügers Arch Eur J Physiol* **354,** 203–212.

Bartlett JD, Close GL, Drust B & Morton JP (2014). The emerging role of p53 in exercise metabolism. *Sport Med* **44,** 303–309.

Bartlett JD, Hwa Joo C, Jeong T-S, Louhelainen J, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B & Morton JP (2012). Matched work high-intensity interval and continuous running induce similar increases in PGC-1 mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol* **112,** 1135–1143.

Bartlett JD, Louhelainen J, Iqbal Z, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B & Morton JP (2013). Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. *AJP Regul Integr Comp Physiol* **304,** R450–R458.

Bass JJ, Wilkinson DJ, Rankin D, Phillips BE, Szewczyk NJ, Smith K & Atherton PJ (2017). An overview of technical considerations for Western blotting applications to physiological research. *Scand J Med Sci Sport* **27,** 4–25.

Benton CR, Nickerson JG, Lally J, Han XX, Holloway GP, Glatz JFC, Luiken JJFP, Graham TE, Heikkila JJ & Bonen A (2008). Modest PGC-1α overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. *J Biol Chem* **283,** 4228–4240.

Bergström J, Hermansen L, Hultman E & Saltin B (1967). Diet, muscle glycogen and physical performance. *Acta Physiol Scand* **71,** 140–150.

Bergström J & Hultman E (1966). Muscle glycogen synthesis after exercise: An enhancing factor localized to the muscle cells in man. *Nature* **210,** 309–310.

Bjorkman O, Sahlin K, Hagenfeldt L & Wahren J (1984). Influence of glucose and fructose ingestion on the capacity for long-term exercise in well-trained men. *Clin Physiol* **4,** 483–494.

De Bock K, Derave W, Eijnde BO, Hesselink MK, Koninckx E, Rose AJ, Schrauwen P, Bonen A, Richter EA & Hespel P (2008). Effect of training in the fasted state on metabolic responses during exercise with carbohydrate intake. *J Appl Physiol* **104,** 1045–1055.

De Bock K, Derave W, Ramaekers M, Richter EA & Hespel P (2006). Fiber type-specific muscle glycogen sparing due to carbohydrate intake before and during exercise. *J Appl Physiol* **102,** 183–188.

De Bock K, Richter EA, Russell AP, Eijnde BO, Derave W, Ramaekers M, Koninckx E, Léger B, Verhaeghe J & Hespel P (2005). Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans. *J Physiol* **564,** 649–660.

Bolster DR, Crozier SJ, Kimball SR & Jefferson LS (2002). AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian

target of rapamycin (mTOR) signaling. *J Biol Chem* **277,** 23977–23980.

Boppart MD, Asp S, Wojtaszewski JFP, Fielding RA, Mohr T & Goodyear LJ (2000). Marathon running transiently increase c-Jun NH2-terminal kinase and p38γ activities in human skeletal muscle. *J Physiol* **526,** 663–669.

Borg GA. (1982). Psychophysical bases of percieved exertion. *Med Sci Sport Exerc* **14,** 377–381.

Bosch AN, Noakes TD & Dennis SC (2007). Influence of carbohydrate-loading on fuel substrate turn-over and oxidation during prolonged exercise. *Med Sci Sport Exerc* **24,** S122.

Burke LM, Collier GR, Beasley SK, Davis PG, Fricker PA, Heeley P, Walder K & Hargreaves M (1995). Effect of coingestion of fat and protein with carbohydrate feedings on muscle glycogen storage. *J Appl Physiol* **78,** 2187–2192.

Burke LM, Collier GR, Davis PG, Fricker PA, Sanigorski AJ & Hargreaves M (1996). Muscle glycogen storage after prolonged exercise: Effect of the frequency of carbohydrate feedings. *Am J Clin Nutr* **64,** 115–119.

Burke LM, Collier GR & Hargreaves M (1993). Muscle glycogen storage after prolonged exercise: effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* **75,** 1019–1023.

Burke LM, Hawley JA, Jeukendrup A, Morton JP, Stellingwerff T & Maughan RJ (2018). Toward a common understanding of diet–exercise strategies to manipulate fuel availability for training and competition preparation in endurance sport. *Int J Sport Nutr Exerc Metab* **28,** 451–463.

Burke LM, Ross ML, Garvican-Lewis LA, Welvaert M, Heikura IA, Forbes SG, Mirtschin JG, Cato LE, Strobel N, Sharma AP & Hawley JA (2017). Low carbohydrate, high fat diet impairs exercise economy and negates the performance benefit from intensified training in elite race walkers. *J Physiol* **595,** 2785–2807.

Bussau VA, Fairchild TJ, Rao A, Steele P & Fournier PA (2002). Carbohydrate loading in human muscle: An improved 1 day protocol. *Eur J Appl Physiol* **87,** 290–295.

Cartee GD, Young DA, Sleeper MD, Zierath J, Wallberg-Henriksson H & Holloszy JO (1989). Prolonged increase in insulin-stimulated glucose transport in muscle after exercise. *Am J Physiol - Endocrinol Metab* **256,** 494–499.

Casey A, Mann R, Banister K, Fox J, Morris P, Macdonald I & Greenhaff P (2000). Effect of carbohydrate ingestion on glycogen resynthesis in human liver and skeletal muscle, measured by (13)C MRS. *Am J Physiol Endocrinol Metab* **278,** 65–75.

Chan MHS, McGee SL, Watt MJ, Hargreaves M & Febbraio MA (2004). Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of nuclear p38 MAP kinase in human skeletal muscle: association with IL-6 gene transcription during

contraction. *FASEB J* **18,** 1785–1787.

Chen Z, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, Kemp BE & Mcconell GK (2003). Effect of exercise intensity of skeletal muscle AMPK signaling in humans. *Diabetes* **52,** 2205–2212.

Chin ER, Balnave CD & Allen DG (1997). Role of intracellular calcium and metabolites in low-frequency fatigue of mouse skeletal muscle. *Am J Physiol - Cell Physiol*; DOI: 10.1152/ajpcell.1997.272.2.c550.

Chryssanthopoulos C, Williams C, Nowitz A & Bogdanis G (2004). Skeletal muscle glycogen concentration and metabolic responses following a high glycaemic carbohydrate breakfast. *J Sports Sci* **22,** 1065–1071.

Civitarese AE, Hesselink MKC, Russell AP, Ravussin E & Schrauwen P (2005). Glucose ingestion during exercise blunts exercise-induced gene expression of skeletal muscle fat oxidative genes. *Am J Physiol Metab* **289,** E1023–E1029.

Cluberton LJ, McGee SL, Murphy RM & Hargreaves M (2005). Effect of carbohydrate ingestion on exercise-induced alterations in metabolic gene expression. *J Appl Physiol* **99,** 1359–1363.

Cochran AJR, Little JP, Tarnopolsky MA & Gibala MJ (2010). Carbohydrate feeding during recovery alters the skeletal muscle metabolic response to repeated sessions of high-intensity interval exercise in humans. *J Appl Physiol* **108,** 628–636.

Cochran AJR, Percival ME, Tricarico S, Little JP, Cermak N, Gillen JB, Tarnopolsky MA & Gibala MJ (2014). Intermittent and continuous high-intensity exercise training induce similar acute but different chronic muscle adaptations. *Exp Physiol* **99,** 782–791.

Coffey VG & Hawley J a (2007). The Molecular Basis of Training Adaptation. *Sport Med* **37,** 737–763.

Coffey VG, Zhong Z, Shield A, B.J C, Chibalin A V., Zierath JR & Haw (2006). Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB J* **20,** 190–192.

Coggan AR & Coyle EF (1988). Effect of carbohydrate feedings during high-intensity exercise. *J Appl Physiol* **65,** 1703–1709.

Coggan AR, Kohrt WM, Spina RJ, Bier DM & Holloszy JO (1990). Endurance training decreases plasma glucose turnover and oxidation during moderate-intensity exercise in men. *J Appl Physiol* **68,** 990–996.

Combes A, Dekerle J, Webborn N, Watt P, Bougault V & Daussin FN (2015). Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CaMKII phosphorylation in human skeletal muscle. *Physiol Rep* **3,** 1–8.

Costill DL, Bowers R, Branam G & Sparks K (1971). Muscle glycogen utilization during prolonged exercise on successive days. *J Appl Physiol* **31,** 834–838.

Costill DL, Flynn MG, Kirwan JP, Houmard J, Mitchell JB, Thomas R & Han Park S (1988). Effects of repeated days of intensified training of muscle glycogen and swimming performance. *Med Sci Sport Exerc* **20,** 249–254.

Coyle EF, Coggan AR, Hemmert MK & Ivy JL (1986). Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *J Appl Physiol* **61,** 165–172.

Coyle EF, Coggan AR, Hemmert MK, Lowe RC & Walters TJ (1985). Substrate usage during prolonged exercise following a preexercise meal. *J Appl Physiol* **59,** 429–433.

Coyle EF, Coggan AR, Hopper MK & Walters TJ (1988). Determinants of endurance in well-trained cyclists. *J Appl Physiol* **64,** 2622–2630.

Coyle EF, Hagberg JM, Hurley BF, Martin WH, Ehsani AA & Holloszy JO (1983). Carbohydrate feeding during prolonged strenuous exercise can delay fatigue. *J Appl Physiol* **55,** 230–235.

Currell K & Jeukendrup AE (2008). Superior endurance performance with ingestion of multiple transportable carbohydrates. *Med Sci Sports Exerc* **40,** 275–281.

Davies SP, Helps NR, Cohen PTW & Hardie DG (1995). 5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2Cα and native bovine protein phosphatase-2Ac. *FEBS Lett* **377,** 421–425.

Décombaz J, Jentjens R, Ith M, Scheurer E, Buehler T, Jeukendrup A & Boesch C (2011). Fructose and galactose enhance postexercise human liver glycogen synthesis. *Med Sci Sports Exerc* **43,** 1964–1971.

Dimauro I, Pearson T, Caporossi D & Jackson M (2012). A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. *BMC Res Notes* **5,** 513.

Egan B, Carson BP, Garcia-Roves PM, Chibalin A V., Sarsfield FM, Barron N, McCaffrey N, Moyna NM, Zierath JR & O'Gorman DJ (2010). Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor γ coactivator-1α mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. *J Physiol* **588,** 1779–1790.

Egan B, O'Connor PL, Zierath JR & O'Gorman DJ (2013). Time course analysis reveals gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human skeletal muscle. *PLoS One* **8,** e74098.

Ekblom B (2017). The muscle biopsy technique. Historical and methodological considerations. *Scand J Med Sci Sport* **27,** 458–461.

Erickson MA, Schwarzkopf RJ & McKenzie RD (1987). Effects of caffeine, fructose, and glucose ingestion on muscle glycogen utilization during exercise. *Med Sci Sports Exerc* **19,** 579–583.

Essen B & Henriksson J (1974). Glycogen content of individual muscle fibres in man. *Acta*

Physiol Scand **90,** 645–647.

Essén B, Jansson E, Henriksson J, Taylor AW & Saltin B (1975). Metabolic Characteristics of Fibre Types in Human Skeletal Muscle. *Acta Physiol Scand* **95,** 153–165.

Fan M, Rhee J, St-Pierre J, Handschin C, Puigserver P, Lin J, Jäeger S, Erdjument-Bromage H, Tempst P & Spiegelman BM (2004). Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1α: Modulation by p38 MAPK. *Genes Dev* **18,** 278–289.

Fares EJM & Kayser B (2011). Carbohydrate mouth rinse effects on exercise capacity in pre- and postprandial states. *J Nutr Metab*; DOI: 10.1155/2011/385962.

Febbraio MA, Chiu A, Angus DJ, Arkinstall MJ & Hawley JA (2000). Effects of carbohydrate ingestion before and during exercise on glucose kinetics and performance. *J Appl Physiol* **89,** 2220–2226.

Fiorenza M, Gunnarsson TP, Hostrup M, Iaia FM, Schena F, Pilegaard H & Bangsbo J (2018). Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle. *J Physiol* **596,** 2823–2840.

Frøsig C, Jørgensen SB, Hardie DG, Richter EA & J JFPW (2004). 5-AMP-activated protein kinase activity and protein expression are regulated by endurance training in human skeletal muscle. *AJP Endocrinol Metab* **286,** 411–417.

Fuchs CJ, Gonzalez JT, Beelen M, Cermak NM, Smith FE, Thelwall PE, Taylor R, Trenell MI, Stevenson EJ & van Loon LJC (2016). Sucrose ingestion after exhaustive exercise accelerates liver, but not muscle glycogen repletion compared with glucose ingestion in trained athletes. *J Appl Physiol* **120,** 1328–1334.

Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A & Goodyear LJ (2000). Exercise induces isoform-specific increase in 5' AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* **273,** 1150–1155.

Gejl KD, Hvid LG, Frandsen U, Jensen K, Sahlin K & Ørtenblad N (2014). Muscle glycogen content modifies SR Ca2+ release rate in elite endurance athletes. *Med Sci Sports Exerc* **46,** 496–505.

Gejl KD, Thams LB, Hansen M, Rokkedal-Lausch T, Plomgaard P, Nybo L, Larsen FJ, Cardinale DA, Jensen K, Holmberg HC, Vissing K & Ørtenblad N (2017). No superior adaptations to carbohydrate periodization in elite endurance athletes. *Med Sci Sport Exerc* **49,** 2486–2497.

Gibala MJ, McGee SL, Garnham AP, Howlett KF, Snow RJ & Hargreaves M (2009). Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1α in human skeletal muscle. *J Appl Physiol* **106,** 929–934.

Gollnick PD, Piehl K, Saubert IV C, Armstrong R & Saltin B (1972). Diet, exercise, and glycogen changes in human muscle fibers. *J Appl Physiol* **33,** 4–8.

Gonzalez JT, Fuchs CJ, Smith FE, Thelwall PE, Taylor R, Stevenson EJ, Trenell MI, Cermak NM & van Loon LJC (2015). Ingestion of glucose or sucrose prevents liver but not muscle glycogen depletion during prolonged endurance-type exercise in trained cyclists. *Am J Physiol - Endocrinol Metab* **309,** E1032–E1039.

Graham TE, Yuan Z, Hill AK & Wilson RJ (2010). The regulation of muscle glycogen: The granule and its proteins. *Acta Physiol* **199,** 489–498.

Granata C, Jamnick NA & Bishop DJ (2018). Training-induced changes in mitochondrial content and respiratory function in human skeletal muscle. *Sport Med* **48,** 1809–1828.

Granata C, Oliveira RSF, Little JP, Renner K & Bishop DJ (2016*a*). Training intensity modulates changes in PGC-1α and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. *FASEB J* **30,** 959–970.

Granata C, Oliveira RSF, Little JP, Renner K & Bishop DJ (2016*b*). Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. *FASEB J* **30,** 3413–3423.

Granata C, Oliveira RSF, Little JP, Renner K & Bishop DJ (2017). Sprint-interval but not continuous exercise increases PGC-1α protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle. *Sci Rep* **7,** 44227.

Gray EA, Green TA, Betts JA & Gonzalez JT (2019). Postexercise Glucose-Fructose Coingestion Augments Cycling Capacity During Short-Term and Overnight Recovery From Exhaustive Exercise, Compared With Isocaloric Glucose. *Int J Sport Nutr Exerc Metab*1–8.

Hammond KM, Sale C, Fraser W, Tang J, Shepherd SO, Strauss JA, Close GL, Cocks M, Louis J, Pugh J, Stewart C, Sharples AP & Morton JP (2019). Post-exercise carbohydrate and energy availability induce independent effects on skeletal muscle cell signalling and bone turnover: implications for training adaptation. *J Physiol* **597,** 4779–4796.

Handschin C, Rhee J, Lin J, Tarr PT & Spiegelman BM (2003). An autoregulatory loop controls peroxisome proliferator-activated receptor coactivator 1 expression in muscle. *Proc Natl Acad Sci* **100,** 7111–7116.

Hansen AK, Fischer C, Plomgaard P, Andersen J, Saltin B & Pedersen BK (2005). Skeletal muscle adaptation: training twice every second day vs. training once daily. *J Appl Physiol* **98,** 93–99.

Hardie G (2011). AMP-activated protein kinase - an energy sensor that regulates all aspects of cell function. *Genes Dev* **25,** 1895–1908.

Hawley J, Schabort E, Noakes TD & Dennis S (1997). Carbohydrate loading and exercise performance: An update. *Sport Med* **24,** 73–81.

Hawley JA, Hargreaves M, Joyner MJ & Zierath JR (2014). Integrative biology of exercise. *Cell* **159,** 738–749.

Hawley JA, Hargreaves M & Zierath JR (2006). Signalling mechanisms in skeletal muscle: Role in substrate selection and muscle adaptation. *Essays Biochem* **42,** 1–12.

Hawley JA & Leckey JJ (2015). Carbohydrate dependence during prolonged, intense endurance exercise. *Sport Med* **45,** 5–12.

Henriksson J (1977). Training induced adaptation of skeletal muscle and metabolism during submaximal exercise. *J Physiol* **270,** 661–675.

Hermansen L, Hultman E & Saltin B (1967). Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand* **71,** e12803.

Hickner RC, Fisher JS, Hansen PA, Racette SB, Mìer CM, Turner MJ & Holloszy JO (1997). Muscle glycogen accumulation in trained and untrained individuals. *FASEB J* **11,** 897–903.

Hochachka PW & Matheson GO (1992). Regulating ATP turnover rates over broad dynamic work ranges in skeletal muscles. *J Appl Physiol* **73,** 1697–1703.

Holloszy J (1967). Biochemical Adaptations in Muscle. *J Biol Chem* **242,** 2278–2282.

Holloszy JO & Coyle EF (1984). Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol* **56,** 831–838.

Holloszy JO, Oscai LB, Don IJ & Molé PA (1970). Mitochondrial citric acid cycle and related enzymes: Adaptive response to exercise. *Biochem Biophys Res Commun* **40,** 1368–1373.

Hood DA (2001). Contractile activity-induced mitochondrial biogensis in skeletal muscle. *J Appl Physiol* **90,** 1137–1157.

Hood DA, Tryon LD, Carter HN, Kim Y & Chen CCW (2016). Unravelling the mechanisms regulating muscle mitochondrial biogenesis. *Biochem J* **473,** 2295–2314.

Hoppeler H, Luthi P, Claassen H, Weibel ER & Howald H (1973). The ultrastructure of the normal human skeletal muscle. *Pflugers Arch Eur J Physiol* **344,** 217–232.

Hornberger TA (2016). Commentary on viewpoint: The rigorous study of exercise adaptations: Why mRNA might not be enough. *J Appl Physiol* **121,** 597.

Horowitz JF, Mora-Rodriguez R, Byerley LO & Coyle EF (1997). Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. *Am J Physiol* **273,** E768-775.

Houmard JA, Shinebarger MH, Dolan PL, Leggett-Frazier N, Bruner RK, McCammon MR, Israel RG & Dohm GL (1993). Exercise training increases GLUT-4 protein concentration in previously sedentary middle-aged men. *Am J Physiol - Endocrinol*

Metab **264,** 0–5.

Howarth KR, Moreau NA, Phillips SM & Gibala MJ (2009). Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *J Appl Physiol* **106,** 1394–1402.

Hulston CJ & Jeukendrup AE (2009). No placebo effect from carbohydrate intake during prolonged exercise. *Int J Sport Nutr Exerc Metab* **19,** 275–284.

Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, Baar K & Jeukendrup AE (2010). Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. *Med Sci Sports Exerc* **42,** 2046–2055.

Hulston CJ, Wallis GA & Jeukendrup AE (2009). Exogenous CHO oxidation with glucose plus fructose intake during exercise. *Med Sci Sports Exerc* **41,** 357–363.

Hultman E (1967). Muscle glycogen in man determined in needle biopsy specimens. *Scand J Clin Lab Invest* **19,** 209–217.

Hurley BF, Nemeth PM, Martin WH 3rd, Hagberg JM, Dalsky GP & Holloszy JO (1986). Muscle triglyceride utilization during exercise: effect of training. *J Appl Physiol (Bethesda, Md 1985)* **60,** 562–567.

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

Impey SG, Hearris MA, Hammond KM, Bartlett JD, Louis J, Close GL & Morton JP (2018). Fuel for the work required: A theoretical framework for carbohydrate periodization and the glycogen threshold hypothesis. *Sport Med* **48,** 1031–1048.

Ingjer F (1979). Effects of Endurance Training on Muscle Fibre ATP-ase Activity, Capillary Supply & Mitochondrial Content in Man. *J Physiol* **294,** 419–432.

Ivy JL, Katz AL, Cutler CL, Sherman WM & Coyle EF (1988). Muscle glycogen synthesis after exercise: Effect of time of carbohydrate ingestion. *J Appl Physiol* **64,** 1480–1485.

Ivy JL & Kuo H (1998). Regulation of GLUT4 protein and glycogen synthase during muscle glycogen synthesis after exercise. *Acta Physiol Scand* **162,** 295–304.

Jager S, Handschin C, St.-Pierre J & Spiegelman BM (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 . *Proc Natl Acad Sci* **104,** 12017–12022.

Jentjens R & Jeukendrup AE (2003). Determinants of post-exercise glycogen synthesis during short-term recovery. *Sport Med* **33,** 117–144.

Jeukendrup A (2014). A step towards personalized sports nutrition: Carbohydrate intake during exercise. *Sport Med*; DOI: 10.1007/s40279-014-0148-z.

Jeukendrup AE & Jentjens R (2006). Oxidation of carbohydrate feedings during prolonged

exercise. *Sport Med* **29,** 407–424.

Jeukendrup AE, Moseley L, Mainwaring GI, Samuels S, Perry S & Mann CH (2005). Exogenous carbohydrate oxidation during ultraendurance exercise. *J Appl Physiol* **100,** 1134–1141.

Jeukendrup AE, Wagenmakers AJ, Stegen JH, Gijsen AP, Brouns F & Saris WH (1999). Carbohydrate ingestion can completely suppress endogenous glucose production during exercise. *Am J Physiol* **276,** E672-83.

Jeukendrup AE & Wallis GA (2005). Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* **26,** S28–S37.

Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ & Thompson CB (2005). AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* **18,** 283–293.

Karlsson J, Nordesjö L -O & Saltin B (1974). Muscle glycogen utilization during exercise after physical training. *Acta Physiol Scand* **90,** 210–217.

Karlsson J, Nordesjo L, Jorfeldt L & Saltin B (1972). Muscle lactate, ATP, and CP levels during exercise after training in man. *J Appl Physiol* **33,** 199–203.

Karlsson J & Saltin B (1971). Diet, muscle glycogen, and endurance performance. *J Appl Physiol* **31,** 203–206.

Keizer HA, Kuipers H, van Kranenburg G & Geurten P (1987). Influence of liquid and solid meals on muscle glycogen resynthesis, plasma fuel hormone response, and maximal physical working capacity. *Int J Sports Med* **8,** 99–104.

Khong TK, Selvanayagam VS, Hamzah SH & Yusof A (2018). Effect of quantity and quality of pre-exercise carbohydrate meals on central fatigue. *J Appl Physiol* **125,** 1021–1029.

Kiens B, Éssen-Gustravsson B, Christensen N & Saltin B (1993). Skeletal muscle substrate utilisation during submaximal exercise in man: Effect of endurance training. *J Physiol* **469,** 459–478.

Kirwan JP, Costill DL, Mitchell JB, Houmard JA, Flynn MG, Fink WJ & Beltz JD (1988). Carbohydrate balance in competitive runners during successive days of intense training. *J Appl Physiol* **65,** 2601–2606.

Kristensen DE, Albers PH, Prats C, Baba O, Birk JB & Wojtaszewski JFP (2015). Human muscle fibre type-specific regulation of AMPK and downstream targets by exercise. *J Physiol* **593,** 2053–2069.

Kuang J, Yan X, Genders AJ, Granata C & Bishop DJ (2018). An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS One* **13,** e0196438.

Lane SC, Areta JL, Bird SR, Coffey VG, Burke LM, Desbrow B, Karagounis LG & Hawley

JA (2013). Caffeine ingestion and cycling power output in a low or normal muscle glycogen state. *Med Sci Sports Exerc* **45,** 1577–1584.

Lane SC, Camera DM, Lassiter DG, Areta JL, Bird SR, Yeo WK, Jeacocke NA, Krook A, Zierath JR, Burke LM & Hawley JA (2015). Effects of sleeping with reduced carbohydrate availability on acute training responses. *J Appl Physiol* **119,** 643–655.

LeBlanc PJ, Peters SJ, Tunstall RJ, Cameron-Smith D & Heigenhauser GJF (2004). Effects of aerobic training on pyruvate dehydrogenase and pyruvate dehydrogenase kinase in human skeletal muscle. *J Physiol* **557,** 559–570.

Lee-Young RS, Palmer MJ, Linden KC, LePlastrier K, Canny BJ, Hargreaves M, Wadley GD, Kemp BE & McConell GK (2006). Carbohydrate ingestion does not alter skeletal muscle AMPK signaling during exercise in humans. *Am J Physiol Endocrinol Metab* **291,** E566–E573.

Levine S, Gordon B & Derick C (1924). Some changes in the chemical constituents of the blood following a marathon race. *J Am Med Assoc* **82,** 1778–1779.

Lin J, Wu H, Tarr PT, Zhang C-Y, Wu Z, Boss O, Michael L, Puigserver P, Isotanl E, Olson EN, Lowell B, Bassel-Duby R & Spiegelman BM (2002). Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. *Nature* **418,** 797–801.

Little JP, Safdar A, Bishop D, Tarnopolsky MA & Gibala MJ (2011). An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1a and activates mitochondrial biogenesis in human skeletal muscle. *Am J Physiol Endocrinol Metab* **300,** 1303–1310.

Little JP, Safdar A, Cermak N, Tarnopolsky MA & Gibala MJ (2010). Acute endurance exercise increases the nuclear abundance of PGC-1α in trained human skeletal muscle. *Am J Physiol - Regul Integr Comp Physiol* **298,** 912–917.

van Loon LJ, Saris W, Kruijshoop M & Wagenmakers AJ (2000). Maximizing postexercise muscle glycogen synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr*106–111.

Van Loon LJC, Koopman R, Manders R, Van Der Weegen W, Van Kranenburg GP & Keizer HA (2004). Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *Am J Physiol - Endocrinol Metab* **287,** 558–565.

MacDougall JD, Hicks AL, MacDonald JR, McKelvie RS, Green HJ & Smith KM (1998). Muscle performance and enzymatic adaptations to sprint interval training. *J Appl Physiol* **84,** 2138–2142.

Marchand I, Chorneyko K, Tarnopolsky M, Hamilton S, Shearer J, Potvin J & Graham TE (2002). Quantification of subcellular glycogen in resting human muscle: granule size, number, and location. *J Appl Physiol* **93,** 1598–1607.

Marquet LA, Brisswalter J, Louis J, Tiollier E, Burke LM, Hawley JA & Hausswirth C (2016*a*). Enhanced endurance performance by periodization of carbohydrate intake: "Sleep Low" strategy. *Med Sci Sports Exerc* **48,** 663–672.

Marquet LA, Hausswirth C, Molle O, Hawley JA, Burke LM, Tiollier E & Brisswalter J (2016*b*). Periodization of carbohydrate intake: Short-term effect on performance. *Nutrients* **8,** 1–13.

Mathai AS, Bonen A, Benton CR, Robinson DL & Graham TE (2008). Rapid exercise-induced changes in PGC-1α mRNA and protein in human skeletal muscle. *J Appl Physiol* **105,** 1098–1105.

Maunder E, Podlogar T & Wallis GA (2018). Postexercise Fructose-Maltodextrin Ingestion Enhances Subsequent Endurance Capacity. *Med Sci Sports Exerc* **50,** 1039–1045.

McBride A, Ghilagaber S, Nikolaev A & Hardie DG (2009). The glycogen-binding domain on the AMPK β subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9,** 23–34.

McBride A & Hardie DG (2009). AMP-activated protein kinase - A sensor of glycogen as well as AMP and ATP? *Acta Physiol* **196,** 99–113.

McConell GK, Lee-Young RS, Chen ZP, Stepto NK, Huynh NN, Stephens TJ, Canny BJ & Kemp BE (2005). Short-term exercise training in humans reduces AMPK signalling during prolonged exercise independent of muscle glycogen. *J Physiol* **568,** 665–676.

McConell GK, Wadley GD, Le plastrier K & Linden KC (2020). Skeletal muscle AMPK is not activated during 2 hours of moderate intensity exercise at ∼65% VO 2 peak in endurance trained men. *J Physiol*1–34.

Mcgee SL & Hargreaves M (2008). AMPK and transcriptional regulation. *Front Biosci* **13,** 3022–3033.

McGee SL, Howlett KF, Starkie RL, Cameron-Smith D, Kemp BE & Hargreaves M (2003). Exercise increases nuclear AMPKα2 in human skeletal muscle. *Diabetes* **52,** 926–928.

Mckinsey TA, Zhang CL & Olson EN (2001). Identification of a signal-responsive nuclear export sequence in class II histone deacetylases. *Mol Cell Biol* **21,** 6312–6321.

McKinsey TA, Zhang CL & Olson EN (2000). Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. *Proc Natl Acad Sci U S A* **97,** 14400–14405.

Melendez-Hevia E, Waddell TG & Shelton ED (1993). Optimization of molecular design in the evolution of metabolism: the glycogen molecule. *Biochem J* **295,** 477–483.

Merrill GF, Kurth EJ, Hardie DG & Winder WW (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol - Endocrinol Metab* **273,** 1107–1112.

Montain SJ, Hopper MK, Coggan AR & Coyle EF (1991). Exercise metabolism at different time intervals after a meal. *J Appl Physiol* **70,** 882–888.

Morgan TE, Cobb LA, Short FA, Ross R & Gunn DR (1971). Effects of long-term exercise on human muscle mitochondria. *Muscle Metab Dur Exerc Adv Exp Med Biol* **11,** 87–95.

Morton JP, Croft L, Bartlett JD, MacLaren DPM, Reilly T, Evans L, McArdle A & Drust B (2009). Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulate oxidative enzyme activity in human skeletal muscle. *J Appl Physiol* **106,** 1513–1521.

Neufer PD, Costill DL, Flynn MG, Kirwan JP, Mitchell JB & Houmard J (1987). Improvements in exercise performance: effects of carbohydrate feedings and diet. *J Appl Physiol* **62,** 983–988.

Nielsen J, Holmberg HC, Schr??der HD, Saltin B & ??rtenblad N (2011). Human skeletal muscle glycogen utilization in exhaustive exercise: Role of subcellular localization and fibre type. *J Physiol* **589,** 2871–2885.

Nielsen J & Ørtenblad N (2013). Physiological aspects of the subcellular localization of glycogen in skeletal muscle. *Appl Physiol Nutr Metab* **38,** 91–99.

Nielsen JN, Mustard KJW, Graham D a, Yu H, MacDonald CS, Pilegaard H, Goodyear LJ, Hardie DG, Richter E a & Wojtaszewski JFP (2003). 5'-AMP-activated protein kinase activity and subunit expression in exercise-trained human skeletal muscle. *J Appl Physiol* **94,** 631–641.

Nybo L (2003). CNS fatigue and prolonged exercise: effect of glucose supplementation. *Med Sci Sports Exerc* **35,** 589–594.

Nybo L, Pedersen K, Christensen B, Aagaard P, Brandt N & Kiens B (2009). Impact of carbohydrate supplementation during endurance training on glycogen storage and performance. *Acta Physiol* **197,** 117–127.

Ørtenblad N, Nielsen J, Saltin B & Holmberg H-C (2011). Role of glycogen availability in sarcoplasmic reticulum Ca^{2+} kinetics in human skeletal muscle. *J Physiol* **589,** 711–725.

Ørtenblad N, Westerblad H & Nielsen J (2013). Muscle glycogen stores and fatigue. *J Physiol* **591,** 4405–4413.

Parkin J, Carey M, Martin I, Stojanovska L & Febbraio MA (1997). Muscle glycogen storage following prolonged exercise: effect of timing of ingestion of high glycemic index food. *Med Sci Sport Exerc* **29,** 220–224.

Perry CGR, Lally J, Holloway GP, Heigenhauser GJF, Bonen A & Spriet LL (2010). Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* **588,** 4795–

4810.

Pfeiffer B, Stellingwerff T, Zaltas E & Jeukendrup AE (2010*a*). Oxidation of solid versus liquid CHO sources during exercise. *Med Sci Sports Exerc* **42,** 2030–2037.

Pfeiffer B, Stellingwerff T, Zaltas E & Jeukendrup AE (2010*b*). CHO oxidation from a CHO gel compared with a drink during exercise. *Med Sci Sports Exerc* **42,** 2038–2045.

Phillips SM, Green HJ, Tarnopolsky MA, Heigenhauser GJ & Grant SM (1996). Progressive effect of endurance training on metabolic adaptations in working skeletal muscle. *Am J Physiol Metab* **270,** E265–E272.

Philp A, Hargreaves M & Baar K (2012). More than a store: regulatory roles for glycogen in skeletal muscle adaptation to exercise. *AJP Endocrinol Metab* **302,** E1343–E1351.

Pilegaard H, Keller C, Steensberg A, Helge JW, Pedersen BK, Saltin B & Darrell Neufer P (2002). Influence of pre-exercise muscle glycogen content on exercise-induced transcriptional regulation of metabolic genes. *J Physiol* **541,** 261–271.

Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B & Neufer PD (2005). Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolism* **54,** 1048–1055.

Price TB, Rothman DL, Taylor R, Avison MJ, Shulman GI & Shulman RG (1994). Human muscle glycogen resynthesis after exercise: Insulin-dependent and - independent phases. *J Appl Physiol* **76,** 104–111.

Van Proeyen K, Szlufcik K, Nielens H, Ramaekers M & Hespel P (2011). Beneficial metabolic adaptations due to endurance exercise training in the fasted state. *J Appl Physiol* **110,** 236–245.

Psilander N (2014). *The Effect of Different Exercise Regimens on Mitochondrial Biogenesis and Performance* (thesis). Karolinska Institutet.

Psilander N, Frank P, Flockhart M & Sahlin K (2013). Exercise with low glycogen increases PGC-1α gene expression in human skeletal muscle. *Eur J Appl Physiol* **113,** 951–963.

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB & Spiegelman BM (2001). Cytokine Stimulation of Energy Expenditure through p38 MAP Kinase Activation of PPARγ Coactivator-1. *Mol Cell* **8,** 971–982.

Putman CT, Jones NL, Hultman E, Hollidge-Horvat MG, Bonen A, McConachie DR & Heigenhauser GJF (1998). Effects of short-term submaximal training in humans on muscle metabolism in exercise. *Am J Physiol - Endocrinol Metab* **275,** 132–139.

Reed MJ, Brozinick JT, Lee MC & Ivy JL (1989). Muscle glycogen storage postexercise: effect of mode of carbohydrate administration. *J Appl Physiol* **66,** 720–726.

Richter EA & Ruderman NB (2009). AMPK and the biochemistry of exercise. *Biochem J* **418,** 261–275.

Riis S, Møller AB, Dollerup O, Høffner L, Jessen N & Madsen K (2019). Acute and

sustained effects of a periodized carbohydrate intake using the sleep-low model in endurance-trained males. *Scand J Med Sci Sport* **29,** 1866–1880.

Roepstorff C, Halberg N, Hillig T, Saha AK, Ruderman NB, Wojtaszewski JFP, Richter EA & Kiens B (2005). Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. *Am J Physiol Endocrinol Metab* **288,** E133–E142.

Romijn JA, Coyle EF, Hibbert J & Wolfe RR (1992). Comparison of indirect calorimetry and a new breath 13C/12C ratio method during strenuous exercise. *Am J Physiol Metab* **263,** E64–E71.

Rose AJ, Kiens B & Richter EA (2006). Ca2+-calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. *J Physiol* **574,** 889–903.

Rowe GC, El-Khoury R, Patten IS, Rustin P & Arany Z (2012). PGC-1α is dispensable for exercise-induced mitochondrial biogenesis in skeletal muscle. *PLoS One*; DOI: 10.1371/journal.pone.0041817.

Rowlands DS, Thorburn MS, Thorp RM, Broadbent S & Shi X (2008). Effect of graded fructose coingestion with maltodextrin on exogenous 14 C-fructose and 13 C-glucose oxidation efficiency and high-intensity cycling performance . *J Appl Physiol* **104,** 1709–1719.

Safdar A, Little JP, Stokl AJ, Hettinga BP, Akhtar M & Tarnopolsky MA (2011). Exercise increases mitochondrial PGC-1α content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis. *J Biol Chem* **286,** 10605–10617.

Saha AK & Ruderman NB (2003). Malonyl-CoA and AMP-activated protein kinase: An expanding partnership. *Mol Cell Biochem* **253,** 65–70.

Saleem A, Adhihetty PJ & Hood DA (2009). Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. *Physiol Genomics* **37,** 58–66.

Saleem A & Hood DA (2013). Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle. *J Physiol* **591,** 3625–3636.

Schmittgen TD & Livak KJ (2008). Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* **3,** 1101–1108.

She QB, Bode AM, Ma WY, Chen NY & Dong Z (2001). Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res* **61,** 1604–1610.

Sherman W, Doyle A, Lamb D & Strauss R (1993). Dietary carbohydrate, muscle glycogen, and exercise performance during 7 d of training. *Ann N Y Acad Sci*27–31.

Sherman WM, Brodowicz G, Wright D, Allen W, Simonsen J & Dernbach A (1989). Effects of 4 h preexercise carbohydrate feedings on cycling performance. *Med Sci Sport Exerc* **21,** 598–604.

Sherman WM, Costill DL, Fink WJ & Miller JM (1981). Effect of exercise-diet manipulation on muscle glycogen and its subsequent utilization during performance. *Int J Sports Med* **2,** 114–118.

Sherman WM, Peden CM & Wright D a (1991). Carbohydrate feedings 1 h before improves cycling performance exercise. *Am J Clin Nutr* **54,** 866–870.

Smith BK, Mukai K, Lally JS, Maher AC, Gurd BJ, Heigenhauser GJF, Spriet LL & Holloway GP (2013). AMP-activated protein kinase is required for exercise-induced peroxisome proliferator-activated receptor γ co-activator 1α translocation to subsarcolemmal mitochondria in skeletal muscle. *J Physiol* **591,** 1551–1561.

Steenberg DE, Jørgensen NB, Birk JB, Sjøberg KA, Kiens B, Richter EA & Wojtaszewski JFP (2019). Exercise training reduces the insulin-sensitizing effect of a single bout of exercise in human skeletal muscle. *J Physiol* **597,** 89–103.

Steinberg GR, Watt MJ, McGee SL, Chan S, Hargreaves M, Febbraio MA, Stapleton D & Kemp BE (2006). Reduced glycogen availability is associated with increased AMPKα2 activity, nuclear AMPKα2 protein abundance, and GLUT4 mRNA expression in contracting human skeletal muscle. *Appl Physiol Nutr Metab* **31,** 302–312.

Stellingwerff T (2012). Case study: Nutrition and training periodization in three elite marathon runners. *Int J Sport Nutr Exerc Metab* **22,** 392–400.

Stellingwerff T, Boon H, Gijsen AP, Stegen JHCH, Kuipers H & Van Loon LJC (2007). Carbohydrate supplementation during prolonged cycling exercise spares muscle glycogen but does not affect intramyocellular lipid use. *Eur J Physiol* **454,** 635–647.

Stellingwerff T & Cox GR (2014). Systematic review: Carbohydrate supplementation on exercise performance or capacity of varying durations. *Appl Physiol Nutr Metab* **39,** 998–1011.

Stellingwerff T, Morton JP & Burke LM (2019). A framework for periodized nutrition for athletics. *Int J Sport Nutr Exerc Metab* **11,** 1–29.

Stephens TJ, Chen Z-P, Canny BJ, Michell BJ, Kemp BE & McConell GK (2002). Progressive increase in human skeletal muscle AMPKα2 activity and ACC phosphorylation during exercise. *Am J Physiol Metab* **282,** E688–E694.

Stocks B, Dent JO, Ogden HB, Zemp M & Philp A (2019). Post-exercise skeleal muscle signalling responses to moderate- to high-intensity steady-state exercise in the fed or fasted state. *AJP Endocrinol Metab*; DOI: 10.1152/ajpendo.00311.2018.

Tachtsis B, Smiles WJ, Lane SC, Hawley JA & Camera DM (2016). Acute endurance exercise induces nuclear p53 abundance in human skeletal muscle. *Front Physiol* **7,** 1–10.

Talanian JL, Holloway GP, Snook LA, Heigenhauser GJF, Bonen A & Spriet LL (2010). Exercise training increases sarcolemmal and mitochondrial fatty acid transport proteins

in human skeletal muscle. *AJP Endocrinol Metab* **299,** 180–188.

Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC & Hamadeh MJ (2007). Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol - Regul Integr Comp Physiol* **292,** 1271–1278.

Taylor C, Bartlett JD, Van De Graaf CS, Louhelainen J, Coyne V, Iqbal Z, MacLaren DPM, Gregson W, Close GL & Morton JP (2013). Protein ingestion does not impair exercise-induced AMPK signalling when in a glycogen-depleted state: Implications for train-low compete-high. *Eur J Appl Physiol* **113,** 1457–1468.

Thomas D, Erdman K & Burke LM (2018). American College of Sports Medicine joint position statement. Nutrition and athletic performance. *Med Sci Sport Exerc* **48,** 543–568.

Thomson D, Herway S, Filmore N, Kim H, Brown J, Barrow J & Winder WW (2008). AMP-activated protein kinase phosphorylates transcription factors of the CREB family. *J Appl Physiol* **104,** 429–438.

Triplett D, Doyle JA, Rupp JC & Benardot D (2010). An isocaloric glucose-fructose beverage's effect on simulated 100-km cycling performance compared with a glucose-only beverage. *Int J Sport Nutr Exerc Metab* **20,** 122–131.

Trommelen J, Beelen M, Pinckaers PJM, Senden JM, Cermak NM & Van Loon LJC (2016). Fructose coingestion does not accelerate postexercise muscle glycogen repletion. *Med Sci Sports Exerc* **48,** 907–912.

Tsintzas O, Williams C, Boobis L & Greenhaff P (1995). Carbohydrate ingestion and glycogen utilization in different muscle fibre types in man. *J Physiol* **489,** 243–250.

Tsintzas O, Williams C, Boobis L & Greenhaff P (1996). Carbohydrate ingestion and single muscle fiber glycogen metabolism during prolonged running in men. *J Appl Physiol* **81,** 801–809.

Vandenbogaerde TJ & Hopkins WG (2011). Effects of acute carbohydrate supplementation on endurance performance: A meta-analysis. *Sport Med* **41,** 773–792.

Varnauskas E, Björntorp P, Fahlén M, Přerovský I & Stenberg J (1970). Effects of physical training on exercise blood flow and enzymatic activity in skeletal muscle. *Cardiovasc Res* **4,** 418–422.

Vollestad M, Vaage O & Hermansen L (1984). Muscle glycogen depletion patterns in type I and subgroups of type II fibres during prolonged severe exercise in man. *Acta Physiol Scand* **122,** 433–441.

Wallis GA, Hulston CJ, Mann CH, Roper HP, Tipton KD & Jeukendrup AE (2008). Postexercise muscle glycogen synthesis with combined glucose and fructose ingestion.

Med Sci Sports Exerc **40,** 1789–1794.

Wibom R, Hultman E, Johansson M, Matherei K, Constantin-Teodosiu D & Schantz PG (1992). Adaptation of mitochondrial ATP production in human skeletal muscle to endurance training and detraining. *J Appl Physiol* **73,** 2004–2010.

Widrick JJ, Costill DL, Fink WJ, Hickey MS, McConell GK & Tanaka H (1993). Carbohydrate feedings and exercise performance: effect of initial muscle glycogen concentration. *J Appl Physiol* **74,** 2998–3005.

Winder WW & Hardie DG (1996). Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol Metab* **270,** E299–E304.

Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M & Holloszy JO (2000). Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol* **88,** 2219–2226.

Wojtaszewski JFP, MacDonald C, Nielsen JN, Hellsten Y, Hardie DG, Kemp BE, Kiens B & Richter EA (2003). Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. *Am J Physiol Metab* **284,** E813–E822.

Wojtaszewski JFP, Nielsen P, Hansen BF, Richter EA & Kiens B (2000). Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol* **528,** 221–226.

Wright DA, Sherman WM & Dernbach AR (1991). Carbohydrate feedings before, during, or in combination improve cycling endurance performance. *J Appl Physiol* **71,** 1082–1088.

Wright DC, Geiger PC, Han DH, Jones TE & Holloszy JO (2007*a*). Calcium induces increases in peroxisome proliferator-activated receptor γ coactivator-1α and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. *J Biol Chem* **282,** 18793–18799.

Wright DC, Han DH, Garcia-Roves PM, Geiger PC, Jones TE & Holloszy JO (2007*b*). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1α expression. *J Biol Chem* **282,** 194–199.

Wu Z, Puigserver P, Adelmant G, Mootha VK, Troy A, Cinti S, Lowell BB, Scarpulla R & Spiegelman BM (1999). Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. *Cell* **98,** 115–124.

Xiao B, Heath R, Saiu P, Leiper FC, Leone P, Jing C, Walker PA, Haire L, Eccleston JF, Davis CT, Martin SR, Carling D & Gamblin SJ (2007). Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature* **449,** 496–500.

Xiao B, Sanders MJ, Underwood E, Heath R, Mayer F V., Carmena D, Jing C, Walker PA,

Eccleston JF, Haire LF, Saiu P, Howell SA, Aasland R, Martin SR, Carling D & Gamblin SJ (2011). Structure of mammalian AMPK and its regulation by ADP. *Nature* **472,** 230–233.

Yeo WK, McGee SL, Carey AL, Paton CD, Garnham AP, Hargreaves M & Hawley JA (2010). Acute signalling responses to intense endurance training commenced with low or normal muscle glycogen. *Exp Physiol* **95,** 351–358.

Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL & Hawley JA (2008). Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. *J Appl Physiol* **105,** 1462–1470.

Yu M, Stepto NK, Chibalin A V., Fryer LGD, Carling D, Krook A, Hawley JA & Zierath JR (2003). Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. *J Physiol* **546,** 327–335.