Carbohydrate Periodisation via a "Sleep Low – Train Low" Model and the Impact of Environmental Heat Stress: A Practical and Metabolomic Exploration

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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

This research programme was carried out in collaboration with Institut National du Sport de l'Expertise et de la Performance (INSEP), Paris, France

and

Fédération Française de Triathlon, Saint Denis, France

24th June 2022

Acknowledgements

First, to my privilege, for this has allowed me to achieve a terminal degree, in comfort and with confidence, support and security.

Secondly, I would like to acknowledge my supervisory team, Dr Julien Louis, Dr Daniel Owens, and Prof. James Morton. Aside from the academic support that I have received throughout my studies, my greatest appreciation is for the personal and professional mentorship; your willingness to listen to and challenge has helped me immeasurably. Additionally, appreciation must also be given to my collaborators at INSEP, Dr Franck Brocherie, Dr Eve Tiollier, Dr Antonio Morales, and Francois Chiron, you welcomed me to the INSEP lab and exercised great patience throughout my language and cultural journey. Elodie Guibert, for tirelessly working beside me, never a crossed word, without your support and friendship during my time at INSEP, my experience would not have been the same.

Marie Phelan and the NMR Metabolomics group at the University of Liverpool. Your support and assistance throughout my very short whirlwind metabolomics career have been exemplary. Your passion and knowledge for the subject area are contagious, you have exposed me to experiences far beyond my original PhD scheme of work.

My Family and friends, I've avoided naming people specifically for fear of forgetting somebody, without your collective support, which far pre-dates this PhD, I would not have been able to pursue this academic path.

Lastly, Heather, my voice of reason, a soundboard for all my frustrations, your contribution to this work is immeasurable. Thank you for your sacrifice, support, and commitment.

Declaration

I Declare that the work in this thesis was carried out per the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work presented herein 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

Endurance athletes have traditionally been advised to consume high carbohydrate intake before, during and after exercise to support high training loads and facilitate recovery. Accumulating evidence suggests periodically training with low carbohydrate availability, termed “train-low”, augments skeletal oxidative adaptations. Comparably, to account for increased carbohydrate utilisation during exercise in hot environmental conditions, nutritional guidelines advocate high carbohydrate intake. Recent evidence suggest heat stress induces oxidative adaptation in skeletal muscle, augmenting mitochondrial adaptation during endurance training. This thesis aimed to assess the efficacy of training with reduced carbohydrate and the impact of elevated ambient temperatures on performance and metabolism. **Chapter 4** demonstrated 3-weeks of Sleep Low-Train Low (SL-TL) improves performance when prescribed and completed remotely. **Chapter 5** implemented SL-TL in hot and temperate conditions, confirming SL-TL improves performance and substrate metabolism, whilst additional heat stress failed to enhance performance in hot and temperate conditions following the intervention. **Chapters 6 and 7** optimised and implemented a novel *in vitro* skeletal muscle exercise model combining electrical pulse stimulation and heat stress. Metabolomics analysis revealed an ‘exercise’-induced metabolic response, with no direct metabolomic impact of heat stress. **Chapter 8** characterised the systemic metabolomic response to acute exercise in the heat and following SL-TL and heat stress intervention revealing distinct metabolic signatures associated with exercise under heat stress. In summary, this thesis provides data supporting the application of the SL-TL strategy during endurance training to augment adaptation. Data also highlights the impact of exercise, environmental temperature and substrate availability on skeletal muscle metabolism and the systemic metabolome. Together, these data provide practical support of the efficacy of the SL-TL strategy to improve performance

and adaptation whilst casting doubt on the utility of this approach in hot environments in endurance trained athletes.

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List of Abbreviations

β-HAD – 3-hydroxyacyl-CoA dehydrogenase
ACC – Acetyl-CoA Carboxylase
ADP – Adenosine Di-Phosphate
AICAR – 5-Aminoimidazole-4-Carboxamide Ribonucleotide
AMP – Adenosine Monophosphate
AMPK – Adenosine Monophosphate-Activated Protein Kinase
ANOVA – Analysis of Variance
ATF2 – Cyclic AMP-dependent transcription factor -2
ATP – Adenosine Triphosphate
BM – Body Mass
BSA – Bovine Serum Albumin
Ca^{2+} - Calcium
CaMKII – Calmodulin Dependent Protein Kinase II
CD36 – Cluster of Differentiation 36
CHO – Carbohydrate
CON – Control
COVID-19 – Novel Coronavirus-19
COX IV – Cytochrome c Oxidase subunit 4
CPT1 – Carnitine Palmitoyl Transferase 1
CREB – cAMP Response Element Binding Protein
CRS – Correlation Reliability Score
CS – Citrate Synthase
CV – Coefficient of Variation
dH_2O – Distilled Water
DNA – Deoxyribose Nucleic Acid
FFA – Free Fatty Acid
FTP – Functional Threshold Power
g – grams
GADPH – Glyceraldehyde 3-phosphate Dehydrogenase
GLUT4 – Glucose Transporter Type 4
HAT – Histone Acetyl Transferase
HIT – High-Intensity Training
HR – Heart Rate
HSP – Heat Shock Protein
IMCL – Intramyocellular Lipid
IMP – Inosine Monophosphate
Kg – Kilograms
KJ – Kilojoules
Km – Kilometres
KO – Knockout
LCHF – Low Carbohydrate, High Fat diet
LIT – Low-Intensity Training
LPS - Lipopolysaccharide
MAP – Maximal Aerobic Power
MEF2 – Myocyte Enhancer Factor 2
mRNA – Messenger Ribonucleic acid
mtDNA – mitochondrial Deoxyribonucleic Acid
mTOR – Mechanistic Target of Rapamycin Complex
NAD(H) – Nicotinamide Adenine Dinucleotide (reduced)
NDMA – N-Nitrosodimethylamine
NEFA - non-esterified Fatty Acids
NMR – Nuclear Magnetic Resonance
NRF1/2 – Nuclear Respiratory Factor 1/2
OXPHOS – Oxidative Phosphorylation
p38-MAPK – Mitogen-Activated Protein Kinase
P53 –Tumour-suppressor protein 53
P70S6K – Ribosomal Protein S6 Kinase beta
PCA – Principal Component Analysis
PCR – Polymerase Chain Reaction
PDH – Pyruvate Dehydrogenase
PDK4 – Pyruvate Dehydrogenase Kinase 4
PGC-1α – Peroxisome proliferator-activated receptor γ coactivator-1α
Pi – Inorganic Phosphate
PLS-DA – Partial Least Squares Discriminant Analysis
PPARγ – Peroxisome Proliferator-Activated Receptor Gamma
PPO – Peak Power Output
PRO – Protein
RER – Respiratory Exchange Ratio
RPE – Rating of Perceived Exertion
RPM – revolutions per minute
rt-qRT PCR – Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction
SDH – Succinate Dehydrogenase
Ser – Serine
SIRT-1- Sirtuin 1
SL – Sleep Low
SL_{Heat} – Sleep low in Hot Conditions
SL-TL - Sleep-low, Train-low
TCA – Tricarboxylic Acid
T_{core} – Core Body Temperature
Tfam – Mitochondrial transcription factor A
Thr – Threonine
T_{mus} – Muscle Temperature
T_{skin} – Skin Temperature
$\dot{V}O_{2max}$ – Maximal Oxygen Uptake
$\dot{V}O_{2peak}$ – Peak Oxygen Uptake
W – Watts
W_{max} – Watt max

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Chapter 1 – General Introduction

This chapter introduces the theories of exercise adaptation, and how intracellular perturbations induced by physical activity promote exercise adaptation and the opportunity to augment post-exercise adaptive responses. Critically, when stressors and stimuli are induced in excess, impairments in adaptation and performance occur.

1.1 Background

Inducing adaptation through exercise training requires repeated exposures to physiological and biomechanical stressors that destabilise the internal cellular environment, forcing a compensatory, adaptive response to occur within the affected tissue (Egan and Zierath, 2013). The notion that living systems possess internal stability that buffers and protects against an ever-changing external environment was first proposed by Claude Bernard in 1865 (Bernard, 1965). Considered the founder of modern experimental physiology, Bernard recognised that the human body could maintain a constant internal environment (milieu interieur) that was vital for the individual's health. This theory later developed into the concept of homeostasis (Cannon, 1926) and was popularised by Walter B. Cannon (Cannon, 1932). A critical distinction between Bernard's Milieu Interieur and Cannon's Homeostasis is the appreciation that internal conditions are variable within a given range rather than fixed, allowing the organism to adapt to environmental changes. Homeostasis highlights the tendency of a system to maintain internal stability through coordinated responses of its parts to a given situation that disturbs normal conditions or function (Billman, 2020).

Limited by the notion that all physiological parameters must be maintained within a specific homeostatic range and any deviations from this constant are corrected automatically by local, negative feedback mechanisms, an appreciation of contextual fluctuation of the internal set point is required. To maintain stability, an organism must vary the internal set point to appropriately match the environmental demands (Sterling, 2012) as per the principle of allostasis, which can be summarised as "stability through change" (Sterling and Eyer, 1988). For instance, when transitioning from rest to exercise, metabolic mobilisation as energy demand increases, and critically during steady state exercise, energy production will eventually

meet demand. Thus balance is restored, albeit this 'balance' is starkly different to the homeostatic set-point during rest (Figure 1.1). Additionally, the concept of allostatic load has been proposed to describe how repeated exposures to stress and cycles between allostatic set points may induce "wear and tear" on biological systems, predisposing the individual to greater risk of injury or disease (McEwen and Stellar, 1993; McEwen, 1998).

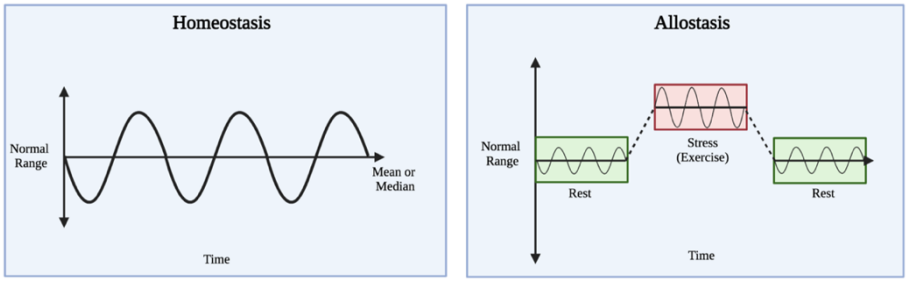

Figure 1.1 – Visual comparison of the principle of homeostasis compared to Allostasis.

Although a useful concept and a considerable progression from early homeostatic models, Allostasis fails to consider the long-term effects of repeated stress required to induce chronic adaptation to biological systems. For instance, repeated bouts or prolonged periods of stress, such as exercise or environmental stress, will extend the challenge a physiological system faces. Prolonged or elevated physiological stress is not sustainable *in infinitum,* and rest periods must occur to avoid injury or disease. General Adaptation Syndrome (GAS) was first proposed in 1936 by Hans Selye following the observation that organisms became habituated to repeated exposures to "noxious agents", (*e.g.,* muscular exercise and temperature changes) as a rationale for stress-induced adaptation (Selye, 1936). Integrating Claude Bernard's 'milieu interieur' and Walter B. Cannon's 'homeostasis', Selye's model comprised three stages, an initial 'alarm reaction' (AR), the stage of resistance and the stage of exhaustion (Selye, 1951) (Figure 1.2). Alarm reactions were characterised as acute effects of "noxious agents", e.g., exercise, hypoglycaemia, tissue catabolism, gastrointestinal issues, and adrenaline secretion. During the stage of resistance, symptoms of alarm reactions are reduced and eventually disappear, indicating a state of 'habituation' indicative of a positive adaptative response to

stressors. Crucially, Selye included the caveat that too high a magnitude or duration of stress induces a state of exhaustion (Selye, 1950), leading to a return of symptoms of earlier alarm reactions. Additionally, Hormesis, a development of the GAS model, is arguably the founding principle of exercise adaptation and is defined as "*a process in which a low dose of a chemical agent or environmental factor that induces an adaptive beneficial effect on the cell or organism but is damaging at high doses*," (Southam, 1943; Mattson, 2008). In exercise, repeated deliberate exposures to physical activity coupled with adequate recovery lead to beneficial skeletal muscle adaptation and phenotypic changes, characteristic of trained individuals.

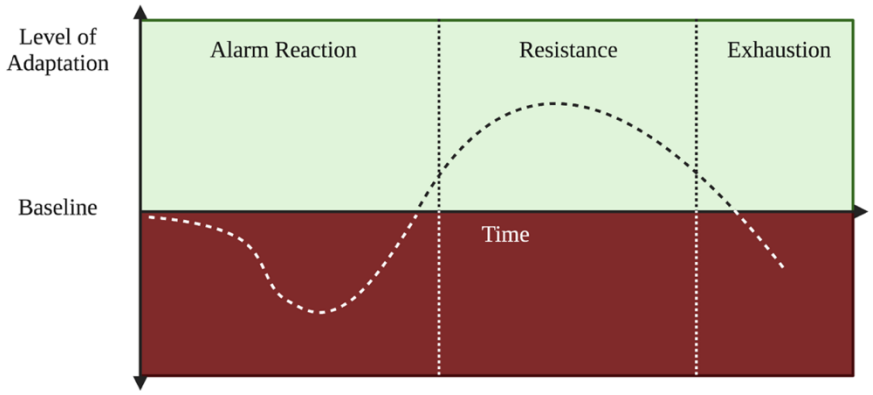

Figure 1.2 – Graphical representation of General Adaptation Syndrome. Initially, adaptation is impaired and declines during the alarm phase. During the "resistance phase", adaptation is improved. Exhaustion occurs when the prolonged application of a stressor or dose is too high in magnitude. Created with biorender.com

Elite endurance athletes possess extremely high maximal oxygen uptake ($\dot{V}O_{2max}$) (Saltin and Astrand, 1967), allowing high, sustained rates of metabolic activity through carbohydrate (CHO) and fatty acid metabolism (Romijn et al., 1993; van Loon et al., 2001) permitting higher sustained workloads over long distances (Sanders, van Erp and de Koning, 2019) and durations (Lucia et al., 1998; Lucia et al., 2002), all of which, critical factors in endurance sport competitive success. At the skeletal muscle level, a key hallmark of endurance training adaptation is an increase in the number and capacity of mitochondria (Holloszy, 1967; Holloszy et al., 1970, 1977; Oscai and Holloszy, 1971; Holloszy and Coyle, 1984), which is

highly correlated to whole-body $\dot{V}O_{2max}$, a proxy for metabolic function and endurance capacity (van der Zwaard, Brocherie and Jaspers, 2021).

As a result of improved metabolic capacity following endurance training, exercise at the same absolute intensity requires less of the muscles' maximal respiratory capacity to sustain a given workload. Consequently, a lesser cellular homeostatic disturbance occurs (Holloszy et al., 1977), including lower carbohydrate oxidation relative to fat (Brooks and Mercier, 1994) and significantly improved lactate disposal and threshold (Messonnier et al., 2013). Collectively, these metabolic adaptations may account for the reduction in intensity-dependent cell signalling responses (Nielsen et al., 2003; Wojtaszewski et al., 2003) and adaptation leading athletes and coaches to search for strategies to maximise post-exercise cell signalling and adaptive responses (Hawley et al., 2018). Several approaches have been proposed and implemented by endurance athletes, including but not limited to; strategic training with low carbohydrate availability (exo- and endogenous) and training in hot ambient conditions and/or altitude (Hawley et al., 2018).

Periodically training with reduced carbohydrate availability has been shown to augment endurance training adaptation and improve performance in trained individuals (Yeo et al., 2008b; Marquet et al., 2016a). Principally, endurance exercise completed with reduced carbohydrate availability, or "train low", induces significant disturbance to the intra-muscular metabolic milieu, augmenting the molecular signalling responses associated with endurance training adaptation. Typically, the metabolic impact of commencing exercise with low carbohydrate availability is reduced muscle glycogenolytic rate (Arkinstall et al., 2004), increased circulating free fatty acid (FFA) availability and oxidation rates (Wojtaszewski et al., 2003) and elevated circulating catecholamines (Hansen et al., 2005). The resultant metabolic

challenge invokes a compensatory adaptive response through the up-regulation of several molecular signalling processes responsible for orchestrating endurance training adaptation. Whilst improved aerobic oxidative capacity and lipid utilisation are desirable endurance training outcomes, the potential for reduced maximal rates of carbohydrate metabolism associated with metabolic remodelling following "train low" interventions require pragmatic application in athletes.

Whilst "train-low" may reduce maximal rates of carbohydrate oxidation, multiple studies have highlighted the stimulatory effect of heat stress on carbohydrate metabolism during exercise (Febbraio et al., 1994a; Febbraio et al., 1996a; Hargreaves et al., 1996a; Hargreaves et al., 1996b; Howlett, Febbraio and Hargreaves, 1999; Parkin et al., 1999; Starkie et al., 1999; Febbraio, 2001). Although not currently studied comprehensively, the additional physiological strain associated with completed endurance exercise in elevated environmental temperatures may augment metabolic training adaptations (Hawley et al., 2018). Indeed, both *in vitro* and *in vivo* models have observed comparable molecular responses to heat stress and mitochondrial adaptations as have been observed during exercise (Liu and Brooks, 2012; Tamura et al., 2014; Tamura and Hatta, 2017; Patton et al., 2018) albeit with scant human evidence to support this hypothesis (Hafen et al., 2018; Hafen et al., 2019) and even less in combination with exercise (Maunder et al., 2021c).

Given the similarities in molecular responses to exercise, carbohydrate availability, and heat stress, combining multiple stressors during exercise may augment endurance training adaptation and performance. However, applying additional stimuli during exercise may lead to reduced exercise capacity during training sessions and, as a result, impaired training outcomes.

1.2 Aims and Objectives

The over-arching aim of this thesis is to characterise the performance and metabolic effects of training with low carbohydrate availability and to determine whether the addition of environmental heat stress could augment any adaptive response or performance. Through applying the sleep-low, train-low (SL-TL) model of carbohydrate periodisation in conjunction with a heat acclimation protocol, this thesis also endeavours to investigate the efficacy of SL-TL in elevated ambient temperatures. A secondary aim of this thesis is to characterise the intra-muscular and serum metabolomic response to exercise, heat stress and carbohydrate availability using a novel ^{1}H-NMR metabolomics approach. These aims will be achieved by the completion of the following objectives:

1. The implementation of a large-scale, home-based exercise-nutrition intervention to investigate the effect of 3 weeks of SL-TL on performance outcomes in trained individuals compared to exercise completed in a consistently high carbohydrate state (Study 1, Chapter 4).
2. To examine the effects of periodising carbohydrate intake (SL-TL) during two weeks of heat acclimation on exercise adaptation and performance compared to a high carbohydrate control group and a SL-TL group in temperate conditions (Study 2 (Chapter 5).
3. To understand the metabolomic impact of exercise completed under heat stress conditions, *in vivo* and *in vitro,* using a combination of electrically stimulated C2C12 myotubes and human serum (Study 2 and 3, Chapters 7 and 8).

1.3 Deviations from Planned Scheme of Work

Due to the COVID-19 pandemic, the works presented within this thesis represent an adapted approach implementing adaptable methodological approaches that could be completed in line with highly changeable COVID-19 restrictions. Therefore, the order in which studies are presented herein does not reflect the order in which data was collected. To resolve the chronology of events, a rationale for an adapted thesis will be discussed here and supported by a Gant chart from the programme start date (September 2018) to thesis defence (September 2022), inclusive of planned and existing schemes of work (Figure 1.3).

The initial thesis workflow included three human trials, including a chronic training intervention at the French National Institute of Sport (INSEP) and two further human studies completed at LJMU. Studies undertaken at LJMU were to include muscle biopsy techniques to allow for greater mechanistic insight into the data generated during the first study. Study 1 was completed as planned, albeit over a longer duration. However, during the preparation of an ethics application for the following study, the -19 pandemic began. With prolonged periods of lockdown and the closure of the university and laboratories, it became apparent that restrictions were likely to prevent the timely completion of any laboratory-based human interventions. As such, the decision was made to reassess the proposed scheme of work and identify feasible data collection strategies during the lockdown, providing sufficient flexibility to accommodate any potential changes in restrictions. We identified home-based, remote data collection and *in vitro* C2C12 cell culture models as feasible strategies. Both approaches satisfied the following requirements:

1. Avoided exposing researchers or participants to unnecessary risk of contracting or transmitting COVID-19.
2. Provided sufficient flexibility to allow for changes in restrictions and could be halted and resumed as necessary.
3. Proved appropriate data collection approaches within the context of this thesis.

Ultimately, the decision was made to capitalise on government restrictions to implement a home-based exercise training intervention whereby the entirety of the study was completed remotely. As restrictions were lifted, access to research labs was permitted. Although face-to-face research was still prohibited, *in vitro* C2C12 cell culture models were implemented as they could be completed in isolation and within the restrictions set out by the government and the university.

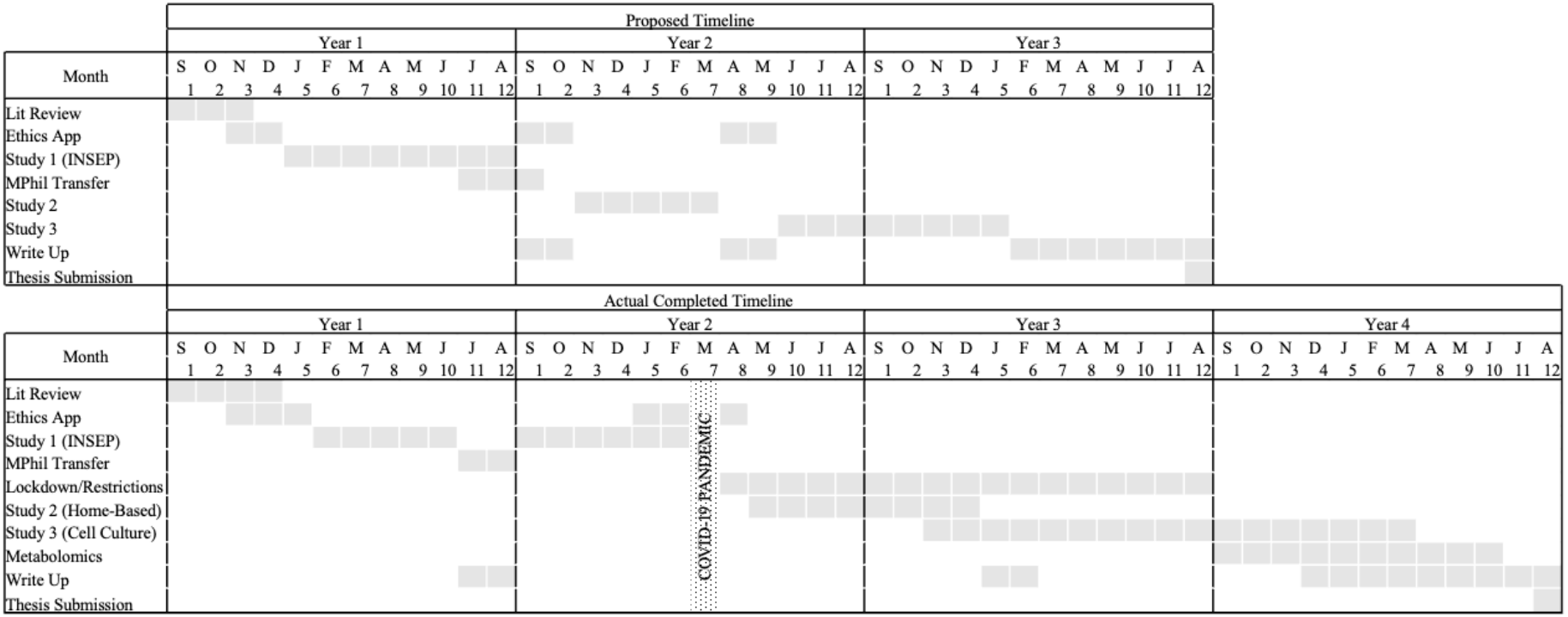

Figure 1.3 – Gant chart representative of planned scheme of work (above) and actual timeline of work (below)

Chapter 2 – Literature Review

This chapter provides a synthesis of the current scientific knowledge that describes the regulation of endurance training adaptation with a focus on substrate metabolism and the importance of CHO and muscle glycogen availability. A review of the literature with regards to the implications of elevated ambient temperatures on substrate metabolism is discussed, and the effects of heat acclimation on physiological adaptations that occur in response to heat stress. Lastly, the potential for crosstalk between molecular responses to exercise, low muscle glycogen and heat stress are considered.

2.1 Introduction

Regular endurance exercise induces significant adaptations within skeletal muscle, underpinned by increased mitochondrial content and number (Holloszy et al., 1970; Phillips et al., 1996; Perry et al., 2010) termed mitochondrial biogenesis (Hood, 2001). Exercise-induced mitochondrial increases result in muscle oxidative capacity improvements (Holloszy, 1967), reduces muscle glycogen utilisation and lactate production with simultaneous increases in fat oxidation at a given workload (Holloszy and Coyle, 1984); as a consequence, endurance exercise capacity improves.

Applying biochemical analytical techniques in exercise science has allowed researchers to investigate the intramuscular conditions induced by exercise and the subsequent molecular adaptation that ensues. In the context of endurance exercise, reduced muscle glycogen content increases AMP-activated protein kinase (AMPK) activity (Polekhina et al., 2005; McBride et al., 2009), up-regulating the expression and activity of peroxisome proliferator-activated receptor 1 co-activator alpha (PGC-1α) (Cantó et al., 2009; Philp et al., 2011), a transcriptional co-activator considered the 'master regulator' of mitochondrial biogenesis (Wu et al., 1999; Lin, Handschin and Spiegelman, 2005). Similarly, changes in reduced nicotinamide adenine dinucleotide: oxidised NAD (NAD^+:NADH) ratio are detected by NAD-dependent protein deacetylase sirtuin-1 (SIRT-1) providing an alternate mechanism of PGC-1α activation via deacetylation (Canto and Auwerx, 2009). AMPK, SIRT1 and PGC-1α provide an energy-sensitive molecular network capable of orchestrating endurance exercise adaptation at the nuclear and mitochondrial levels (Canto and Auwerx, 2009). Given the energy-sensitive nature of the AMPK-SIRT1-PGC-1α pathways, manipulating the energy status of the muscle through reduced CHO availability (specifically muscle glycogen) during exercise is an increasingly

popular strategy to augment post-exercise signalling responses. The manipulation of muscle glycogen through diet and exercise up-regulates post-exercise mRNA expression (Febbraio et al., 2002; Pilegaard et al., 2005) and increases the activity of proteins associated with mitochondrial biogenesis (Helge and Kiens, 1997), including AMPK (Steinberg et al., 2006; Yeo et al., 2008a; McBride et al., 2009) and PGC-1α (Baar et al., 2002; Psilander et al., 2013). The chronic application of "Train-low" provides positive performance outcomes and favourable alterations to body composition in trained individuals (Hansen et al., 2005; Yeo et al., 2008a; Marquet et al., 2016a; 2016b), highlighting the practical benefit of reducing muscle glycogen concentration to promote exercise adaptation.

A by-product of adenosine triphosphate (ATP) breakdown during muscular contraction, muscle temperature (T_{mus}) is increased, leading to higher work intensity and metabolic rate (Saltin, Gagge and Stolwijk, 1968; Brooks et al., 1971). Both internal and environmental heat stress severely impacts exercise capacity and performance (Galloway and Maughan, 1997), inducing a significant shift towards greater CHO oxidation compared to exercise in temperate conditions (Febbraio, 2001). Repeated exposure to heat stress during exercise may provide a potent stimulus for metabolic adaptation, with tentative evidence supporting heat stress-induced mitochondrial adaptation at rest (Hafen et al., 2018; 2019) and following exercise (Maunder et al., 2021c) in humans. Cell and animal models have provided evidence of increased AMPK – SIRT1 - PGC-1α signalling, elevated mitochondrial enzyme activity (Chen et al., 1999; Tamura et al., 2014) and increased PGC-1α and mitochondrial oxidative protein content following chronic heat exposure (Liu and Brooks, 2012) providing a plausible rationale for combined exercise, carbohydrate periodisation and heat stress to maximise endurance training adaptation.

2.2 Endurance Training Induces Skeletal Muscle Adaptation

In the 1960s, John Holloszy was amongst the first researchers to report biochemical adaptations in rat skeletal muscle following 12 weeks of endurance exercise (10 mins per day, 5 days per week). Increases in critical respiratory chain enzymes (cytochrome c and Succinate dehydrogenase [SDH]) resulted in greater mitochondrial pyruvate oxidation and improved exercise capacity (Holloszy, 1967). Further investigation reported a two-fold increase in citrate synthase (CS) and citrate dehydrogenase activity, a 50% increase in citric acid cycle protein activity (α-ketoglutarate dehydrogenase and mitochondria-specific malate dehydrogenase) and a 35% increase in glutamate dehydrogenase. Changes in multiple mitochondrial proteins suggest large-scale remodelling of the mitochondria following 12 weeks of exercise (Holloszy et al., 1970), an adaptive response comparable across all fibre types in rat skeletal muscle (Baldwin et al., 1972). In the same year, evidence of skeletal muscle adaptation and improvements in oxidative capacity was shown following 10 weeks of endurance training with significant increases in maximal succinate dehydrogenase activity (5.6 ± 0.3 vs 4.3 ± 0.2 $\mu g \cdot g \cdot min^{-1}$) and mitochondrial protein (5.6 ± 0.2 vs 4.4 ± 0.3 mg·g) content compared to untrained controls (Gollnick and Ianuzzo, 1972). These biochemical adaptations observed in animal models were later shown in human skeletal muscle with exercise training increasing mitochondrial protein content (4.2 vs 5.4 mg·g) and mitochondrial number (25.6 ± 2.1 vs 21.0 ± 1.8 AU) (Morgan et al., 1971) with cross-sectional analysis revealing greater mitochondrial protein abundance in well-trained endurance athletes (Hoppeler et al., 1973; Nielsen et al., 2010).

A critical adaptation induced during endurance training is increased mitochondrial number and content, a process termed mitochondrial biogenesis (Hood, 2001). Mitochondria

are essential organelles responsible for producing ATP and maintaining energy balance within skeletal muscle (Hood, 2009). Therefore, increases in mitochondrial protein abundance are accountable for ATP production, free fatty acid (FFA) transport, FFA oxidation, glucose transport and oxygen delivery, collectively resulting in greater energy production efficiency (Holloszy and Coyle, 1984) and subsequent improvements in exercise capacity within endurance-trained populations (Talanian et al., 2010). Alongside the changes in mitochondrial adaptations, shifts towards greater oxidative muscle fibre types are observed, with increased capillarisation and angiogenesis (Andersen and Henriksson, 1977). Additionally, an enlargement of the immediate muscular supply of energy substrate for use during exercise is augmented, including increased intramuscular substrate storage of glycogen (Hickner et al., 1997; Putman et al., 1998) and lipids (van Loon et al., 2004; Tarnopolsky et al., 2007). The improved capacity to utilise intramuscular triglycerides in endurance-trained individuals during exercise reduces the demand and contribution of finite muscle glycogen stores to energy production (Hurley et al., 1986), a significant metabolic adaptation attributed to changes in mitochondrial content in response to exercise training.

Mitochondrial adaptations undoubtedly alter the metabolic capacity of the muscle; however, multiple energy systems are necessary for energy production and are similarly changed in response to endurance exercise. There are increases in the Tricarboxylic Acid (TCA) cycle and electron transport chain proteins in the mitochondria (Morgan et al., 1971), as well as increases in proteins responsible for 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 these adaptations are a tighter coupling of ATP supply with demand and reductions in adenosine diphosphate (ADP) concentrations required to achieve the same O_2 consumption per gram of muscle (Holloszy and Coyle, 1984). The greater efficiency of energy production from mitochondria results in smaller increases in ADP, adenosine monophosphate (AMP), and inorganic phosphate (Pi) during exercise at the same absolute intensity. These metabolites have an essential role in the allosteric regulation of glycogenolysis and glycolysis, meaning increased mitochondrial efficiency results in greater lipid utilisation with a proportional decrease in carbohydrate metabolism at the same absolute exercise intensity (Hermansen, Hultman and Saltin, 1967; Karlsson, Nordesjo and Saltin, 1974; Phillips et al., 1996). The combination of reduced muscle glycogen utilisation, tighter ATP coupling, and increased lipid oxidation and transport results in greater fatigue resistance in endurance-trained individuals, ultimately leading to improved endurance capacity and performance.

2.2.1 Mechanisms of Adaptation Following Endurance Exercise

At the onset of exercise, calcium ions (Ca^{2+}) are released from the sarcoplasmic reticulum, muscle glycogenolytic rate and FFA availability are increased, and exercise metabolites are generated, such as lactate as well as reactive oxygen species, all of which serve to destabilise the cellular environment and induce specific signalling responses regulating skeletal muscle adaptation (Hawley, Tipton and Millard-Stafford, 2006). These exercise-induced homeostatic perturbations result in the activation of specific protein kinases, including AMP-dependent protein kinase (AMPK), Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) and p38 mitogen-activated protein kinase (p38 MAPK). Once activated, these kinases combine with and activate transcription factors at the nuclear and mitochondrial levels,

promoting the transcription of genes that derive nuclear and mitochondrial proteins. These proteins are assembled into tertiary complexes containing multiple individual subunits before becoming functional as part of a comprehensive metabolic network.

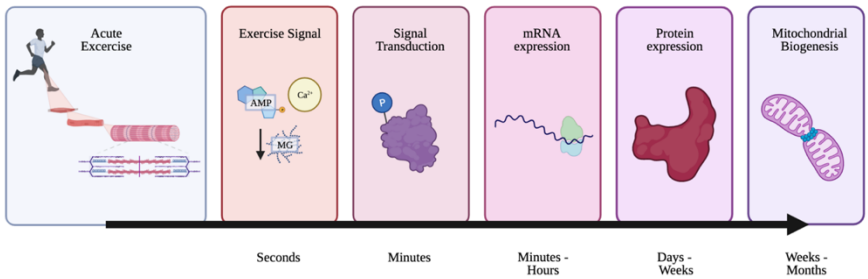

Figure 2.1 – Schematic of the time course of exercise-induced physiological, biochemical, and molecular responses in skeletal muscle leading to training adaptation. Acute exercise changes metabolism, altering the intracellular environment (e.g., increased AMP, Ca2+ and decreased muscle glycogen). These exercise signals activate signalling proteins via phosphorylation, controlling downstream transcription factors and influencing gene expression, the chronic effect of which increases protein synthesis and subsequent phenotypic adaptation. Created with biorender.com

2.2.1 i) 5' AMP Activate Protein Kinase (AMPK) Signalling in Response to Endurance Training

AMPK is a Serine/Threonine Kinase comprised of 3 subunits: an, α - catalytic subunit, and β- and γ-, two regulatory subunits. The α-subunit contains the threonine 172 (Thr^{172}) phosphorylation site that is activated by upstream kinases (Hawley et al., 1997), the β-subunit contains the glycogen binding domain (McBride et al., 2009), and the γ-subunit is responsible for housing the binding sites for AMP and ATP. Due to AMPK's sensitivity to changes in these metabolites and muscle glycogen concentration, it is widely accepted that AMPK is sensitive to metabolic stress, evidenced by its increase in activity when AMP concentrations are increased during periods of rapid ATP hydrolysis during exercise (Drake, Wilson and Yan, 2016) and following exercise-induced muscle glycogen depletion (Polekhina et al., 2005; McBride et al., 2009). Upon Activation, AMPK stimulates energy-generating processes,

increasing glucose uptake via glucose transporter type 4 (GLUT4) translocation and fatty acid oxidation (Merrill et al., 1997) whilst simultaneously suppressing energy-costly cellular processes, including protein synthesis (Bolster et al., 2002) to facilitate the restoration of energy balance to the tissue (Richter and Ruderman, 2009). A mechanism by which AMPK increases lipid oxidation is via the inhibition of acetyl-CoA-carboxylase-ß (ACCß), thus reducing malonyl-CoA concentrations (Saha and Ruderman, 2003). Given the inhibitory effect of malonyl-CoA on fatty acid transporter carnitine palmitoyl transferase-1 (CPT-1), any AMPK-induced reduction in malonyl-CoA availability will only serve to facilitate fatty acid transport into the mitochondria for oxidation (Bezaire, Heigenhauser and Spriet, 2004).

As well as coordinating metabolic alterations during exercise to account for changes in the energy status of the muscle, AMPK is an essential signalling protein when considering endurance training adaptation. AMPK activates the downstream co-activator PGC-1α via phosphorylation of Thr^{177} and Ser^{538} residues (Jager et al., 2007), promoting the expression of nuclear and mitochondrial genes (Hood et al., 2016). Through the chronic application of the chemical AMPK activator AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide), mitochondrial protein expression (CS and SDH) was elevated in comparable amounts to those induced via endurance training (Winder et al., 2000). AMPK as an exercise-sensitive signalling kinase was first evidenced in a rodent model by Winder and Hardie (1996) and later supported in multiple human models across a variety of exercise modalities, including; knee extensor exercise (Frosig 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). Phosphorylation of AMPK occurs in an exercise-dependent manner with higher intensities resulting in greater AMPK activation than lower exercise intensities. For example, exercise completed at 80% $\dot{V}O_{2peak}$ resulted in greater AMPK activation compared to 40% $\dot{V}O_{2peak}$ (Chen et al., 2003) even when exercise

was work matched (Egan et al., 2010) or 75% $\dot{V}O_{2max}$ compared to 50% $\dot{V}O_{2max}$ (Wojtaszewski et al., 2000). Alongside intensity-dependent activation, there is also evidence of isoform-specific responses. AMPK exists in two isoforms, AMPKα1 and AMPKα2, with the latter showing greater sensitivity to exercise with AMPKα1 appearing unresponsive to exercise-induced activation at high and low intensities (Wojtaszewski et al., 2000).

Evidence in favour of endurance training status attenuating AMPK activation is increasingly apparent, with post-exercise AMPK activation decreased following a period of adaptation (Nielsen et al., 2003). This reduction in AMPK activation is likely due to decreased cellular disturbance of homeostasis following endurance exercise (AMP, ADP concentrations, muscle glycogen utilisation). This evidence highlights the need to periodically manipulate training variables, including exercise intensity and duration, to maintain sufficient training stimulus to activate AMPK and achieve progressive adaptations to exercise.

The carbohydrate or specifically muscle glycogen, sensitive nature of AMPK was first proposed by Wojtasewski *et al.* (2003), who reported higher basal activities in both AMPK isoforms (α1 and α2) when muscle glycogen was low (~160 mmol·kg^{-1} DW [millimoles of glycogen per kilogram of dry tissue]) compared with muscle that had undergone glycogen loading (~900 mmol·kg^{-1} DW). Despite significant differences in muscle glycogen concentration, the energy status of the muscle was unaffected with comparable concentrations of adenine nucleotides and creatine phosphate, suggesting that the change in AMPK activation was due to fuel-dependent mechanisms. When exercise is completed with low muscle glycogen concentration, AMPK^{Thr172} phosphorylation is significantly increased (Yeo et al., 2010), alongside expanded nuclear AMPKα2 protein abundance (Steinberg et al., 2006) compared to exercise commenced with high muscle glycogen concentration. AMPK is similarly sensitive

to exogenous CHO availability, with fasted exercise (Stocks et al., 2019) inducing AMPK activation above that of exercise completed in a fed state. Likewise, withholding exogenous CHO ingestion during exercise increases AMPK^{Thr172} phosphorylation and AMPK activation compared to fed conditions.

2.2.1 ii) Peroxisome Proliferator-Activated Gamma Coactivator 1-alpha (PGC-1α)

Referred to as the "master regulator" of mitochondrial biogenesis, PGC1-α exerts control over multiple nuclear and mitochondrial transcription factors in its capacity as a transcriptional co-activator (Hawley and Morton, 2014). To facilitate the transcription of necessary genes, PGC1-α alters the chromosome structure through the recruitment of histone acetyltransferase (HAT) enzymes, which bind to DNA-bound transcription factors, including nuclear respiratory factors 1 and 2 (NRF-1/2) and myocyte enhancer factors 2 (MEF-2) promoting gene transcription. (Wu et al., 1999; Handschin et al., 2003). Notably, in the cases of NRF-1/2, PGC1-α binds to each and promotes nuclear and mitochondrial gene expression, encoding essential mitochondrial proteins necessary for biogenesis.

Since its discovery in 1998 (Puigserver et al., 1998), evidence supporting PGC1-α's role as a regulator of mitochondrial biogenesis has become unequivocal. For example, rodent models using over-expression of PGC1-α have shown increased skeletal muscle oxidative enzyme capacity (Lin et al., 2002) and improvements in exercise capacity (Calvo et al., 2008). Conversely, PGC1-α gene Knockout (KO) rodent models show impaired mitochondrial biogenesis in response to endurance exercise when compared to wildtype (WT) controls (Leick et al., 2010).

Using a single leg extensor model in humans, Pilegaard et al. (2003) reported 4 weeks of training (1 hr per day, 5 days per week) significantly increased pre, and immediately post-exercise PGC1-α activity in the trained limb, whilst PGC1-α mRNA expression was increased in both legs 2 and 6 hrs post-exercise although greater in the trained leg. This increase in PGC1-α expression coincided with increases in mitochondrial transcription factor (Tfam) and Peroxisome Proliferator-Activated Receptor (PPAR) mRNA in the untrained limb. Despite a relative decrease in workload during exercise, PGC1-α expression increased significantly in the trained leg.

A critical study in developing an understanding of the role of PGC1-α during post-exercise adaptive signalling processes was conducted by Perry et al. (2010). The authors provided a time course of both PGC1-α mRNA expression and protein abundance in response to two weeks of high-intensity cycling exercise. Following each bout of exercise, PGC1-α mRNA expression increased 4h post-exercise before returning to baseline at 24h. The cumulative effect of repeated exercise-induced increases in mRNA resulted in increased PGC1-α protein abundance at two weeks, indicative of an exercise training response. Of note, across the two weeks of training, the amplitude of PGC1-α mRNA expression was reduced, suggestive of the participants becoming accustomed to the exercise modality or intensity. A reason for reduced PGC1-α mRNA expression may be the apparent intensity-sensitive nature of PGC1-α mRNA expression, with exercise at 80% $\dot{V}O_{2max}$ inducing a significantly greater transcriptional response compared to exercise at 40% $\dot{V}O_{2max}$ despite trials being matched for total work done (Egan et al., 2010). Interestingly, the expression of PGC1-α mRNA was not expressed to a greater extent when exercise intensity exceeded 100% $\dot{V}O_{2max}$ (Edgett et al., 2013). These are essential observations in the context of exercise training in elite athletes,

whereby progressive overload is an essential component of training prescription to avoid any plateau in exercise adaptation.

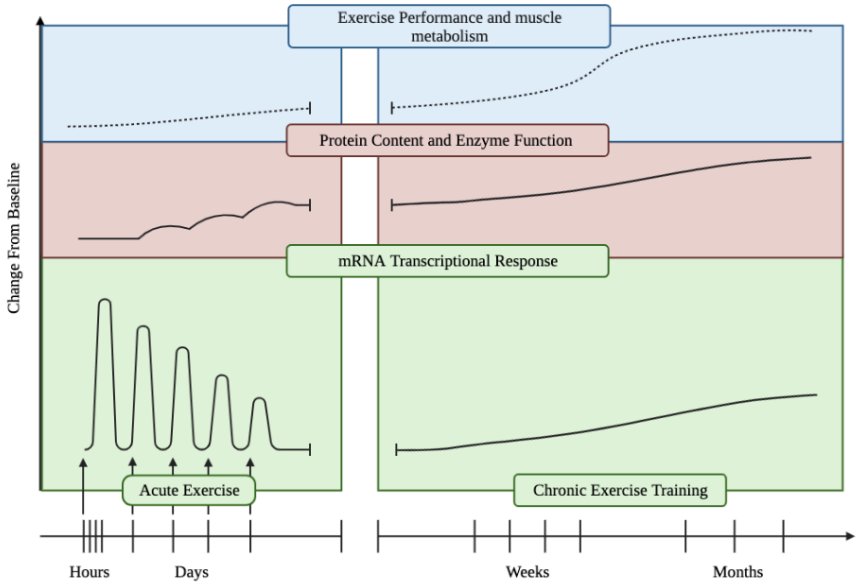

Figure 2.2 – A theoretical overview of the Transcriptional (mRNA) (bottom panel), translational (Protein content) (middle panel) and phenotypic (top panel) responses to acute and chronic exercise training. Adapted from (Egan and Zierath, 2013). Created with biorender.com.

2.2.1 iii) p38 Mitogen-activated Protein Kinase (p38 MAPK)

A member of the mitogen-activated protein kinase family, p38 MAPK is a crucial stress-inducible kinase, critically important within signal transduction pathways associated with PGC1-α activation and subsequent mitochondrial biogenesis. p38 MAPK is activated in response to various stimuli, including mechanical strain, inflammation, oxidative stress, and environmental stress. Akimoto et al. (2005) reported exercise-induced p38 MAPK phosphorylation leading to increased PGC1-α mRNA expression in mice. Additionally, the authors provided evidence that activating the p38 MAPK pathway in C2C12 myotubes increased PGC1-α promoter activity which several inhibitors could block. In addition to the exercise-sensitive response, transgenic mice with over-expressed p38 activator, anti-tumour effector domain MKK6 (MKK6E), had greater PGC1-α and COXIV protein expression in fast-

twitch skeletal muscle. This study was the first to show the contractile activity-induced activation of p38 MAPK and the downstream activation of PGC1-α and resultant mitochondrial adaptations. Follow-up *in vivo* studies by the same research group proposed a mechanism for p38 MAPK-dependent PGC1-α expression via MEF2 and activating transcription factor 2 (ATF2) binding sites on the PGC-1α promotor (Akimoto, Li and Yan, 2008).

In response to exercise, p38-AMPK activation increases following running (Boppart et al., 2000; Bartlett et al., 2012) and both steady-state (Benziane et al., 2008; Egan et al., 2010) and intermittent cycling (Gibala et al., 2009) with exercise commenced under conditions of low muscle glycogen concentration further augmenting this response (Chan et al., 2004). Interestingly, both whole muscle homogenate (Yeo et al., 2010; Bartlett et al., 2013; Stocks et al., 2019) and cytosolic fractions (Chan et al., 2004) do not show any increase in p38MAPK phosphorylation when exercise is completed with low CHO availability, suggesting p38 MAPK phosphorylation post-exercise may be isolated to nuclear fractions only (Chan et al., 2004).

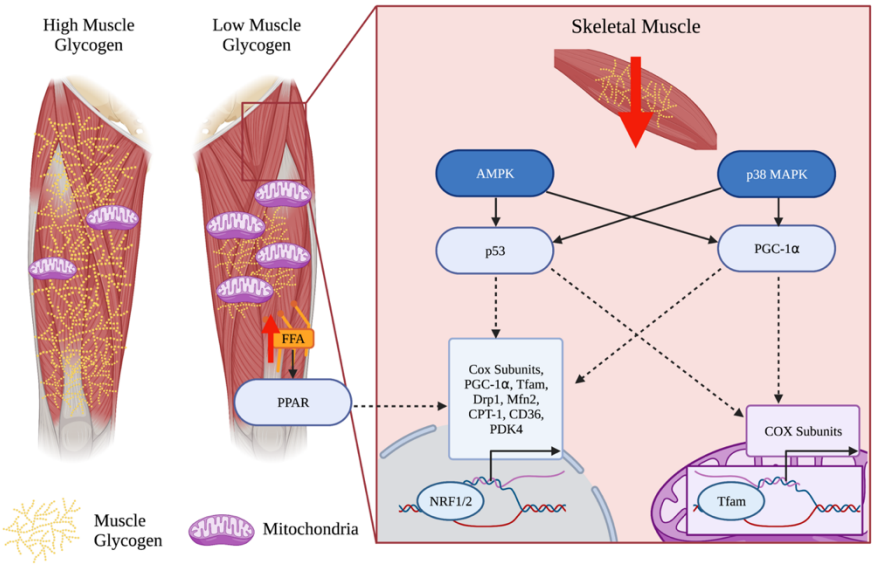

Figure 2.3 – Molecular Response to exercise completed under low muscle glycogen availability. Created with biorender.com.

2.2.1 iv) The Role of Temperature in the Regulation of Endurance Training Adaptation

Beyond training modality, duration, and intensity, local (muscle and core) and environmental temperatures are increasingly considered essential for maximising endurance training adaptation. Temperature can be easily manipulated through multiple strategies, with the impact of cold and heat stress on the regulation of endurance training briefly explained within this section.

Cold Stress

Post-exercise cold exposure has been increasingly investigated as a therapeutic strategy to increase mRNA and protein expression of markers associated with mitochondrial biogenesis and endurance training adaptation. Reducing post-exercise ambient temperatures (~7°C) (Slivka et al., 2012; 2013; Zak et al., 2017) and cold water immersion (Ihsan et al., 2014; 2015; 2020b) have been shown to up-regulate PGC-1α gene transcription and translational responses when compared to exercise alone.

Research has also focused on post-exercise cold water emersion on markers of mitochondrial adaptation. For example, Joo et al. (2016) reported a 6-fold increase in PGC-1α mRNA expression in human skeletal muscle subjected to acute post-exercise cold water immersion compared to control (no cold exposure). Further research from the same research group aimed to understand the mechanistic control of this PGC-1α mRNA up-regulation and evidenced that reduced muscle temperature was not responsible for elevated PGC-1α transcriptional response and attributed increases in mRNA to elevated systemic increases in

noradrenaline, which may have up-regulated AMPK phosphorylation impacting its downstream targets such as PGC-1α (Allan et al., 2017).

When post-exercise cold water immersion was applied to a single limb immediately following endurance exercise three times weekly for four weeks, total AMPK and AMPK phosphorylation were significantly increased compared to the non-cooled limb (Ihsan et al., 2015). In addition, large effect sizes were observed in changes in protein content of p38 MAPK and PGC-1α in the cold limb only, suggesting greater signalling pathways associated with higher mitochondrial biogenesis following post-exercise cold water immersion; however, this did not translate into increases in mitochondrial protein content.

Heat Stress

Repeated, strategic exposure to heat stress is a common practical approach to dealing with the detrimental effects of heat stress on performance (Racinais et al., 2015a) with two weeks of daily heat exposure (heat acclimation) restoring the deleterious effects of heat stress on time trial performance in 37°C vs 8°C (Racinais et al., 2015c). Heat acclimation and the associated intramuscular adaptations will be discussed in more detail later in this chapter.

There is increasing evidence across various experimental models showing an 'endurance-like' adaptive response following repeated heat exposure. C2C12 myotubes subjected to 5-6 days of heat acclimation increased AMPK phosphorylation and subsequent increases in SIRT1 and PGC-1α expression. Downstream transcription factors (NRF1, NRF2 and Tfam) were up-regulated, leading to an increased transcriptional response at the mitochondrial level (Liu and Brooks, 2012; Patton et al., 2018). Following heat acclimation,

myotubes were phenotypically altered, allowing greater peak oxidation rates during a lipopolysaccharide challenge (LPS), an *in vitro* test intended to impair mitochondrial capacity (Patton et al., 2018). Rodent models have shown that pre-exercise heat stress improves exercise capacity (Chen et al., 1999), which was attributed to increased mitochondrial enzyme activity compared to unheated controls (Sammut et al., 2001). Comparing exercise-only, heat-only and their combination (exercising followed by heat stress), Tamura et al. (2014) reported increased adaptive response when animals were heated post-exercise with up-regulated adaptive signalling and protein activity.

In the context of endurance exercise, there is no consensus on the effects of pre-, during or post-exercise heat stress on humans' skeletal muscle adaptive response. The intramuscular impact of heat stress is difficult to methodologically isolate from other exercise-induced stressors. Multiple strategies are available to induce local muscle heat, but the extent to which they exert a systemic effect via increased core body temperature needs to be better understood. Utilising short-wave diathermy (2h per day for 6 consecutive days), Hafen et al. (2018) reported increased PGC-1α and mitochondrial complex I and V enzyme expression in human *vastus lateralis*. Phenotypically, greater mitochondrial respiratory capacity was observed in the heat-treated limb compared to the internal contralateral control.

Understanding the combined benefit of additional heat stress during exercise at the skeletal muscle level requires further investigation and careful consideration of the implications on exercise capacity. Any improvement in signalling responses and local adaptation could be offset by reduced training volume or quality.

2.3 Manipulating Carbohydrate Availability During Exercise to Augment Training Adaptation

Commencing endurance exercise with reduced carbohydrate availability results in significant disturbance to the intra-muscular metabolic milieu, with low carbohydrate availability impacting the molecular signalling responses associated with endurance training adaptation. For example, exercise with low carbohydrate availability reduces muscle glycogen utilisation (Arkinstall et al., 2004), increases circulating free fatty acid (FFA) availability (Wojtaszewski et al., 2003) and circulating catecholamines (Hansen et al., 2005) compared to exercise completed with high carbohydrate availability. The resultant metabolic challenge invokes a compensatory adaptive response through the up-regulation of several molecular signalling processes responsible for orchestrating endurance training adaptation.

At the molecular level, $AMPK^{Thr172}$ (Yeo et al., 2010), ACC^{Ser79} and $p53^{Ser15}$(Bartlett et al., 2013), phosphorylation, AMPK activity (Wojtaszewski et al., 2003) and nuclear abundance of AMPKa2 (Steinberg et al., 2006) are increased following exercise with low carbohydrate availability. Utilising multiple train-low strategies, carbohydrate availability has been shown to impact adaptive signalling responses positively; however, many key signalling kinases, including p38 MAPK and CaMKII (Yeo et al., 2010; Bartlett et al., 2013; Lane et al., 2015; Hearris et al., 2019; Stocks et al., 2019), have shown no carbohydrate dependent response. Alongside increased signalling responses, exercise with low carbohydrate availability induces a significant transcriptional response, including greater PGC-1a expression as well as others involved in substrate utilisation and mitochondrial biogenesis (Pilegaard et al., 2002; Bartlett et al., 2012; 2013; Psilander et al., 2013; Lane et al., 2015; Stocks et al., 2019). Given the up-regulation of molecular signalling events and mRNA, this evidence

suggests that commencing exercise with low muscle glycogen augments AMPK-PGC-1a mediated signalling, leading to increased mRNA expression of various metabolic and mitochondrial proteins. That said, further evidence is needed to link molecular and phenotypic outcomes from changes in signalling response, protein expression, and chronic performance improvement.

Commencing exercise with low carbohydrate availability can be achieved through a variety of exercise, nutrition, and combined interventions. Herein, an overview of each "train low" strategy will be discussed, covering both acute and chronic interventions, and appraising the efficacy of these interventions in practice.

2.3.1 Fasted Training

Performing endurance training following an overnight fast represents one of the most straightforward strategies for training with reduced carbohydrate availability, with breakfast consumed only following exercise. Despite limited alterations to pre-exercise muscle glycogen following an overnight fast, plasma glucose remains lower with increased FFA availability compared to participants who consumed breakfast before exercise (Montain et al., 1991; Horowitz et al., 1997). Based upon this metabolic response, exercise in the fasted state results in increased post-exercise AMPK and CREB signalling (Akerstrom et al., 2006; Stocks et al., 2019) with mRNA expression of genes associated with the regulation of substrate utilisation (GLUT4, CD36, CPT-1, PDK4) and mitochondrial function (UCP3) (Civitarese et al., 2005; Cluberton et al., 2005) elevated compared to groups fed pre-and during exercise.

Utilising an 8-week fasted endurance training intervention, Nybo et al. (2009) reported daily fasted exercise sessions (50-90 mins of high-intensity exercise at 70-85% $\dot{V}O_{2max}$) increased 3-hydroxy acyl-CoA dehydrogenase (β-HAD) activity and muscle glycogen storage capacity compared with a control group that consumed breakfast before exercise. Later, Van Proeyen et al. (2011) reported significantly increased citrate synthase (CS) and β-HAD following six weeks of fasted endurance training (60-90 mins cycling at 70% $\dot{V}O_{2max}$). Mechanistically, fasted exercise significantly impacts substrate metabolism, with periods of regular fasted exercise reducing muscle glycogen utilisation during exercise (De Bock et al., 2008) with an increase in intra-myocellular lipid (IMCL) utilisation in type I fibres (Van Proeyen et al., 2011). Collectively, these data show that restricting exogenous carbohydrate intake pre- and during endurance exercise enhances post-exercise gene expression and oxidative enzyme activity. However, whilst fasted exercise has shown superior biochemical adaptations, the translation to improved exercise capacity has yet to be experimentally shown.

2.3.2 "Sleep-low, Train-low" (SL-TL)

As with fasted training, The SL-TL model utilises a fasted exercise bout in the morning with muscle glycogen intentionally depleted by exercise the previous night, with low muscle glycogen maintained until the following morning through restriction of carbohydrate intake during the intervening overnight period. This strategy extends the cumulative time spent with reduced muscle glycogen (12-14h) depending on the duration of time (including sleep) between the two training sessions. Several acute studies have shown increased signalling kinase activity (AMPK and p38 MAPK), transcription factor (p53) and metabolic gene expression associated with substrate utilisation and mitochondrial biogenesis compared to exercise completed with high glycogen conditions (Wojtaszewski et al., 2003; Bartlett et al., 2013; Lane et al., 2015).

Utilising a whole-body exercise modality (running), Bartlett et al. (2013) required participants to perform a bout of high-intensity training (HIT) running under conditions of high or low carbohydrate availability. To promote low carbohydrate availability for a specific session, participants completed a glycogen-depleting exercise the evening before the trial. They were restricted from consuming exogenous carbohydrates during and in the hours between exercise bouts. p53 phosphorylation was significantly higher in the low carbohydrate condition immediately post and 3h post-exercise compared to the high carbohydrate condition. The post-exercise transcriptional response was also elevated in the low carbohydrate condition with Pyruvate Dehydrogenase Kinase 4 (PDK4), Tfam, and mitochondrial subunit 4 (COXIV) and PGC-1α all up-regulated 3h post-exercise compared to the carbohydrate fed group. Despite evidence in favour of augmented training adaptation when implementing this exercise-nutrition strategy, the carbohydrate restriction resulted in an overall calorie reduction in the low carbohydrate condition; given the similarities in adaptation between carbohydrate and calorie restriction, it is difficult to isolate the effects of reduced carbohydrate availability on the observed adaptive response.

Addressing the energy intake imbalance in the study mentioned above, Lane et al. (2015) implemented an isoenergetic diet of 8 $g \cdot kg^{-1}$ carbohydrate for both exercise conditions. Still, the researchers timed carbohydrate intake differently to promote low carbohydrate availability overnight. During the Sleep Low (SL) condition, participants consumed 8 $g \cdot kg^{-1}$ carbohydrate before the evening hit session, followed by an overnight fast, whereas the control (sleep normal) condition consumed 4 $g \cdot kg^{-1}$ carbohydrate before the HIT and 4 $g \cdot kg^{-1}$ carbohydrate following the HIT session. The following morning, both conditions completed a 2-hr steady-state cycling protocol (50% Peak Power Output [PPO]). Despite increased fat oxidation and PDK4 mRNA expression following SL, this strategy had no additional benefit

when considering markers of mitochondrial biogenesis. This may be due to the trained nature of the participants and the relatively low exercise intensity during the low carbohydrate session. Despite exercise being implemented to reduce muscle glycogen concentration, sufficiently low concentrations necessary to induce these adaptive responses may not have been achieved.

The translation of acute training responses to enhanced performance following the chronic application of SL-TL is limited. Nevertheless, researchers at the French National Institute of Sport (INSEP) conducted a 3-week exercise nutrition intervention implementing the SL-TL approach and reported improved performance in trained triathletes (Marquet et al., 2016a). To replicate a real-world exercise test, the researchers conducted a cycle-run test (to replicate land-based triathlon disciplines) whereby participants completed a 40-min sub-maximal cycling test (70% Maximal aerobic power [MAP]) followed by a 10km track-based time trial. Participants who completed two weeks of SL-TL improved their 10km running time by ~3% compared to no change in the control group. Additionally, the SL group improved supra-maximal cycling performance (150% MAP) compared to the control and showed positive changes to body composition (reduced fat mass and maintained lean muscle mass). The same group also reported that a SL approach induced positive performance adaptation following as little as one week of training, despite no changes in metabolic markers of adaptation (Marquet et al., 2016b), suggesting that changes in pacing strategies during tests and subjective perceived exertion may have been altered following 1 week of SL-TL.

Given the acute metabolic alterations observed during fasted exercise, a four-week SL intervention in trained cyclists aimed to elucidate the skeletal muscle adaptive response to the sleep low strategy with specific attention to genes and proteins associated with fat metabolism (Riis et al., 2019). No differences in intramuscular triglyceride metabolism were observed

between SL and control groups. Despite increases in fat oxidation during sleep low sessions, there was no increase in proteins associated with fatty acid transport or metabolism, casting doubt on the transferability of acute adaptive responses to longer-term training adaptation. Unfortunately, this study did not measure "conventional" adaptive markers (AMPK/p38 MAPK phosphorylation, PGC-1α mRNA and protein expression and transcriptional responses) used in previous low carbohydrate literature. Despite positive data associated with mitochondrial biogenesis and energy-sensing kinases in response to low carbohydrate availability, this study failed to add any further information to the evidence base in this regard, making it difficult to place this study within the literature.

Most recently, Salokannel, Hakulinen and Ahtiainen (2021) implemented a 4-week SL-TL intervention in recreationally, endurance-trained males (n = 5) and females (n = 12). The authors published data indicating comparable increases in $\dot{V}O_{2peak}$ within both groups, with maximal running velocity and supra-maximal time to exhaustion lactate concentrations improved in the SL group only. Despite performance improvements, no changes in sub-maximal substrate utilisation were observed following the intervention, but heart rate was reduced in SL only. It must be noted that the HIT protocol employed only included 4x 4mins of running at or above 95% $\dot{V}O_{2peak,}$ which is considerably fewer intervals and duration typically reported during glycogen depletion protocols; as such, muscle glycogen may not have been depleted sufficiently enough to induce positive metabolic responses to exercise.

2.3.3 Twice per Day Training

Amongst the earliest proposed strategies to manipulate carbohydrate availability around specific exercise bouts, the Twice per Day (TD) strategy shares similarities to the SL

approach, i.e., the initial exercise bout serves to reduce muscle glycogen concentration, and carbohydrate ingestion is prohibited until after the completion of the second exercise bout albeit, the intervening period is not overnight. As per the name, the TD approach typically involves completing the first session in the morning, and the second, later the same day.

The first authors to implement this strategy of carbohydrate manipulation reported greater oxidative adaptations and endurance performance following 10 weeks of single-leg knee extensor exercise (Hansen et al., 2005). Whilst these results were promising, the divergent exercise regimens between limbs must be considered. Specifically, exercise was completed 5 days per week, with one limb completing daily exercise under normal glycogen conditions, while the opposite limb completed exercise twice every other day. The second daily session is completed under low glycogen conditions (Figure 2.4). This strategy allowed both limbs to achieve the same total work throughout the training. However, one limb would complete 50% of the sessions with low muscle glycogen availability. Interestingly, the "low" trained limb had greater performance improvements than the control participants. Alongside performance improvements, maximal citrate synthase activity was also increased in the train low group, highlighting mitochondrial remodelling. It improved oxidative capacity as a potential mechanism of adaptation following this strategy.

Despite the positive data generated by this group, the population and exercise choices were noteworthy, as knee extensor exercise does not generalise well to real-world endurance training practices. The untrained participants may see a different response from active or trained participants. Additionally, using a contralateral SL extensor model does not allow for the dissociation of local and systemic effects of exercise.

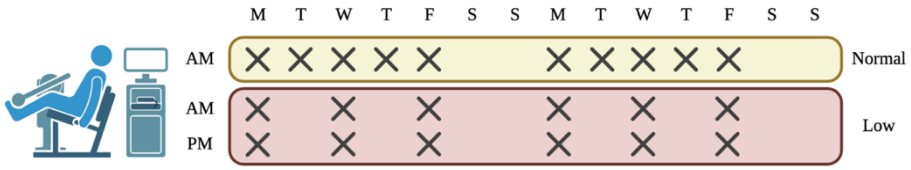

Figure 2.4 – Adapted schematic representation of the twice-per-day model implemented by Hansen et al. (2005). The "Normal" leg completed daily exercise bouts with high muscle glycogen, and the "low" leg completed 2 bouts of exercise every 2 days, with the first bout of exercise intended to deplete muscle glycogen before the following session. X represents individual training sessions. Created with biorender.com

With greater practical "real world" applicability, Yeo et al. (2008b) utilised a TD training regimen in well-trained male cyclists. During a 3-week training intervention, participants completed exercise once daily, alternating between steady-state or HIT, with high muscle glycogen (control) or twice every second day. The second session was conducted with low muscle glycogen concentration. In the "low" glycogen group, steady-state exercise was performed in the morning, followed by a 2h carbohydrate-restricted recovery period before completing a HIT session. Muscle biopsies were collected pre-and post-training intervention to assess intra-muscular adaptation, and a time trial (TT) was used to measure changes in performance. Improvements in TT performance were comparable between groups despite significant increases in CS activity, β-HAD activity, COXIV and fat oxidation rate in the low carbohydrate group. However, these adaptations occurred despite decreases in exercise capacity in response to exercise with low carbohydrate availability. This suggests that low muscle glycogen concentration may induce superior adaptive reaction even when training volume is reduced. Utilising the same study design, Hulston et al. (2010) reported increased lipid oxidation alongside increased Cluster of Differentiation 36 (CD36) and β-HAD expression in the train low group compared to the control group. Studies implementing the TD approach have provided robust evidence to support the hypothesis that training with low carbohydrate availability likely induces beneficial skeletal muscle adaptation in the context of metabolism, whilst the impact on performance remains equivocal. It is important to remember

that whilst both groups completed the same total exercise, the timing of each group's exercise bouts was different; recovery between bouts was not controlled. Comparing groups within these studies are therefore hampered by divergent training programmes.

2.3.4 Periodised Approach and an Amalgamation of Train Low Strategies

Applying laboratory-based interventions within real-world endurance training settings requires a pragmatic approach to carbohydrate manipulation and integration into an athletic training programme. As such, an amalgamation of the aforementioned train-low strategies is needed to allow optimal integration of any nutritional intervention with training programmes by coaches, sport scientists and nutritionists rather than a one-dimensional strategy in isolation (Stellingwerf, 2012). Given the dynamic nature of applied exercise and nutritional requirements, it is, therefore, necessary to investigate the effects of multiple strategies of carbohydrate periodisation during an athlete's training programme. There is no evidence of improved performance outcomes or adaptive response after several weeks of carbohydrate periodisation. Incorporating fasted, twice daily and post-exercise carbohydrate replenishment in elite race walkers failed to induce additional performance benefits compared to traditional high carbohydrate provision (Burke et al., 2017). Additionally, comparable muscular adaptations were observed, including increased CS activity and performance between periodised carbohydrate training (twice daily) and exogenous carbohydrate provision around all training sessions (Gejl et al., 2017). A consistent issue with both studies may be due to the magnitude of muscle glycogen depletion. As the athletes recruited in these studies were highly trained and habitually consuming recommended carbohydrate intakes before and during exercise training (Burke et al., 2017), their resting muscle glycogen was significantly greater than lesser trained populations used in early low carbohydrate training literature (Areta and

Hopkins, 2018), requiring greater exercise intensity and duration to deplete muscle glycogen sufficiently. In a study by Gejl et al. (2017), the authors reported muscle glycogen concentrations remained above 400 mmol·kg dw^{-1} following the second bout of exercise during the twice-daily strategy, failing to induce sufficient muscle glycogen depletion for beneficial adaptation. Conversely, within the literature reporting positive training adaptations, lower muscle glycogen values (around 100 mmol·kg dw^{-1}) appeared to be sufficient to induce positive training adaptations (Yeo et al., 2008b; Morton et al., 2009). This data may provide evidence of an absolute critical level of muscle glycogen that must be achieved to induce significant activation of signalling pathways and subsequent muscular adaptations.

2.3.1 Glycogen Threshold Hypothesis

Muscle glycogen availability is increasingly accepted as a critical factor for promoting endurance training adaptation. An increasing body of evidence suggests the training response is augmented when exercise is commenced with low carbohydrate availability. Critically, studies that have reported pre-exercise muscle glycogen concentrations in the range of 100-300 mmol·kg dw^{-1} report increased the activity of crucial cell signalling kinases, transcription factors and gene expression of metabolic proteins relative to exercise with muscle glycogen above 300 mmol·kg dw^{-1} (Winder, Taylor and Thomson, 2006; Cochran et al., 2010; Yeo et al., 2010; Bartlett et al., 2013; Impey et al., 2016). Furthermore, when sessions are repeatedly performed with low muscle glycogen concentrations, several markers of skeletal muscle adaptation are increased (Hansen et al., 2005; Yeo et al., 2008b; Morton et al., 2009), whilst exercise that fails to meet this threshold does not show improved adaptation (Gejl et al., 2017). This data, taken with evidence from studies utilising exogenous carbohydrate supplementation during exercise, suggests a glycogen threshold exists. Interestingly, AMPK activation was

attenuated during exercise with exogenous carbohydrate supplementation (Akerstrom et al., 2006); however, when exogenous carbohydrate failed to maintain muscle glycogen concentration above 200 mmol·kg dw^{-1}), AMPK activation occurred (Lee-Young et al., 2006). Collectively, this data suggests the existence of a glycogen threshold, whereby exceeding this absolute level of muscle glycogen induces greater acute and chronic skeletal muscle adaptation associated with train-low models.

The potential existence of a glycogen threshold does not diminish the adaptive response to endurance exercise without exceeding this critical concentration. This threshold highlights the possibility of enhanced training adaptations associated with train low models when this specific muscle glycogen depletion is achieved. Manipulating muscle glycogen availability may provide a time-efficient strategy to promote training adaptation compared to exercise commenced with high muscle glycogen concentrations. Impey et al. (2016) reported comparable signalling and gene expression responses following endurance exercise commenced with high (600 mmol·kg dw^{-1}) and low (100 mmol·kg dw^{-1}) muscle glycogen concentrations; however, these responses were achieved following considerably less work (~60 min) in the low glycogen condition. This data suggests that train-low exercise induces work/time-efficient signalling responses to exercise.

Table 2.1 – Overview of the methodological details and study outcomes of both acute and chronic train low studies according to the type of carbohydrate periodisation used.

Reference	Participants	Duration	Exercise Protocol & Glycogen Status (mmol·kg dw^{-1})	Skeletal Muscle Adaptations (Compared to High CHO)	Exercise Performance Outcomes (compared to high CHO)
Twice per day model					
Hansen et al. (2005)	7 untrained men (SL P_{max} = 74 ± 7 W)	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^{-1} respectively.	Protein Activity: ↑ CS ↑ β-HAD	↑ TTE during SL knee extensor exercise
Yeo et al. (2008b)	14 trained male cyclists/triathletes ($\dot{V}O_{2peak}$ = ~ 61 ml·kg·min^{-1}, PPO = ~ 374 W)	3 weeks 4 x week	100 min steady state cycling (63% PPO) followed by 8 x 5 mins intervals at maximal pace either 2 hrs (LOW) or 24 h (HIGH) later. Pre interval exercise glycogen: (LOW: 256, HIGH: 390). Post exercise glycogen (LOW: 124, HIGH: 229).	Protein Activity: ↑ CS ↑ β-HAD ↑ COXIV protein content	↔ in 60 min TT (↑ ~ 10% both groups)
Morton et al. (2009)	23 active men ($\dot{V}O_{2max}$ = ~55 ml·kg·min^{-1})	6 weeks 4 x week	6 x 3 mins running (90% $\dot{V}O_{2max}$). 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).	↑ SDH protein activity	↔ in $\dot{V}O_{2max}$ & intermittent exercise performance (YoYoIR2) (↑ in both groups)
Yeo et al. (2008a)	12 trained male cyclists/triathletes ($\dot{V}O_{2peak}$ = 61.5 ± 1.5 ml·kg·min^{-1}, PPO = 340 ± 5.8 W)	Acute exercise	100 mins steady state cycling (63% PPO) followed by 8 x 5 min intervals at maximal pace either 2 hrs (LOW) or 24 hrs (HIGH) later. Pre-interval exercise glycogen: (LOW: 256, HIGH: 390). Post exercise glycogen (LOW: 124, HIGH: 229).	↑ AMPK^{Thr172} phosphorylation	N/A
Hulston et al. (2010)	14 trained male cyclists ($\dot{V}O_{2peak}$ = 65.3 ± 4.8 ml·kg·min^{-1}, PPO = 377 ± 28 W)	3 weeks 6 x week	90 mins cycling at 70% $\dot{V}O_{2max}$ followed by (2 hrs apart) HIT (8 x 5 mins) in LOW group. HIGH group performed alternate days of either steady state or HIT cycling. Acute glycogen status not measured.	↑ β-HAD protein content ↑ fat utilisation from muscle triglycerides	↔ in 60 min TT (↑ both groups)
Cochran et al. (2010)	10 active men ($\dot{V}O_{2peak}$ = 51.0 ± 1.6 ml·kg·min^{-1})	Acute exercise	HIT cycling (5 x 4-mins at 90-95% heart rate reserve) twice per day (separated by 3 hrs). 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).	↑ p38MAPK phosphorylation Gene Expression: ↔ PGC-1α (↑ both groups) ↔ COXIV (↑ both groups)	N/A
Cochran et al. (2015)	18 active men ($\dot{V}O_{2peak}$ = 44 ± 9 ml·kg·min^{-1}, PPO = 318 ± 47 W)	2 weeks 3 days x week	HIT cycling (5 x 4 mins at 60% PPO) twice per day (separated by 3 hrs). One group consumed CHO (2.3 g·kg) between sessions (HIGH) whereas the other restricted CHO intake (LOW). Acute glycogen status not measured.	↔ CS activity (↑ both groups) Protein Content: ↔ CS (↑ both groups) ↔ COXIV (↑ both groups)	↑ 250 kJ TT performance

(Andersson-Hall et al., 2018)	12 moderately trained women ($\dot{V}O_{2max}$ = 45 ± 6 ml·kg·min^{-1})	Acute exercise	LIT exercise (60 mins at 75% $\dot{V}O_{2max}$) followed by placebo (PLA) (0g CHO, 0g PRO), protein (PRO) (0g CHO, 20g PRO) or CHO ingestion (40g CHO, 0g PRO). 120 mins recovery before incremental peak fat oxidation (FO) test (3mins at 30, 40, 50, 60, 70 and 80% $\dot{V}O_{2max}$). Acute glycogen status not measured.	Metabolomics (Serum) ↑ 3-Hydroxybutyrate (PLA only) ↑ Acetoacetate (PLA Only) ↑ Acetone (PLA and PRO) ↑ Leucine, Isoleucine, Valine, and Alanine (PRO)	↔ FO (Placebo vs. Protein) ↓ FO in CHO ↑ RER in CHO
Fasted training model					
Akerstrom et al. (2006)	9 active men ($\dot{V}O_{2peak}$ and PPO not reported)	Acute exercise	2 hrs 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^{-1}**)** in both groups). Post exercise glycogen: (300 & 200 in FED and FAST respectively).	Protein Activity: ↔ AMPKα2 ↓ AMPKα2 in FED	N/A
Lee-Young et al. (2006)	9 active men ($\dot{V}O_{2peak}$ = 48.5 ± 2.6 ml·kg·min^{-1})	Acute exercise	120 mins cycling (65% $\dot{V}O_{2peak}$) exercise in either a fasted (FAST) or fed (exogenous CHO during) state. Pre exercise glycogen: (500 mmol·kg dw^{-1} in both groups). Post exercise glycogen: (150 & 100 in FED and FAST respectively).	↔ AMPKα2 activity (↑ both groups) Protein Phosphorylation: ↔ AMPKα2^{Thr172} (↑ both groups) ↔ ACC-β^{Ser222} (↑ both groups)	N/A
De Bock et al. (2008)	20 active men ($\dot{V}O_{2peak}$ = 52.9 ± 1.5 ml·kg·min^{-1})	6 weeks 3 x week	1 – 2 hrs cycling (75% $\dot{V}O_{2peak}$). One group trained in the fasted state (FAST) with the other consuming CHO before and during exercise (FED). Acute glycogen status not measured	↑ FABPm	N/A
Nybo et al. (2009)	15 untrained men ($\dot{V}O_{2peak}$ = 38 ± 1 ml·kg·min^{-1}, PPO = 294 ± 10 W)	8 weeks 3-4 x week	3-6 mins of high intensity intervals (70-85% $\dot{V}O_{2max}$). Participants either received CHO or placebo (PLA) during exercise. Acute glycogen status not measured.	↑β-HAD activity ↑ basal muscle glycogen content	↔ peak power output, $\dot{V}O_{2max}$ & 15 min TT performance (↑ both groups)
Van Proeyen et al. (2010)	20 active men ($\dot{V}O_{2peak}$ = 60.9 ± 1.9 ml·kg·min^{-1})	6 weeks 4 x week	1 – 1.5 hrs cycling (70% $\dot{V}O_{2max}$). One group trained in the fasted state (FAST) with the other consuming CHO before and during exercise (FED). Acute glycogen status not measured.	Protein Activity: ↑ CS ↑ β-HAD	Similar improvements in 1 h TT performance in both groups
Andersson Hall et al. (2016)	Thirteen highly trained athletes (nine men and four women; $\dot{V}O_{2max}$ 66 ± 1 ml·min^{-1}·kg^{-1})	Acute exercise	One condition fasted (FAST), 1 condition having consumed 1.2 g·kg^{-1} CHO 3 hrs prior to exercise (CON) followed by incremental peak fat oxidation (FO) test (3mins at 30, 40, 50, 60, 70 and 80% $\dot{V}O_{2max}$). In fed group, FO was followed by 60 mins exercise at 75% $\dot{V}O_{2max}$ (10 mins cycling, 10 mins rowing). 120 min recovery before 2nd FO test (EXER). Acute glycogen status not measured.	↑ Plasma FFA in FAST and EXER ↑ Plasma Cortisol in EXER ↓ Plasma insulin in EXER Metabolomics: ↓ BCAA in EXER ↑FFA in EXER and FAST ↑ Ketone Bodies in EXER & FAST ↑Glycerol in EXER & FAST	↑ FO in FAST & EXER ↑ Fat_{max} in FAST & EXER ↓ RER in FAST & EXER
Sleep low model					
Pilegaard et al. (2005)	Study A: 6 active men ($\dot{V}O_{2peak}$ = 57.5 ± 2.7 ml·kg·min^{-1})	Acute exercise Acute exercise	Study A: 1-legged glycogen depleting exercise followed by 2-legged cycling (2 hrs at 45% $\dot{V}O_{2max}$) the subsequent day. Pre-exercise glycogen: (LOW: 337, HIGH: 609). Post exercise glycogen (LOW: 306, HIGH: 423). Study B: 3 hrs of 2-legged knee extensor exercise with either normal (NORM) or LOW glycogen. Pre-exercise glycogen:	Study A: Gene Expression: ↑ PDK4 ↑LPL ↑HKII Study A&B:	N/A N/A

	Study B: 6 active men ($\dot{V}O_{2peak}$ = 57.5 ± 2.7 ml·kg·min^{-1})		(LOW: 240, HIGH: 398). Post exercise glycogen (LOW: 101, HIGH: 153).	Gene Expression: ↑ PDK4 ↑ UCP3	
Wojtaszewski et al. (2003)	8 trained men ($\dot{V}O_{2peak}$ = 65 ± 1 ml·kg·min^{-1})	Acute exercise	60 mins cycling at 70% $\dot{V}O_{2peak}$ 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).	↑ AMPKα2 protein activity ↑ ACC^{Ser221} phosphorylation	N/A
Chan et al. (2004)	8 active men ($\dot{V}O_{2peak}$ = 49.0 ± 3.2 ml·kg·min^{-1})	Acute exercise	60 mins cycling (70% $\dot{V}O_{2peak}$) 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).	↑ p38 MAPK phosphorylation ↑IL-6 gene expression	N/A
Steinberg et al. (2006)	7 active men ($\dot{V}O_{2peak}$ = 49.0 ± 3.7 ml·kg·min^{-1})	Acute exercise	60 mins cycling at 70% $\dot{V}O_{2max}$ with either reduced (LOW) or normal (NORM) muscle glycogen. Pre exercise glycogen: (LOW: 150, HIGH: 390). Post exercise glycogen: (LOW: 17, HIGH: 111).	↑ AMPKα2 protein activity ↑ nuclear translocation of AMPKα2 Protein Phosphorylation: ↑ AMPKα2 ↑ ACC^{Ser221} ↑ GLUT4 gene transcription	N/A
Bartlett et al. (2013)	8 active men ($\dot{V}O_{2peak}$ = 55 ± 6 ml·kg·min^{-1})	Acute exercise	HIT running (6 x 3 mins at 90% $\dot{V}O_{2max}$). 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).	Protein Phosphorylation: ↑ ACC^{Ser79} ↑ p53^{Ser15} Gene Expression: ↑ PGC-1α ↑ PDK4 ↑ Tfam ↑ COXIV	N/A
Psilander et al. (2013)	10 trained male cyclists ($\dot{V}O_{2max}$ 65 ± 1 ml·kg·min^{-1}, PPO = 387 ± 8 W)	Acute exercise	6 x 10 mins cycling (64% $\dot{V}O_{2max}$) with either HIGH or LOW glycogen (achieved by exercise/diet manipulation 14 hrs previously). Pre exercise glycogen: (LOW: 166, HIGH: 478). Post exercise glycogen: (LOW: 130, HIGH: 477).	Gene Expression: ↑ PGC-1α ↑PDK4 ↑ COXI	N/A
Lane et al. (2015)	7 trained male cyclists ($\dot{V}O_{2max}$ = 67 ± 4 ml·kg·min^{-1}, PPO = 422 ± 39 W)	Acute exercise	Evening bout of high intensity cycling (8 x 5 mins at 82.5% PPO) followed by 120 mins 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).	Protein Phosphorylation: ↑ACC^{Ser79} Gene Expression ↑ CD36 ↑FABP3 ↑PDK4	N/A
Marquet et al. (2016a)	21 male triathletes ($\dot{V}O_{2max}$ = 60.2 ± 5.7 ml·kg·min^{-1}, PPO = 387 ± 29 W)	3 weeks 6 x week	HIT (8 x 5 mins cycling at 85% MAP or 6 x 5 mins running at individual 10 km intensity) in the evening followed by LIT (60 mins 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	↑ 10 km running performance ↑ TTE cycling (150% peak aerobic power)

Marquet et al. (2016b)	11 trained male cyclists ($\dot{V}O_{2max}$ = 64.2 ± 6 ml·kg·min^{-1}, PPO = 342 ± 38 W)	1 week 6 x week	HIT (8 x 5 mins 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	↑ 20 km cycling TT performance
Riis et al. (2019)	13 trained males ($\dot{V}O_{2max}$ = 63.9 ± 4.7 ml·kg·min^{-1}, PPO = 376 ± 36 W)	4 weeks 6 x week	HIT (10 x 5 mins cycling at 90% HRmax) in the evening followed by LIT (75 mins 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.	Protein Expression: ↔ ATGL ↔ HSL ↔ CGI-58 ↔ G0S2	↔ 30 min cycling TT (↑ both groups)
Salokannel, Hakulinen and Ahtiainen (2021)	Study A: 5 trained males, 1 trained female ($\dot{V}O_{2max}$ = 48.1 ± 4.3 ml·kg·min^{-1}) Study B: 5 trained males, 12 trained females ($\dot{V}O_{2max}$ = 47 ± 1.4 ml·kg·min^{-1})	Acute Exercise 4 weeks 2 x week	Participants performed HIT (4 x 4mins at 95% $\dot{V}O_{2peak}$) followed by LIT Exercise the following morning (60 mins treadmill running at 60% $\dot{V}O_{2peak}$). LOW group restricted CHO intake overnight whilst HIGH group consumed CHO following evening and prior to morning exercise. Acute glycogen status not measured, no information on dietary intake.	N/A	Study A: ↑ Fat oxidation Study B: ↔ $\dot{V}O_{2peak}$ (↑ both groups) ↔ anaerobic threshold velocity (↑ both groups) ↑ $\dot{V}O_{2peak}$ Velocity ↑TTE during anaerobic test (125% $\dot{V}O_{2peak}$) ↔ substrate oxidation
(Margolis et al., 2021b)	12 non-obese, recreationally active males ($\dot{V}O_{2max}$ = 44 ± ml·kg·min^{-1})	Acute Exercise	48hrs prior to exercise, participants completed initial glycogen depletion (GD) followed by 24hr iso-caloric diet to elicit adequate (AD) or LOW CHO availability. Participants completed 80 mins cycling at ~65% $\dot{V}O_{2max}$ and consumed ~146g CHO throughout. BASELINE : LOW; 467 ± 95, AD; 472 ± 109 GD: LOW; 207 ± 99, AD; 210 ± 145; PRE: LOW; 217 ± 103; AD; 396 ± 70	Serum Metabolomics: ↑ FA Metabolites in LOW ↑ Valine ↑ Leucine ↑ Isoleucine	N/A
Recover low model					
Pilegaard et al. (2000)	9 active men ($\dot{V}O_{2max}$ = 57.5 ± 2.7 ml·kg·min^{-1})	Acute exercise	75 mins cycling (75% $\dot{V}O_{2max}$) followed by 24 hrs 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: ↑ PDK4 ↑ UCP3 ↑ LPL ↑ CPT1	N/A
Jensen et al. (2015)	15 male triathletes ($\dot{V}O_{2max}$ = 66 ± 2 ml·kg·min^{-1})	Acute exercise	4 hrs cycling (56% $\dot{V}O_{2max}$) followed by 4 hrs recovery feeding with either HIGH (1 g·kg·h^{-1}) or LOW (water only) CHO. Post exercise glycogen: (LOW: 234, HIGH: 245). 4 hrs glycogen: (LOW: 264, HIGH: 444).	Gene Expression: ↔ PGC-1α ↔ Tfam ↔ NRF-1 ↔ COXIV ↔ PDK4 ↔ LPL ↔ PPAR ↔ UCP3 ↔ GLUT4	N/A

High fat feeding					
Hammond et al. (2016)	10 active men ($\dot{V}O_{2max}$ = 60 ± 3.6 ml·kg·min^{-1})	Acute exercise	High intensity running (8 x 5 mins @ 85% $\dot{V}O_{2peak}$) followed by steady state running (60 mins at 70% $\dot{V}O_{2peak}$) 3.5 hrs 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^{-1}) post steady state running.	Protein Activity: ↓ p70S6K (with high fat feeding) Gene Expression: ↔ PGC-1α ↔p53 ↔ CS ↔ Tfam ↔ PPAR ↔ ERRα	N/A
Periodised model					
Impey et al. (2016)	11 amateur male cyclists ($\dot{V}O_{2max}$ = 60 ± 3.6 ml·kg·min^{-1})	Acute exercise	Based on the principle of fuel for the work required. 4 x 30 s HIT cycling (150% PPO) & 45 mins 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 hrs previously). HIGH consumed CHO before, during & after exercise whereas LOW consumed leucine enriched protein.	Gene Expression: ↑ p53 ↑ SIRT1 ↑Tfam Protein Activity: ↑ AMPK (immediately exercise) ↓ p70S6K (3hr post exercise)	↓ Exercise capacity (1 min efforts at 80% PPO) (158 vs. 100 mins)
Burke et al. (2017)	22 international male race walkers ($\dot{V}O_{2max}$ = 53.6 ± 7.0 ml·kg·min^{-1}, PPO = 285 ± 20 W)	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	↔$\dot{V}O_{2peak}$ (↑ all groups) ↔10 km race times (↑ in high CHO and periodised CHO groups ↔ LCHF ↑ increased O_2 cost of race walking in LCHF
Gejl et al. (2017)	26 elite male endurance athletes ($\dot{V}O_{2max}$ = 65 ± 1.4 ml·kg·min^{-1}, PPO = 395 ± 11 W)	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^{-1} following LOW carbohydrate availability training session.	Protein Activity: ↔ CS (↑ both groups) ↔ β-HAD	↔ $\dot{V}O_{2max}$ (↑ both groups) ↔30 min TT performance (↑ both groups)
Burke et al. (2020)	26 international race walkers (19 males, 7 females) ($\dot{V}O_{2max}$ = 59.1 ± 4.3 ml·kg·min^{-1})	3 weeks	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	↑ $\dot{V}O_{2peak}$ in LCHF group, ↔ $\dot{V}O_{2peak}$ in PCHO or HCHO. Greater improvement in 20km race walk in HCHO than PCHO. ↓ 20km race walk LCHF.

β-HAD, 3-hydroxyacyl-CoA dehydrogenase; ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ATGL, Adipose triglyceride lipase; BCAA, Branched chain amino acid; CD-36, Cluster of differentiation 36; CGI-58, Comparative gene identification-58; CHO, Carbohydrate; COXIV, Cytochrome c oxidase subunit 4 isoform 1; CPT-1, Carnitine palmitoyltransferase I; CS, Citrate Synthase ERRα, Estrogen-related receptor alpha; FABP, Fatty acid binding protein; FFA, free fatty acid; G0S2, G0.G1 switch gene 2; GLUT4, Glucose transporter type 4; HCHO, High Carbohydrate; HIT, High intensity training; HKII, Hexokinase 2; HSL, hormone sensitive lipase; IL-6, Interleukin-6; LCHF, Low carbohydrate, high fat; LPL, Lipoprotein Lipase; P53, Tumour suppressor protein; P70S6K, Ribosomal protein S6 kinase beta-1; PCHO, Periodised carbohydrate; PDK4, pyruvate dehydrogenase kinase 4; PGC-1α, Peroxisome proliferator-activated receptor-gamma coactivator – 1 alpha; P_{max}, maximal power; PPAR, peroxisome proliferator-activated receptor; PPO, Peak power output; RER, Respiratory exchange ratio; SDH, succinate dehydrogenase; SIRT1, Sirtuin-1; TTE, Time to exhaustion; Tfam, Mitochondrial transcription factor; TT, time-trial; UCP3, uncoupling protein 3 $\dot{V}O_{2max}$, Maximal oxygen uptake, YoYoIR2, Yo-Yo Intermittent Recovery Test Level 2

2.4 Impact of Elevated Ambient Temperature on Exercise Capacity

Intra-muscular, core and environmental temperatures are increasingly researched in the context of endurance training adaptation. Nevertheless, the impact of heat stress on exercise performance is well characterised, with performance significantly impaired in hot ambient temperatures. Heat acclimation and acclimatisation strategies consistently induce performance improvements in hot conditions; albeit, the translation to improved exercise capacity in temperate environments remains equivocal. Regarding metabolism, the acute impact of heat stress on substrate utilisation and metabolic adaption following acclimation requires further investigation, with little known about the time course of metabolic adaptations caused by environmental heat stress. Throughout the second half of this chapter, the impact of heat stress on performance, metabolism, and strategies to reduce the negative effect of heat stress on performance will be discussed before parallels between intramuscular adaptive responses are considered following endurance training adaptation, manipulation of carbohydrate availability and heat stress.

2.5 Acute Responses to Additional Heat Stress During Endurance Exercise

Exercise undertaken in high ambient temperature reduces the capacity to perform prolonged, endurance-type exercise. For Example, in their seminal study, Galloway and Maughan (1997) provided evidence reporting that as ambient temperature increased from 11 to 31 °C, time to exhaustion in cyclists working at ~70% $\dot{V}O_{2max}$ was significantly decreased (93.5 to 51.6mins, respectively) (Figure 2.5). The mechanisms of accelerated fatigue under conditions of elevated heat stress during exercise are complex, with multiple integrative

mechanisms proposed for being responsible for compromised performance capacity relative to ambient conditions.

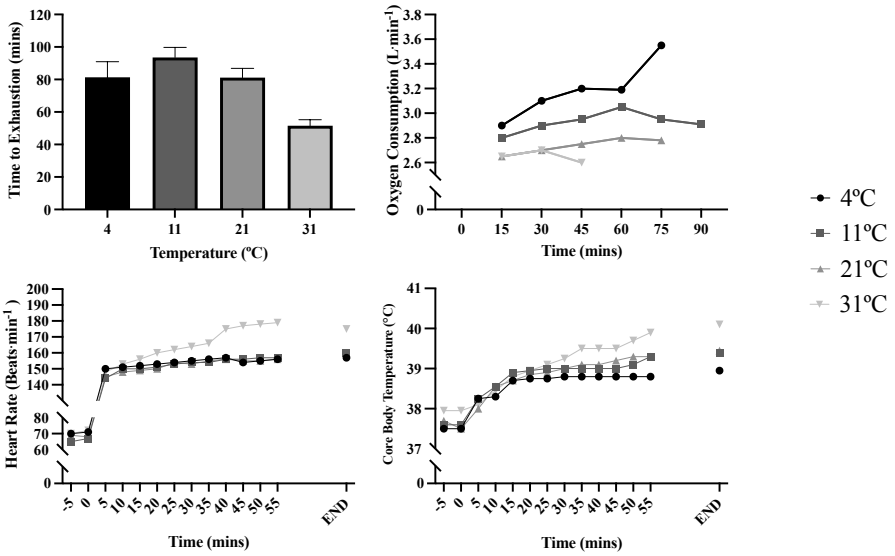

Figure 2.5 – Time to exhaustion and physiological measures across different environmental temperatures at 70% $\dot{V}O_{2max}$ in 8 healthy males. Adapted from Galloway and Maughan (1997).

It is well documented that exercise in a warm environment poses a significant thermal challenge to humans and ultimately results in reduced exercise performance (Febbraio et al., 1996b; Galloway and Maughan, 1997; Gonzalez-Alonso et al., 1999; Parkin et al., 1999). Multiple mechanisms have been proposed for impaired exercise performance in hot conditions, including reduced maximal aerobic capacity, increased physiological demand and elevated cardiovascular strain. Increased cardiovascular demand results from competing demands for blood flow to the working skeletal muscle for oxygen supply and skin blood flow requirements for heat dissipation (Sawka et al., 2011). As blood flow to the active muscles is not limited by heat stress (Gonzalez-Alonso, Crandall and Johnson, 2008), the skin becomes under-perfused as cardiac output is not sufficient to meet the competing blood flow demands due to a reduction in stroke volume (Gonzalez-Alonso, Mora-Rodriguez and Coyle, 2000). The resultant under-perfusion of skin capillaries is heightened due to exercise-induced vasoconstrictor activity, increasing cutaneous vasoconstriction in warm and cool conditions to maintain central blood

volume (Johnson and Kellogg, 2010). As exercise progresses and core temperature increases, active cutaneous vasodilation occurs, facilitating skin blood flow. When skin temperatures are already high, cutaneous vasodilation is blunted and results in a plateau long before the maximum skin blood flow that could be achieved at rest under the same thermal drive (Gonzalez-Alonso et al., 1999). As vasodilator function is compromised, skeletal muscle heat production increases and the rate of heat loss from the body is reduced due to high ambient temperatures and humidity; the onset of hyperthermia (increased core temperature) is inevitable at any exercise intensity. Whilst some increase in core body temperature during exercise is expected, the increase is proportional to absolute power output (Nielsen, 1938). Reducing the temperature gradient between the body and the environment by increasing environmental temperature will increase the rate of heat gain at a given intensity (Galloway and Maughan, 1997). Whilst hyperthermia can impair exercise performance via reduced power output (Nybo, Rasmussen and Sawka, 2014), heat stress has been shown to improve neurological function during exercise as a result of altered brain activity (Nielsen et al., 1990), reduced voluntary activation of muscle during sustained contractions (Nybo and Nielsen, 2001a) and increased perceived exertion (Nybo and Nielsen, 2001b). The evidence of a central nervous system component in fatigue during high thermal-load exercise is supported by literature utilising centrally acting pharmacological agents that improve exercise performance in hot but not in temperate conditions (Watson et al., 2005; Roelands et al., 2008). The role of the central nervous system and the central integration of core and skin temperature undoubtedly impact exercise capacity at a given workload in the heat (Nybo, 2010).

Despite the mechanisms of the increased rate of fatigue in hot conditions being unclear, it is evident that fatigue is induced through heat-related mechanisms and not due to reduced substrate availability (Parkin et al., 1999), nor the metabolic mechanisms of fatigue that are

attributed to exercise in temperate conditions (Maughan, Shirreffs and Watson, 2007). Although unlikely to be a limiting factor, rates of carbohydrate oxidation are significantly increased in high ambient conditions during exercise (Febbraio et al., 1994b; 1996b; Hargreaves et al., 1996a; Starkie et al., 1999). This increase in carbohydrate utilisation is attributed to increased glycogenolysis (+25%) alongside a reduction in exogenous carbohydrate utilisation (Jentjens, Wagenmakers and Jeukendrup, 2002). Whilst muscle glycogenolytic rate is increased during exercise in the heat due to reduced time to exhaustion, higher concentrations of muscle glycogen are observed at exhaustion compared to temperate conditions (Nielsen et al., 1990); Parkin et al., 1999), supporting the notion that glycogen depletion is not a limiting factor during exhaustive exercise in the heat (Maughan, Shirreffs and Watson, 2007).

The combination of the elevated requirement of oxygen provided to the working skeletal muscle, blood flow to internal organs and high thermoregulatory demand of skin blood flow produces a severe cardiovascular demand during exercise in the heat (Sawka et al., 2011). These factors are exacerbated during dehydration and result in a greater reduction in exercise capacity in the heat (Casa et al., 2010). Multiple mechanisms are responsible for reduced exercise capacity in the heat, spanning cardiovascular, metabolic, and central components.

2.6 Metabolic Alterations Associated With Elevated Ambient Temperature

Much of the traditional exercise metabolism literature has been conducted in temperate ambient conditions. However, exogenous heat stress significantly impacts skeletal muscle energy metabolism (Febbraio, 2001). Exposure to heat stress during prolonged endurance

exercise augments whole-body and intramuscular endogenous carbohydrate utilisation with concomitant decreases in fat oxidation rates, with literature summarised in Table 2.3.

2.6.1 Carbohydrate Utilisation

As early as 1975, Fink, Costill and Van Handel (1975) investigated the effect of elevated environmental temperature (41°C vs 9°C) on intracellular substrate utilisation during intermittent exercise. Oxygen consumption, heart rate and rectal temperature were greater in the hot condition, with elevated blood lactate. Additionally, biopsy analysis revealed greater muscle glycogen depletion in the heat, with a concomitant decrease in muscle triglyceride utilisation despite comparable absolute workloads.

Since this initial study, researchers have consistently shown increased intramuscular carbohydrate utilisation during 40 mins of exercise at 70% $\dot{V}O_{2max}$ in hot conditions compared to cooler environments (Febbraio et al., 1994a; 1994b; Hargreaves et al., 1996a). Interestingly, when strategies are implemented to reduce muscle temperature, including heat acclimation (King et al., 1985; Kirwan et al., 1987; Febbraio et al., 1994a), preventing dehydration (Hargreaves et al., 1996b; Gonzalez-Alonso, Calbet and Nielsen, 1999), reducing ambient temperature (Febbraio et al., 1996c; Parkin et al., 1999) or via external cooling (Kozlowski et al., 1985), glycogenolytic rate and carbohydrate utilisation are decreased. Whilst the trend toward greater intramuscular carbohydrate utilisation is clear, it is noteworthy that not all studies observed increased muscle glycogen utilisation during exercise in the heat (Young et al., 1985; Nielsen et al., 1990; Yaspelkis et al., 1993; Maxwell, Gardner and Nimmo, 1999). This may be explained by methodological differences and the circumstances under which data was collected. For example, a critical factor for metabolic alterations during exercise in the

heat may be the temperature increase between conditions; Yaspelkis et al. (1993) used acclimatised athletes with only a 10°C difference between conditions resulting in a modest 0.4°C difference in core body temperature between groups. As exercise generally results in local hypothermia (Febbraio et al., 1996c), a 0.4°C in core temperature may be insufficient to induce a substantial physiological response. Pre-exercise muscle glycogen concentration is also critically important, as increased availability results in increased muscle glycogenolysis during exercise (Hespel and Richter, 1992; Chesley, Hultman and Spriet, 1995; Hargreaves, McConell and Proietto, 1995). Unsurprisingly, muscle glycogen utilisation was greater in studies/conditions where pre-exercise muscle glycogen concentration was greatest (Young et al., 1985; Nielsen et al., 1990). In their cool condition (exact temperature not reported), Young et al. (1985) reported greater muscle glycogen concentration, resulting in greater muscle glycogen utilisation. Despite this, the hot condition had a 25% greater rate of muscle glycogen breakdown (not significant) compared to temperate conditions [21°C]) accompanied by consistently elevated lactate production, suggesting a greater contribution of anaerobic glycolysis to energy production during exercise in the heat (49°C). Exercise intensity is a similarly crucial variable when considering glycogenolysis during exercise. Maxwell, Gardner and Nimmo (1999) reported no difference between muscle glycogen breakdown between hot and cool environments during supra-maximal exercise. The authors suggested that increased energy turnover rendered the heat stress unimportant due to the exercise intensity, as muscle lactate concentration was comparable in each condition.

Increased muscle glycogen utilisation during endurance exercise in the heat involves oxidative and non-oxidative energy pathways. Muscle lactate accumulation is augmented in humans during exercise in the heat (Young et al., 1985; Febbraio et al., 1994a; 1994b; Hargreaves et al., 1996b; Gonzalez-Alonso et al., 1999; Parkin et al., 1999), indicative of

greater flux through anaerobic glycolysis. Nielsen et al. (1990) reported no difference in arteriovenous or muscle lactate between exercise in the cool and the heat. However, the non-counterbalanced nature of the exercise protocol makes it difficult to draw conclusions based on this evidence. This, taken with data from Rowell et al. (1968) showing increased arterial lactate in heat-stressed individuals, suggests that exercise in hot conditions increases lactate production at the muscular level. The precise impact of this phenomenon on the rate of glycogenolysis during exercise in the heat requires further investigation.

Researchers have consistently observed that respiratory exchange ratio (RER) is increased during exercise in the heat (Febbraio et al., 1994a; 1994b; Hargreaves et al., 1996a), suggesting a greater utilisation of carbohydrates, specifically muscle glycogen, at the expense of lipid oxidation (Hargreaves et al., 1996a; Gonzalez-Alonso et al., 1999). Furthermore, when infusing glucose using either a stable isotope tracer technique or arteriovenous difference, evidence suggests a greater contribution of muscle glycogen during exercise to meet the energy demands than temperate conditions (Hargreaves et al., 1996a; Gonzalez-Alonso, Calbet and Nielsen, 1999). Mechanistically, an up-regulated pyruvate dehydrogenase (PDH) pathway may be responsible as pyruvate oxidation is increased because of increased glycolytic flux; it remains to be investigated whether PDH activity is increased during exercise in the heat. The collective evidence suggests that sub-maximal exercise in the heat must induce a marked increase in core body temperature (> 0.5°C) to augment intramuscular carbohydrate utilisation. If this critical temperature value is not met, it is unlikely that any measurable metabolic differences between exercise and heat stress will be observed.

2.6.2 Heat Stress Blunts Lipid Metabolism

Typically, plasma FFA concentrations are unchanged during exercise in hot environments relative to temperate exercise (Fink, Costill and Van Handel, 1975; Nielsen et al., 1990; Yaspelkis et al., 1993). FFA availability within the blood only represents the net balance between whole-body lipolysis and uptake by other tissues. However, Fink, Costill and Van Handel (1975) did provide evidence of reduced intramuscular triglyceride (IMTG) utilisation during exercise performed at 41°C. This remains the sole study to report IMTG utilisation, likely due to high variability in current analytical techniques (Wendling et al., 1996b).

Only two studies have investigated the effect of heat stress during exercise on lipid uptake. Nielsen et al. (1990) reported no difference in FFA uptake between exercise in hot compared to temperate conditions. However, the lack of counterbalancing in this study is likely to play a role in the metabolic response to exercise and environmental conditions. Meanwhile, it has been shown that FFA uptake is reduced during the latter stages of exercise with dehydration-induced hyperthermia (Gonzalez-Alonso et al., 1999). Reduced FFA uptake into the limb, coupled with an elevated RER during exercise in the heat, is suggestive of lower total lipid oxidation in the working muscle, evidence consistent with the initial findings by Fink, Costill and Van Handel (1975).

Table 2.3 – Overview of the methodological details and study outcomes of exercise studies investigating the effect of heat stress on prolonged exercise substrate metabolism.

Reference	Participants	Protocol	Metabolic Effect (compared to temperate condition)	Thermoregulatory Effects (compared to temperate)
Dolny and Lemon (1988)	8 Healthy Males, ($\dot{V}O_{2max}$, 55 ± 8 $mL \cdot kg^{-1} \cdot min^{-1}$), 4 hr post absorptive	90-mins cycle at 65% $\dot{V}O_{2max}$ in 5, 20, 30°C	↑ RER ↑ CHO oxidation (~6.4%) ↑ Blood Lactate ↑ serum adrenaline: noradrenaline ↔ NEFA	↑ T_{rec} (~0.25°C)
Febbraio et al. (1996a)	7 endurance trained males, ($\dot{V}O_{2max}$, 65 ± 13 $mL \cdot kg^{-1} \cdot min^{-1}$), overnight fast	40 mins cycle at 70% $\dot{V}O_{2max}$ in 20 & 40°C	↑ RER ↓ $\dot{V}O_2$ ↑ CHO oxidation (~16.3%) ↑ net muscle glycogenolysis ↑ Blood and Muscle Lactate ↑ Blood Glucose ↑ Plasma adrenaline	↑ T_{rec} (~1°C) ↑ T_{mus} (~1.3°C)
Febbraio et al. (1996b)	12 endurance trained males, ($\dot{V}O_{2max}$, 65 ± 7 $mL \cdot kg^{-1} \cdot min^{-1}$), overnight fast	40 mins cycle at 70% $\dot{V}O_{2max}$ in 20 & 40°C	↑ RER ↔ $\dot{V}O_2$ ↑ net muscle glycogenolysis ↑ net muscle CrP degradation ↑ net muscle Cr accumulation ↑ Blood and Muscle Lactate ↑ Blood Glucose ↔ Muscular ATP, ADP, AMP, IMP ↑ Muscular AMP pre to post ↑ Muscular NH_3	↑ T_{rec} (~1°C) ↑ T_{mus} (~1.7°C)
Fernandez-Elias et al. (2015)	7 endurance trained males, ($\dot{V}O_{2max}$, 55 ± 3 $mL \cdot kg^{-1} \cdot min^{-1}$), controlled pre-trial diet	Dehydrating exercise in heat, 4-hrs rehydration, 40-mins cycle at 75% $\dot{V}O_{2max}$ in 25, 36°C	↑ CHO oxidation ↑ net muscle glycogenolysis ↑ Blood Lactate	↑ T_{rec} (~0.7°C)
Galloway and Maughan (1997)	8 active males, ($\dot{V}O_{2max}$, ~56 ± 7 $mL \cdot kg^{-1} \cdot min^{-1}$), overnight fast	Cycle TTE at 75% $\dot{V}O_{2max}$ in 4, 11, 21, 31°C	↔ CHO Oxidation ↔ Blood Lactate ↔ Blood Glucose ↔ Glycerol ↔ NEFA	↑ T_{rec} (~0.3-0.5°C)

Hargreaves et al. (1996a)	6 endurance trained males, ($\dot{V}O_{2max}$, ~64 mL·kg^{-1}·min^{-1}), overnight fast	40 mins cycle at 65% $\dot{V}O_{2max}$ in 20 & 40°C	↑ RER ↑ CHO Oxidation (~19.8%) ↔ $\dot{V}O_2$ ↑ muscle glycogenolysis oxidation (~16.8%) ↑ Hepatic Glucose Production ↑ Plasma Glucose & Lactate ↑ Plasma Adrenaline: noradrenaline ↑ Plasma Cortisol ↑ Plasma Glucagon ↑ Plasma GH	↑ T_{rec} (~0.9°C)
Hettinga et al. (2007)	6 well-trained males, ($\dot{V}O_{2max}$, ~66 mL·kg^{-1}·min^{-1}), 2 hr fast	20 mins cycle at 60% MAP in 15.5 & 35.5°C	↔ RER ↑ $\dot{V}O_2$ ↓ Gross Efficiency ↑ Blood Lactate	↑ T_{rec} (~0.3°C)
Jentjens et al. (2006)	9 endurance trained males, ($\dot{V}O_{2max}$, 65 ± 3 mL·kg^{-1}·min^{-1}), overnight fast	90 mins cycle at 55% Wmax in 16 & 35°C with ~1.5g·min^{-1} CHO ingestion	(Data from between 60-90mins of exercise) ↔ RER ↔ $\dot{V}O_2$ ↔ Total CHO Oxidation ↑ Exogenous CHO Oxidation ↔ Fat Oxidation ↑ Muscle Glycogenolysis ↑ Plasma Lactate ↔ Plasma Glucose ↔ Insulin ↔ NEFA	↑ T_{rec} (~0.8°C)
Marino et al. (2001)	9 endurance trained males, ($\dot{V}O_{2max}$, 66 ± 4 mL·kg^{-1}·min^{-1}), nutritional status unknown	30 mins run at 70% PTRS in 15 & 35°C	↑ RER ↔ $\dot{V}O_2$ ↑ CHO Oxidation ↔ Plasma Lactate	↑ T_{rec} (~0.6°C)
Maunder et al. (2020)	Part A: 9 Endurance Trained Males ($\dot{V}O_{2max}$, 57 ± 5 mL·kg^{-1}·min^{-1}), overnight fast Part B: 11 Endurance Trained Males ($\dot{V}O_{2max}$, 57 ± 5 mL·kg^{-1}·min^{-1}), 4 hr fast	Part A: 60 mins cycling at absolute power output at VT_1 in 18 & 35°C. Part B: 20 mins cycling at absolute power output at VT_1 in 18, 28, 34 & 40°C	Part A: ↑ CHO Oxidation at High intensity only ↓ Fat Oxidation at High intensity only ↑ Plasma Lactate ↔ Plasma Glucose Part B: ↑ Plasma Adrenaline ↑ CHO Oxidation at High and moderate intensity ↓ Fat Oxidation at High intensity	Part A: ↑ T_{rec} (data not reported) ↑ T_{mus} (data not reported) Part B: ↑ T_{rec} (~ 0.2 and 0.3°C at moderate and high intensity respectively) ↑ T_{mus}
Nielsen et al. (1990)	7 healthy males, ($\dot{V}O_{2max}$, ~54 mL·kg^{-1}·min^{-1}), overnight fast	30 mins incline walk in 18°C followed immediately by 60 min incline walk in 40°C	↑ $\dot{V}O_2$ ↔ Arterial & Venous Lactate ↔ Plasma Glucose ↔ Plasma FFA ↔ Muscle Glycogenolysis	↑ T_{es} (~1.1 °C) ↑ T_{sk} (~4.8 °C)

Parkin et al. (1999)	8 endurance trained males, ($\dot{V}O_{2max}$, 55 ± 8 mL·kg^{-1}·min^{-1}), overnight fast	Cycle TTE at 70% $\dot{V}O_{2max}$ in 3, 20, 40°C	↔ RER ↔ $\dot{V}O_2$ ↑ Net Muscle Glycogenolysis ↑ Plasma Adrenaline ↔ Noradrenaline	↑ T_{rec} (~0.5°C)
Baldwin, Snow and Febbraio (2000)	7 untrained males, ($\dot{V}O_{2max}$, 51 ± 4 mL·kg^{-1}·min^{-1}), overnight fast	40 mins cycle at 70% $\dot{V}O_{2max}$ in 20 & 40°C	↔ RER ↔ $\dot{V}O_2$ ↑ Muscle and Plasma NH_3	↑ T_{rec} (~0.6°C)
Yaspelkis et al. (1993)	9 endurances trained, heat acclimatised males, ($\dot{V}O_{2max}$, 69 ± 1 mL·kg^{-1}·min^{-1}), overnight fast	60 mins cycle at ~74% $\dot{V}O_{2max}$ in 24 & 34°C	↔ RER ↔ $\dot{V}O_2$ ↔ CHO Oxidation ↔ Net muscle glycogenolysis ↑ Plasma Glucose &Lactate ↔ Glycerol ↔ NEFA	↑ T_{rec} (~0.4°C)
Young (1990)	13 untrained males, ($\dot{V}O_{2max}$, 45 ± 5 mL·kg^{-1}·min^{-1}), 4hr post liquid meal	30 mins cycle at 70% $\dot{V}O_{2max}$ in 21 & 49°C	↑ RER ↓ $\dot{V}O_2$ ↑ CHO Oxidation (~3.8%) ↔ Net muscle glycogenolysis ↑ Plasma and Muscle Lactate	↑ T_{rec} (~0.7°C)

Oesophageal Temperature (T_{es}), Muscle Temperature (T_{mus}), Rectal Temperature (T_{rec}), Skin Temperature (T_{sk})

2.6.3 Availability and Requirements for Glucose During Exercise and Heat Stress

Hyperglycaemia, or elevated blood glucose, is consistently observed during exercise in heat (Fink, Costill and Van Handel, 1975; Yaspelkis et al., 1993; Febbraio et al., 1994a; Febbraio et al., 1996b; Hargreaves et al., 1996a) suggesting an imbalance between glucose production and utilisation. Utilising a dye infusion technique, Rowell et al. (1968) reported augmented hepatic glucose production (HGP) in heat-stressed humans, a finding later confirmed via isotopic tracer dilution (Hargreaves et al., 1996a) and arteriovenous balance (Gonzalez-Alonso, Calbet and Nielsen, 1999) techniques. The regulation of hepatic fuel metabolism has been reviewed by Kjaer (1998) and involves a complex network of neural and hormonal feedback loops. Typically, during sub-maximal exercise in ambient conditions, euglycemia is maintained via hepatic glucose production (HGP) and is regulated by the metabolic glucose requirements. When blood glucose is increased via exogenous ingestion (McConell et al., 1994; Jeukendrup et al., 1999) or infusion (Howlett et al., 1998), HGP is blunted. Conversely, a study by Angus et al. (2001) reported that when glucose was ingested and appeared in the blood, HGP was unaffected during exercise in the heat, highlighting the strength of the feed-forward mechanism imposed by thermal stress.

2.7 Mechanisms of Altered Substrate Metabolism During Exercise in the Heat

Multiple mechanisms have been proposed to explain the shift towards greater carbohydrate utilisation during exercise in heat (Rowell et al., 1968; Kozlowski et al., 1985; Sawka et al., 1985; Young et al., 1985). Potential mechanisms include reduced oxygen provision to muscles due to competing blood flow demands with the skin during exercise in

the heat (Rowell et al., 1968), altered recruitment patterns favouring greater use of fast-twitch muscle fibres (Sawka et al., 1985; Young et al., 1985), direct temperature effects on enzyme-mediated reactions (Q10 effect) (Kozlowski et al., 1985; Young et al., 1985) and increased circulating adrenaline levels (King et al., 1985; Yaspelkis et al., 1993).

2.7.1 Effect of Catecholamines

Adrenaline increases during exercise (Galbo, 1986) which is increased further when exercise is completed in hot environmental conditions (Galbo et al., 1979; Powers, Howley and Cox, 1985; Nielsen et al., 1990; Febbraio et al., 1994a; 1996c; Hargreaves et al., 1996a; 1996b; Gonzalez-Alonso, Calbet and Nielsen, 1999; Parkin et al., 1999). As glycogen phosphorylase is sensitive to the β-adrenergic receptor stimulation (Richter et al., 1982), increased circulating adrenaline levels likely increases intramuscular glycogen utilisation. Whilst plasma adrenaline concentrations match changes in muscle glycogen utilisation during heat stress (Febbraio et al., 1994a; 1996c; Hargreaves et al., 1996b; Gonzalez-Alonso et al., 1997), these finds are not unanimous (Nielsen et al., 1990). Furthermore, studies investigating the relationship between circulating adrenaline and muscle glycogen utilisation have shown a positive effect when supra-physiological doses are infused (Jansson, Hjemdahl and Kaijser, 1986; Spriet, Ren and Hultman, 1988). Contrastingly, studies using adrenaline infusions have shown no increased muscle glycogen utilisation during exercise (Chesley, Hultman and Spriet, 1995; Wendling et al., 1996a). Justifications for the diminished effect of adrenaline on glycogen phosphorylase during exercise include Ca^{2+} release, substrate availability, and changes in AMP and free inosine monophosphate (IMP) (Ren and Hultman, 1990; Chesley, Hultman and Spriet, 1995; Wendling et al., 1996a). The measurements of energy metabolites by Chesley, Hultman and Spriet (1995), combined with high exercise intensity (85% $\dot{V}O_{2max}$), would likely have

activated glycogen phosphorylase irrespective of adrenaline levels via local increases in Ca^2 from the sarcoplasmic reticulum and changes in free AMP, ADP and inorganic phosphate. When the intra-muscular milieu remains relatively undisturbed during exercise, muscle glycogen utilisation is still augmented during exercise in the heat (Febbraio et al., 1994b), suggesting a regulatory role of adrenaline on carbohydrate metabolism during prolonged exercise. Trained men infused with adrenaline during exercise at 70% $\dot{V}O_{2max}$ increased muscle glycogen utilisation and lactate accumulation (Febbraio et al., 1998) despite no change in the energy status of the muscle (Febbraio et al., 1994b). The observation of increased RER during exercise in hot conditions is ubiquitous compared to exercise in thermoneutral (Young et al., 1985; Dolny and Lemon, 1988; Febbraio et al., 1994a; 1994b; Hargreaves et al., 1996a); similar metabolic responses are seen following exercise with adrenaline infusion (Wendling et al., 1996a; Febbraio et al., 1998). A proposed mechanism for the increase in carbohydrate metabolism is the activation of PDH (Febbraio, 2001), which depends on the balance of PDH kinase inhibition via increased pyruvate and ADP and PDH phosphatase activation via increased Ca^{2+} (Putman et al., 1993). The measurement of pyruvate production during exercise in the heat has not yet been investigated, nor following adrenaline infusion, so it remains to be confirmed whether pyruvate is increased and subsequently metabolised.

Increased circulating adrenaline may also have a regulatory role on HGP. Whilst evidence to support this hypothesis in humans is limited, Kjaer et al. (1993) infused physiologically normal concentrations of adrenaline in individuals who had undergone local anaesthesia of the sympathetic coeliac ganglion (responsible for the liver, pancreas and adrenal medulla innervation) and found no effect of adrenaline on glucose production. More recently, however, Howlett et al. (1999) have demonstrated increased circulating glucose and HGP when adrenaline is infused in physiological concentrations in endurance-trained (Howlett, Febbraio

and Hargreaves, 1999) and bi-laterally adrenalectomized humans (Howlett et al., 1999). This evidence shows that adrenaline may have a regulatory role in controlling hyperglycaemia and HGP during exercise and heat stress.

Whilst carbohydrate metabolism is significantly altered during exercise and heat stress, there appears to be minimal impact on plasma fatty acid levels (Fink, Costill and Van Handel, 1975; Nielsen et al., 1990; Yaspelkis et al., 1993; Gonzalez-Alonso, Calbet and Nielsen, 1999) despite overall reductions in FFA uptake in working leg skeletal muscle (Gonzalez-Alonso, Calbet and Nielsen, 1999). This suggests that FFA release from adipocytes is reduced during exercise and heat stress, a counterintuitive physiological response given the potent lipolytic nature of adrenaline (Martin, 1996). A potential mechanism for this phenomenon may be reduced blood flow to adipose tissue during exercise and heat stress, reducing the available albumin and promoting fatty acid re-esterification within adipocytes. However, the similar plasma glycerol levels reported by Yaspelkis et al. (1993) during exercise and heat stress indicate that it is unlikely that fatty acid esterification accounts for the similar FFA levels and is more likely due to a reduction in FA lipolysis during exercise in the heat. Although not experimentally confirmed, the direct inhibitory effect of heat on hormone-sensitive lipase and other enzymes responsible for fatty acid liberation and metabolism should be considered.

2.7.2 Effect of Increased Muscle Temperature

Exercise increases intramuscular temperature (T_{mus}) in a workload-dependent manner (Saltin and Hermansen, 1966; Saltin, Gagge and Stolwijk, 1968), which is augmented during exercise and heat stress (Febbraio et al., 1994a; 1994b; Hargreaves et al., 1996a). It is hypothesised that elevated T_{mus} may directly alter enzyme activity, ultimately affecting

substrate metabolism (Kozlowski et al., 1985; Young, 1990). The temperature coefficient (Q_{10}) is the factor by which the rate of an enzymatic reaction changes in response to increased/decreased temperature. The typical Q_{10} value for a biological process is between 2 and 3 (Reyes, Pendergast and Yamazaki, 2008), meaning that for every 10°C increase in temperature, there is a 2 to 3-fold increase in enzyme reaction rate. Physiologically a 10°C increase in T_{mus} is impossible; however, a modest 2°C increase in T_{mus} could result in a 30 to 40% increase in enzyme activity.

Direct investigations of increased T_{mus} on intramuscular metabolism are scarce; however, Edwards et al. (1972) demonstrated that glycogen utilisation and lactate accumulation were augmented during exhaustive isometric exercise following lower-limb hot water immersion (44°C). Despite only local muscle heating taking place, it is noteworthy that core temperature was increased relative to temperate conditions. As core temperature increases, increased plasma adrenaline and glycogenolysis may have been caused by hormonal influence instead of heat alone.

Utilising heat pads to increase T_{mus} without elevating the core temperature, Febbraio et al. (1996a), reported increased glycogenolysis and lactate accumulation in the absence of elevated core temperature or plasma adrenaline concentrations during 2 mins of supra-maximal exercise (115% $\dot{V}O_{2max}$). The authors did observe reductions in the total adenine nucleotide (TAN) pool and IMP accumulation which could be attributed to changes in carbohydrate metabolism. Reductions in TAN can allosterically mediate enzyme activity, specifically via the activation of phosphofructokinase (PFK) (Uyeda, 1979) and phosphorylase (Ren and Hultman, 1990), key enzymes in glycogenolysis and glycolysis, increasing glycolytic flux. A more recent study by the same research group aimed to heat one leg whilst cooling the other

before and during exercise at 70% $\dot{V}O_{2max}$ using water-perfused cuffs (Starkie et al., 1999). The initial difference in T_{mus} in the heated vs cooled leg was reduced during exercise however remained significantly elevated at the termination of exercise. Whilst there was an increase in muscle glycogenolytic rate in the heated limb, no differences in high-energy phosphagen metabolism were noted between legs, providing evidence that temperature *per se* has a role in regulating carbohydrate metabolism during exercise and heat stress.

2.7.3 Central Nervous System and Neuromuscular Recruitment Change

The hypothesis that exercise in the heat results in differential utilisation of muscle fibres (greater fast-twitch fibre recruitment) (Sawka et al., 1985; Young et al., 1985) was first suggested following evidence of different muscle lactate accumulation between muscle fibres following exercise in the heat (Young et al., 1985). Exercise at 49°C resulted in greater lactate accumulation in type II muscle than exercise in a cool environment (21°C). However, further studies investigating the correlation between muscle glycogen utilisation and fibre type-specific utilisation following exercise in the heat failed to highlight a relationship and showed no difference in lactate accumulation (Febbraio et al., 1994a). This suggests that neuromuscular patterns observed in the heat are consistent with previous observations during prolonged exercise in temperate conditions (Gollnick et al., 1973).

2.7.4 Changes in Skeletal Muscle Blood Flow

During exercise under heat stress, to facilitate skin blood flow for thermoregulation, a vascular shunt prioritises blood flow to working muscles, skin, and essential organs. Splanchnic (Rowell et al., 1968), hepatic (Rowell et al., 1965), renal (Radigan and Robinson, 1949) and inactive

muscle blood flow reduces during exercise and heat stress. Due to competing demands for blood supply, cardiovascular demand exceeds the maximal cardiac output capacity leading to potentially altered substrate metabolism and ultimately impaired performance through reduced O_2 supply to the working muscle (Fink, Costill and Van Handel, 1975; Kozlowski et al., 1985; Nielsen et al., 1990; Young, 1990). The issue of altered blood supply to the working muscle during exercise and heat stress is contentious (Bell et al., 1983; Nielsen et al., 1990), with little understanding of how changes in skeletal muscle blood flow alters local O_2 extraction. Using quantitative measures of contracting muscle blood flow during exercise in an animal model (Sheep), Bell et al. (1983) demonstrated reduced blood flow during exercise in the heat using radioactive microspheres. In human models, direct measures of active limb blood flow via thermodilution plethysmography heat (Savard et al., 1988; Nielsen et al., 1990; 1993; 1997) and doppler flowmetry techniques were used (Smolander and Louhevaara, 1992) revealed unaltered blood flow during exercise in hot conditions. The discrepancies between findings may be explained by species differences as heat dissipation (sweating vs panting) and hydrostatic pressures (2 *vs* 4-legged exercise) differ between animal models, but it may also be attributed to the insufficient degree of thermal regulation to compromise cardiac output capacity. When hyperthermia was coupled with dehydration, muscle blood flow was attenuated during exercise (Gonzalez-Alonso, Calbet and Nielsen, 1999). Importantly, the adverse effects of dehydration on cardiovascular function must be considered here as function is severely impaired when compared to hyperthermia alone or exercise in temperate conditions (Gonzalez-Alonso et al., 1997). A combination of hyperthermia and dehydration during exercise led to a ~1.0 $L \cdot min^{-1}$ decrease in contracting limb blood flow compared with similar, euhydrated exercise, with leg O_2 consumption unaffected (Gonzalez-Alonso, Calbet and Nielsen, 1999). Despite altered O_2 consumption, metabolic analysis highlighted an increase in muscle glycogen utilisation and lactate accumulation with exercise and heat stress (Gonzalez-Alonso, Calbet

and Nielsen, 1999). The data generated by this research group suggests that even if muscle blood flow is reduced during exercise and heat stress, arteriovenous O_2 difference is adjusted accordingly to ensure that O_2 supply is not compromised. Whilst this evidence shows that O_2 supply is unlikely to play a significant role in altered muscle metabolism during exercise in the heat, it does not rule out the influence of decreased blow flow on these metabolic processes. The functional vascular shunt in skeletal muscle has already been shown to alter substrate metabolism due to an altered supply rate of nutrients and removal of metabolic by-products (Clark et al., 1995). However, the importance of nutrient and non-nutrient supply and removal during heat stress has not been investigated directly.

2.8 Chronic Responses to Additional Heat Stress During Endurance Exercise

Repeated heat exposure during sub-maximal exercise, or heat acclimation (discussed in greater detail in section 2.9), is a common strategy for mitigating the physiological and negative performance implications of elevated ambient temperatures. A recent meta-analysis identified 52 studies examining the effect of heat acclimation or acclimatisation on the psycho-physiological responses to heat exposure (n = 26) and subsequent exercise performance (n = 26) (Tyler et al., 2016). Heat acclimation had an ergogenic effect on performance, irrespective of the strategy employed (i.e., constant work rate or clamped hyperthermia), with extended programmes having greatest efficacy. Regarding the physiological impact of heat acclimation, core temperature is decreased at rest and during exercise, coupled with reduced heart rate, increased sweat rate and skin blood flow with improvements in subjective ratings of perceived exertion and thermal sensation.

Cycling time trial (TT) performance is consistently improved following a period of heat acclimation in both laboratory (Lorenzo et al., 2010; Keiser et al., 2015; Rendell et al., 2017) and field-based scenarios (Racinais et al., 2015c). For example, Racinais et al. (2015c) utilised a 43 km (~25 miles) cycling TT in hot ambient conditions (~37 °C) in the field as a 'real-world' performance test to investigate the impact of a 2-week heat acclimatisation protocol on cycling TT performance. At the onset of exercise, initial power outputs were comparable between hot and cool ambient conditions. Still, power output rapidly decreased in the hot condition throughout the test compared to a slight decrease in the cool conditions. The 43km time trial was repeated weekly, with 1-week heat acclimation restoring ~50% of performance and 2 weeks nearly completely restoring exercise capacity compared to temperate conditions.

2.9 Heat Acclimation to Mitigate the Detrimental Effects of Heat Stress

There are multiple strategies to acutely mitigate the detrimental effects of heat stress; the most common strategy to prepare for exercise in high ambient conditions is heat acclimatisation/acclimation, usually completed in the weeks before. Natural heat acclimatisation in the competition environment is considered the optimal strategy to prepare for competition in hot environmental conditions (Racinais et al., 2015b). However, logistical limitations mean heat acclimation strategies are often more feasible around athlete training schedules and demands. Heat acclimation is the artificial replication of the competition environment (heat stress), intended to induce physiological strain leading to beneficial adaptation which ultimately benefits performance. There is no consensus on the optimal strategy for heat acclimation; however, all strategies induce numerous integrated physiological adaptations that improve performance and reduce heat illness risk (Periard, Racinais and Sawka, 2015).

Heat acclimation research was pioneered within the South African mining industry, where it was observed that experienced miners had a higher sweat rate than newer workers within hot mines. This observation informed policy change that ultimately led to new recruits completing a heat acclimation protocol before entry into the workforce within the mines (Pogue, 2006). Using a scientific approach, Dresoti (1935) demonstrated that heart rate was reduced within 14 days of heat acclimation, sweat rate increased, and work performed during a rock shovelling exercise improved. Since this early occupational evidence, researchers have aimed to characterise and optimise heat acclimation protocols for the betterment of performance, whether commercially or athletically.

2.9.1 Physiological Adaptations to Heat Acclimation

2.9.1 i) Core and Skin Temperature

Repeated exposure to elevated environmental temperatures has been shown to reduce resting (Sunderland, Morris and Nevill, 2008; Racinais et al., 2015c), mean (Garrett et al., 2012; Neal et al., 2016), and matched-time point (King et al., 1985; Garrett et al., 2009) core temperature (T_{core}) irrespective of the measurement site. Most improvement in T_{core} occurs within the first 7 days of heat exposure (Robinson, 1943; Horvath and Shelley, 1946), with longer heat acclimation protocols showing a greater magnitude of change (Tyler et al., 2016). Similarly, when considering the impact of heat acclimation on skin temperature (T_{skin}), reductions are observed during exercise, with most adaptation occurring within 7 days of exposure (Robinson, 1943; Horvath and Shelley, 1946), with reductions in temperatures associated with improved heat loss mechanisms and potentially changes in skin blood flow. Interestingly, despite changes in resting T_{core} following heat acclimation, there is no difference in resting T_{Skin} at rest (Cheung and McLellan, 1998; Patterson, Stocks and Taylor, 2004a; Kuennen et al., 2011).

2.9.1 ii) Sweat Rate

Improved sweat rate occurs within 3-4 days of initial exposure (Eichna et al., 1950; Ladell, 1951) and permits greater evaporative heat loss in conditions of low vapour gradients (Eichna et al., 1950). Earlier sweat onset accompanied with increased sweat volume allows for greater heat loss following acclimation (Nadel et al., 1974). Whilst sweat rate and total volume increases, sodium concentration decreases following acclimation (Allan and Wilson, 1971); the mechanisms behind this response are reasonably well understood and include both neurological and endocrine components (Sawka et al., 2011). Decreases in sodium concentration within the sweat can occur after as little as 2 days and decrease linearly during the first week of acclimation (Buono et al., 2018). Heat loss is maximised via evaporation through increased cutaneous vasodilation, increasing skin blood flow for a given body temperature (Ladell, 1951).

2.9.1 iii) Blood Volume

Bazett (1940) characterised the haematological adaptations associated with heat acclimation and reported increases in both plasma volume and total circulating haemoglobin, with plasma volume expansion occurring more rapidly, leading to a temporary decrease in measured haemoglobin concentration. Although highly variable between individuals (Nielsen et al., 1993; Patterson, Stocks and Taylor, 2004b; 2014), plasma volume expansion is a consistently reported adaptation in response to heat acclimation and occurs within 3-4 days of initial heat exposure (Glaser, 1949; Senay, Mitchell and Wyndham, 1976; Sawka and Coyle, 1999). It is noteworthy that despite various changes in blood volume, erythrocyte concentration remains broadly consistent among individuals (Sawka et al., 2000). It was later reported that

this increase was only transient and peaked at approximately day 5 (Wyndham et al., 1968; Senay, Mitchell and Wyndham, 1976). However, it remains to be conclusively investigated as to whether plasma volume expansion can be maintained through core temperature-clamped acclimation protocols (Patterson, Stocks and Taylor, 2004b; 2014). It is hypothesised that plasma volume could be maintained through the oncotic effect of increased vascular protein content (Senay, Mitchell and Wyndham, 1976; Senay, 1979; Harrison et al., 1981) via increased protein synthesis (Horowitz and Adler, 1983; Yang et al., 1998) and a concomitant decrease in protein loss as a result of reduced blood flow through cutaneous capillaries (Harrison, 1985), reducing their permeability for large molecules in response to heat acclimation (Senay, 1970; 1972). In addition, increased sodium preservation can facilitate plasma volume maintenance (Patterson, Stocks and Taylor, 2004b; 2014).

Whilst plasma volume expansion is used as a marker of heat acclimation, and the changes in haematocrit during a heat response test correlate to changes in performance (Racinais et al., 2012; 2014), the contribution of this response to improved performance is likely limited. Increased plasma volume does not improve thermoregulatory control (Sawka and Coyle, 1999; Watt et al., 2000).

2.9.1 iv) Cardiovascular Adaptations

During exercise and heat stress, core, skin and muscle temperature increase, increasing cardiovascular strain through competing demands for oxygen supply at the working muscles and cutaneous blood supply to aid heat loss (Gonzalez-Alonso, Crandall and Johnson, 2008). This increase in cardiovascular strain results in increased heart rate and a decline in stroke volume with a potential subsequent reduction in cardiac output (Periard et al., 2011).

The increases in plasma volume and sweat rate improve heat dissipation via evaporation from the skin, leading to a greater internal temperature gradient from internal to external temperature (Eichna et al., 1950; Coyle and Gonzalez-Alonso, 2001). Through increased heat transfer via blood flow, a greater volume of blood is "spared" for circulation (Eichna et al., 1950) and reduces cardiovascular strain during exercise in the heat (Coyle and Gonzalez-Alonso, 2001). Blood pressure and cardiac output are better sustained because of increased plasma volume and reduced heart rate (Sawka and Coyle, 1999), a key indicator of heat acclimation. The mechanisms by which heart rate is reduced are partly attributed to increased plasma volume supporting greater venous return, cardiac pre-loading and improving ventricular filling (Senay, 1986; Nielsen et al., 1993). Changes in heart rate can also be a result of sympathetic activity and changes in core temperature. For this reason, it has been suggested that maintaining heart rate throughout the heat acclimation protocol may be optimal to support adaptation to heat stress (controlled heart-rate protocol) (Periard, Racinais and Sawka, 2015).

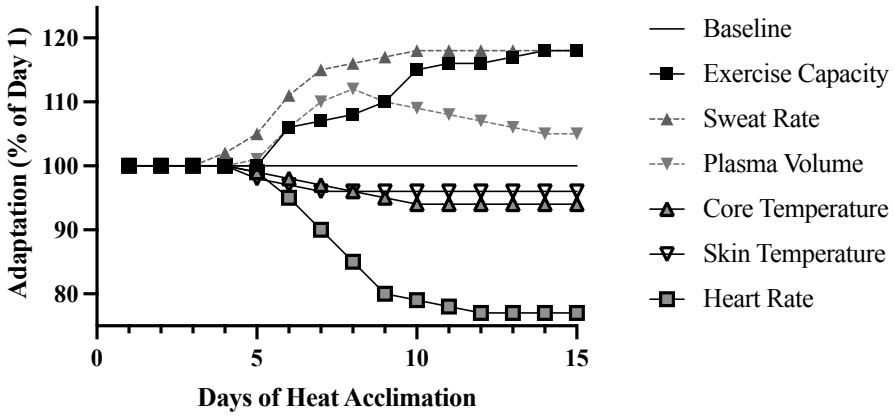

Figure 2.6 – Time course of induction in human adaptations to heat stress, including physiological and performance changes from baseline [based on Periard, Racinais and Sawka (2015)]

2.9.1 v) Thermal Tolerance

At the cellular level, adaptation to heat stress must occur to allow the cells to survive a high level of strain, known as thermal tolerance (Moseley, 1997; Horowitz, 1998) and is dependent on the Heat shock protein (HSP) response (Kuennen et al., 2011), of which HSP72 is highly responsive to heat stress and exercise (Locke, 1997). The function of HSPs is to bind to denatured and nascent cellular polypeptides to protect and facilitate repair from various stressors from heat stress, energy depletion, and acidosis, amongst others (Kregel, 2002). The HSP response is highly complex, as evidenced by several human studies whereby heat acclimation increased HSP72 & 90 from basal levels with, individuals the with greatest physiological adaptation showing reduced post-exercise expression (McClung et al., 2008). Conversely, extracellular HSP72 expression decreased with an increase in post-exercise response following as little as 2 days of heat acclimation (Marshall, Ferguson and Nimmo, 2006) but not after 10 days (Yamada et al., 2007; Magalhaes Fde et al., 2010). Data also suggest a basal increase in HSP following 15 days of heat acclimation (Sandstrom et al., 2008). In addition to the HSP response, heat stress has also been shown to regulate gene expression (Sonna, Sawka and Lilly, 2007), leading to potential global adaptations that allow a "heat acclimated phenotype" that is more tolerant to heat and other stressors (Horowitz, 2010). The role of HSPs has yet to be empirically investigated in relation to heat acclimation-induced gene transcription and phenotype induction.

The adaptations associated with heat stress span further than cellular thermal tolerance, and it is possible to induce heat acclimation without a significant HSP response (Hom et al., 2012). However, HSP responses likely occur during heat acclimation, and the generation of thermal tolerance occurs in parallel.

2.9.1 vi) Muscle Adaptations

Improved thermal tolerance at the cellular level could also indicate a skeletal muscle-specific response following repeated heat stress bouts. Heat acclimation has been suggested to alter whole-body and local skeletal muscle metabolism (Young et al., 1985; Febbraio et al., 1994a). Heat acclimation has also been shown to decrease oxygen uptake (Sawka et al., 1983), glycogen utilisation (40-50%) (King et al., 1985; Kirwan et al., 1987), muscle and blood lactate accumulation during sub-maximal exercise (Febbraio et al., 1994a) in the heat. A reason for the reduced lactate accumulation may be due to improved removal via increased total body water, increasing splanchnic circulation (Rowell et al., 1968) and delayed lactate accumulation due to improved cardiac output and a decrease in metabolic rate (Sawka et al., 1983; Young et al., 1985). Heat acclimation may induce greater aerobic metabolism via increased muscle capillary growth (Kuhlenhoelter et al., 2016) and mitochondrial adaptations (Tamura et al., 2014). This concept will be discussed in greater detail later in this chapter (2.12). As well as aerobic adaptation, heat acclimation has been shown to improve hypertrophic muscle response in cultured cells (Yamashita-Goto et al., 2002; Goto et al., 2003) and animal tissue (Uehara et al., 2004), translating to increased muscle cross-sectional area in humans (Goto et al., 2011).

2.9.1 vii) Neural Adaptations

Limitations to prolonged exercise performance in the heat are predominantly due to cardiovascular implications rather than neural (Periard, Caillaud and Thompson, 2011; Racinais and Girard, 2012). Despite this, acute hyperthermia decreases neural drive to skeletal muscle (Nybo and Nielsen, 2001a) due to peripheral failures in neural drive transmission and supra-spinal alterations when contractions are prolonged (Racinais, Gaoua and Grantham,

2008). Cell models have shown that heat acclimation may reverse deteriorations in peripheral neural drive transmission (Karunanithi et al., 2002; Kelty et al., 2002).; albeit, this has not been reported in humans (Racinais et al., 2017). This evidence suggests that the decrease in neural drive in humans is potentially a side effect of the increase in axonal conduction velocity, which shortens the depolarisation time (Bolton, Sawa and Carter, 1981; Rutkove, Kothari and Shefner, 1997). Heat acclimation can partly restore the ability to sustain neural drive during prolonged contractions in the heat (Racinais et al., 2017). Still, the lack of change in the spinal or peripheral nervous system suggests a supra-spinal adaptive response to heat acclimation (Racinais et al., 2017).

2.9.1 viii) Perceptual and Cognitive Adaptations

Heat acclimation can protect various cognitive tasks, including psychomotor performance (Walker, Dawson and Ackland, 2001), attention (Radakovic et al., 2007) and planning tasks (Racinais et al., 2017) during acute hyperthermia. However, acute hyperthermia increases thermal discomfort (Racinais et al., 2017), potentially negatively impacting exercise capacity (Stevens et al., 2018). Heat acclimation may not affect thermal sensation/comfort at rest (Tyler et al., 2016; Racinais et al., 2017) but can improve thermal sensation/comfort during training and competition in both teams- and endurance sports (Sunderland, Morris and Nevill, 2008; Kelly et al., 2016; Tyler et al., 2016) however the extent to which this improves performance remains unknown.

2.10 Acclimation Improves Exercise Capacity

2.10.1 Hot Environments

Since the early work by Dresoti (1935), it has been well documented that frequent heat exposure via acclimation or acclimatisation can improve work capacity in hot conditions, with a recent meta-analysis showing a ~15% mean improvement in performance following a heat acclimation protocol (Tyler et al., 2016). Interestingly, the reported percentage improvement in performance was correlated with the duration of heat acclimation protocols, with long-term heat acclimation (LTHA) protocols inducing more significant performance benefit (22 ± 29%) compared to medium-term heat acclimation (MTHA) and short-term heat acclimation (STHA) protocols with +22 ± 28% and +7 ± 8% respectively.

The observed decrease in $\dot{V}O_{2max}$ during hyperthermia is likely one of the main limiting factors during prolonged exercise in the heat (Periard and Racinais, 2015). Heat acclimation can improve $\dot{V}O_{2max}$ in hot and temperate conditions, although it cannot fully restore the initial decrease in $\dot{V}O_{2max}$ imposed by heat stress (Sawka et al., 1985; Lorenzo et al., 2010) and is proportionately associated with improvements in exercise capacity in the heat (Lorenzo et al., 2010) Nevertheless, in some cases, in some cases, this may be sufficient to restore exercise capacity to that observed in temperate conditions completely (Keiser et al., 2015; Racinais et al., 2015c). For example, during a 43km cycling TT at 37°C, power output was reduced by ~16% compared to a 43km time trial completed at 8°C, with half of this performance decrement being restored after 1 week of heat acclimatisation and almost wholly restored following 2 weeks (Racinais et al., 2015c). Whilst it is accepted that heat acclimation consistently improves sub-maximal exercise capacity in the heat, the magnitude of improvement depends on several factors, including the severity of environmental conditions (temperature, wind, and humidity) and type of performance (duration and intensity).

2.10.2 Temperate Environments

It is generally accepted that heat acclimation improves $\dot{V}O_{2max}$ and exercise capacity in the heat; however, the translation to performance benefit in temperate conditions is conflicting. For example, Minson and Cotter (2016) outlined the potential ergogenic effect of exercise training in hot environmental conditions and how the associated thermoregulatory adaptations (specifically plasma volume expansion) induced by high ambient temperatures may improve performance in temperate conditions. Conversely, Nybo and Lundby (2016), whilst acknowledging the ergogenic benefit of heat acclimation in hot conditions, refuted the translational benefits of heat acclimation, suggesting that exercise training offered no additional benefit to performance compared to exercise training in temperate conditions.

Following a period of heat acclimation, no beneficial effect has been reported for temperate performance following 5 (Neal et al., 2016), 10 (Keiser et al., 2015) and 14 days (Karlsen et al., 2015) of heat acclimation. Meanwhile, improvements in $\dot{V}O_{2max}$ have been reported across population groups in unfit (23%), untrained (13%) (Shvartz et al., 1977), and trained populations (3-5%) during exercise in temperate conditions (Sawka et al., 1985; Lorenzo et al., 2010) following a period of heat acclimation. However, the transferability of performance adaptation between environmental conditions remains a contentious topic that requires further investigation.

2.11 Heat Acclimation Time Course and Decay

2.11.1 Kinetics

Motivated by desire to optimise heat acclimation protocols within the mining industry, understanding the kinetics of heat acclimation was highly topical following the early occupational heat acclimation studies of the 20th century (Wyndham and Jacobs, 1957). Characterising the time course of changes in heart rate, skin and rectal temperature during daily walking in a hot room, Robinson (1943) reported a rapid decrease in each within the first 7 days of acclimation, followed by a slowed, gradual decline until 23 days. Ladell (1951) suggested heat acclimation occurs in two phases, the initial phase lasting 2-3 days, characterised by reduced heart rate and decreased temperature sweating threshold, followed by increased sweat rate and fatigue resistance over a more-extended period. Heat acclimation is considered a relatively rapid process, beginning upon initial exposure, with 75-80% of total adaptations occurring within the first 4-7 days (Shapiro, Moran and Epstein, 1998; Periard, Racinais and Sawka, 2015).

Furthermore, the kinetics of heat acclimation adaptations are proportionate to the frequency of heat exposure; for example, the previously reported data is based upon daily heat exposures, whereas reducing the frequency of heat exposure to once every 3 days requires 3 times as long to reach comparable levels of acclimation (Fein, Haymes and Buskirk, 1975). Importantly, when heat exposures are interspersed by 1 week, no heat acclimation induction is observed (Barnett and Maughan, 1993). Therefore, it must be accounted for in preparation for any competition that rest or intermittent heat exposure will slow the rate of heat acclimation compared to a daily heat acclimation protocol (Gill and Sleivert, 2001; Chalmers et al., 2014).

More recently, heat acclimation research has focused on implementing short-term protocols to more closely reflect the logistical constraints observed during international competition (Garrett et al., 2009; 2012; Chalmers et al., 2014). Despite positive adaptive responses occurring after as little as 7 days (Sunderland, Morris and Nevill, 2008; Garrett, Rehrer and Patterson, 2011; Chalmers et al., 2014), athletes may still require between 7-10 days for further cardiovascular and sudomotor adaptations (Nielsen et al., 1993; Lorenzo et al., 2010; Karlsen et al., 2015) with a further two or more weeks for optimal aerobic performance in the heat (Racinais et al., 2015c)

2.11.2 Induction

Classically, heat acclimation was said to be induced by 100 mins of daily exercise in the heat (Lind and Bass, 1963). However, the methods of inducing heat acclimation have diversified since; spanning a range of modalities, including passive, active and a mix of active and passive hyperthermia (Daanen, Racinais and Periard, 2018). Across the multiple strategies, the general rule-of-thumb to induce adaptations requires elevated core temperature, profuse sweating and elevated skin blood flow (Racinais et al., 2015a) and can be induced through passive heat exposure, self-paced exercise, constant work rate exercise, controlled hyperthermia or controlled heart rate approaches.

Studies implementing passive exposure have been used to isolate the effect of repeated heat exposure without exercise (Brazaitis and Skurvydas, 2010; Racinais et al., 2017; Racinais, Wilson and Periard, 2017). However, repeated passive heat exposures only allow partial development of a heat-acclimated phenotype (Takamata et al., 2001; Beaudin et al., 2009) and are likely inefficient at inducing a heat acclimation response appropriate for exercise in the hot

environments, with strategies more closely reflecting competition advisable. Passive heat acclimation extends to maintaining elevated core body temperature post-exercise (Scoon et al., 2007; Zurawlew et al., 2016) or elevating core temperature before active acclimation (Mee et al., 2018).

Traditionally, self-paced exercise in the heat has been used to promote heat acclimation (Armstrong et al., 1986; Barnett and Maughan, 1993) and involves working or training in natural heat. However, typically used in team sports athletes undergoing heat acclimatisation (Buchheit et al., 2011; Racinais et al., 2012; 2014), self-paced exercise has been replaced by more controlled methods in recent years. Constant work rate exercise has been the primary model for developing knowledge of heat acclimation kinetics (Robinson, 1943; Nadel et al., 1974; Nielsen et al., 1993; Nielsen et al., 1997). Here, internal, and external thermal loads are fixed (i.e., cycling at 150W at 35°C and 60% RH), providing a constant thermal load. Of note, the initial thermal load will diminish as acclimation occurs (Eichna et al., 1950; Fox et al., 1963; Rowell et al., 1967; Taylor, 2014) and is a potential justification for reduced plasma volume following its initial increase (Patterson, Stocks and Taylor, 2004b; 2014). Despite relative decreases in training stimulus and force function, a recent meta-analysis highlighted that constant work rate and clamped core temperature acclimation lead to similar adaptations (Tyler et al., 2016).

Acclimation via clamped core temperature (isothermic controlled hyperthermia at 38.5°C) has been proposed to provide constant forcing function through the acclimation process and, thus, maintains acclimation stimulus as adaptations develop (Fox et al., 1963; Regan, Macfarlane and Taylor, 1996; Patterson, Stocks and Taylor, 2004b; Garrett et al., 2009). In the context of athlete preparation for competition, fixed temperature protocols may be

unsuitable as exercise intensity is dramatically reduced or ceased when the target core temperature is achieved (Gibson et al., 2015), negatively impacting one of the central stimuli for heat acclimation, sweating. It has been suggested that completing heat acclimation at a given heart rate may be more suitable for athletes (Periard, Racinais and Sawka, 2015), as heart rate provides an overall index of cardiovascular strain imposed by thermal stress and exercise and decreases with acclimation. Therefore, training with a clamped heart rate accounts for the acute effect of heat stress via reduced power output, maintains cardiovascular strain, and as acclimation develops, power output increases at a pre-determined heart rate (Periard, Racinais and Sawka, 2015).

Responses to heat acclimation show substantial variation between individuals (Racinais et al., 2012; Corbett et al., 2018), with some athletes acclimating within 1 week, whilst others required 2 (Daanen, Racinais and Periard, 2018). Although heat acclimation has been shown to improve performance across many populations, sport-specific strategies are likely necessary to optimise performance truly.

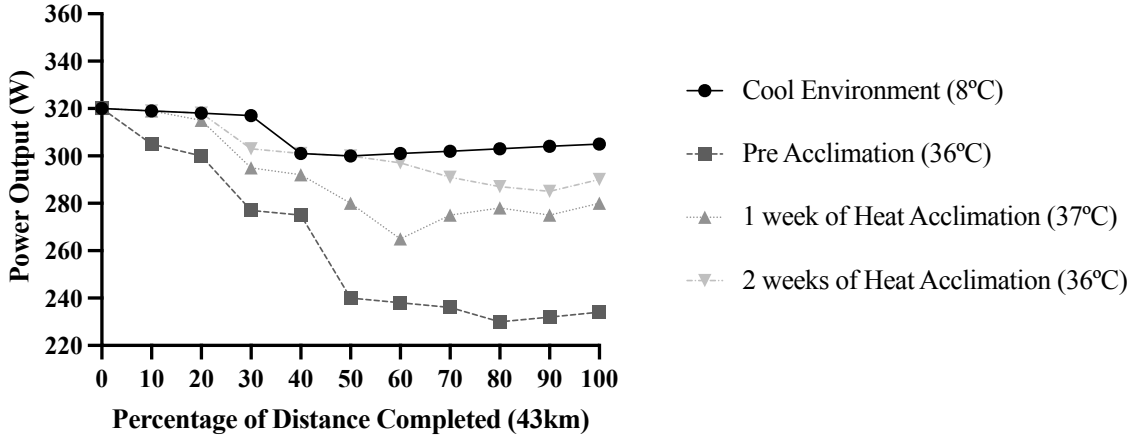

Figure 2.7 – Power output during a 43 km cycling time trial in cool and hot ambient conditions. [Based on data from Racinais et al. (2015c)]

2.11.3 Decay

A recent meta-analysis and systematic review reported that whilst athletes could maintain heat acclimation adaptations for several weeks, these adaptations decreased by a magnitude of ~2.5% per day (Daanen, Racinais and Periard, 2018). Adaptive improvements in sweat rate decayed most rapidly; however, limited data makes drawing meaningful conclusions difficult. The decay rate could be slowed by regular heat exposure post-acclimation. Importantly, re-acclimation during this period appears to be quicker than the initial acclimation rate (Daanen, Racinais and Periard, 2018). Based on this information, 4-5 days of re-acclimation could be sufficient to regain complete heat acclimation if done within one month of the initial acclimation (Ashley, Ferron and Bernard, 2015).

2.12 Heat Stress as a Stimulus for Endurance Training Adaptation

Several recent reviews have provided an overview of the benefits of additional heat stress on therapeutics, performance and adaptation (Hyldahl and Peake, 2020; Kim et al., 2020a). Positive outcomes of heat therapy/stress include improved angiogenesis, muscle mass regulation, mitochondrial biogenesis, glucose metabolism and insulin signalling. Furthermore, multiple experimental models (*i.e.*, cell, animal, and human) across various heating strategies have been used to investigate the potential benefit of heat stress on endurance training adaptation. Of note, the positive effects of heat therapy on mitochondrial quality and quantity and the parallels in signalling response between endurance exercise, muscle glycogen availability and heat stress warrant further investigation to identify potential strategies for promoting mitochondrial biogenesis for the benefit of exercise training adaptation.

2.12.1 Cellular Evidence

Cell models provide highly controlled experimental models permissive to determining signalling responses, protein changes and phenotypic adaptations in response to heat stress. C2C12 myotubes heated (40°C) for 1 hr had an immediate heat-protective response with elevated HSP72 mRNA expression, which remained for 24 hrs compared to cells maintained at 37°C (Liu and Brooks, 2012). AMPK phosphorylation also increased post-heat exposure and preceded increases in SIRT1 and PGC-1α. As a result of this up-regulation of AMPK and PGC-1α, downstream transcription factors increased, including NRF1, NRF2 and Tfam, ultimately up-regulating mitochondrial component mRNA (Cycs, COXII, COXIV) and GLUT 4. Following 5 days of heat exposure, both PGC-1α and mitochondrial subunit protein abundance were significantly increased compared to non-heat acclimated cells (maintained at 37°C). This evidence provided early data suggesting beneficial mitochondrial adaptation in response to heat stress via molecular mechanisms responsible for endurance training adaptation. Supported by Patton et al. (2018), C2C12 myotubes exposed to 40°C for 2 hrs per day for 6 consecutive days increased gene and protein expression of markers associated with mitochondrial biogenesis (AMPK, SIRT1, PGC-1α) as well as transcription factors (NRF1, TFAM). When subject to a lipopolysaccharide (LPS) challenge designed to reduce Citrate synthase activity and fat oxidation, myotubes exposed to 6 days of heat exposure had no impaired peak oxidation rates and greater oxidative reliance than control myotubes suggesting that heat stress may improve aerobic metabolism in muscle cells and protect against LPS mediated energy deficit.

2.12.2 Animal Models

In vivo studies in animal models, specifically rodents, have provided complimentary data supporting heat stress as a possible stressor capable of inducing endurance-like adaptations and mitochondrial biogenesis. Rats who received pre-exercise heat treatment (anaesthetised rats were heated to 41°C 15 mins before exercise) improved time to exhaustion by ~25 mins during an exercise treadmill test at 37°C (Chen et al., 1999). Those in the heat-treated group exhibited greater tolerance to exercise in the heat and a significantly increased HSP72 mRNA response in skeletal muscle. Mitochondrial enzyme activity was increased dramatically in complex I and II/III by 43% and 28%, respectively. Rat cardiac muscle has also been shown to have elevated mitochondrial enzyme activity (complex I, IV, V) following a single bout of heat exposure (Sammut et al., 2001).

In a comprehensive study of acute and chronic exercise and heat stress on mitochondrial biogenesis, Tamura et al. (2014) showed that exercise and heat stress induced positive intra-muscular adaptations, with the greatest adaptive response observed with combined heat stress and exercise. Rats were either exercised (30 mins running), heated (30 mins at 40 ºC) or post-exercise and heated (30 mins running followed by 30 mins at 40 ºC) for 5 days per week for 3 consecutive weeks with *plantaris* (Fast-twitch) and *soleus* (slow-twitch) muscle harvested 40 hrs post completion of the programme. Heat stress and exercise alone induced comparable mitochondrial adaptation (CS and β-HAD activity), with combined exercise and heat stress causing a greater adaptive response. This adaptive response was attributed to increased p38 MAPK signalling, which was increased exercise and heat-stressed animals compared to no significant increases in other conditions. Interestingly, AMPK activation was elevated in response to exercise with, a down-regulation in response to heat stress (- 42.3%) and combined

heat stress and exercise. The authors hypothesised that increased reactive oxygen species resulted in greater p38 MAPK activity independently of AMPK signalling, providing evidence of the role of multiple signalling pathways in mitochondrial biogenesis.

2.12.3 Human Evidence and Applications

A consensus on whole-body heat stress to induce mitochondrial biogenesis is absent in humans. Heat stress alone does not induce transcription of genes associated with mitochondrial biogenesis in humans (Zak et al., 2017). Crucially, no change in gene expression may be due to the heating protocol used. Specifically, the use of hot ambient conditions only increased core by temperature by a modest 0.2°C, with no measurement of muscle temperature, therefore, it may be assumed that the heat stress stimulus was not sufficient to promote an adaptive response. A recent study comparing the short-term adaptive response to whole-body (environmental chamber at 50ºC) and local heat stress (water-perfused suit maintained at 50 ºC) has provided evidence of beneficial heat-stress induced adaptation in humans (Ihsan et al., 2020a). Participants were heated to 39ºC rectal temperature (~1 hr), which induced enhanced anabolic signalling (mTOR/Akt) and phosphorylation of FOXO1 and FOXO3a, potentially indicating a reduction or blunting of the muscular proteolytic response. Up-regulated p38 MAPK activation, elevated HSP mRNA expression and transcriptional markers associated with mitochondrial biogenesis (NRF1, NRF2, COX2 and COX4 mRNA) were observed during whole-body heat stress indicating a potentially beneficial mitochondrial biosynthetic response. Despite positive data regarding whole-body heat stress, local muscle heating was ineffective at inducing a positive adaptive response to local heat stress.

Slivka et al. (2012) subjected 9 recreationally active participants to 3 experimental trials consisting of 1 h cycling at 60% of maximal aerobic power, followed by recovery in either cold (7 ºC), room temperature (20 ºC) and hot (33 ºC) conditions. In contrast to the previous cell- and animal-based studies, they found that PGC-1α expression was attenuated in the hot condition and augmented in the cold condition, likening the response to seasonal cold acclimation in fish species (Egginton and Sidell, 1989; Battersby and Moyes, 1998). The substrate metabolism data in hot conditions were similarly contentious, with carbohydrate oxidation decreased with an increase in fat oxidation compared, a finding in opposition with previous literature summarised in Table 2.3 (Febbraio et al., 1994a; 1994b; 2003; Hargreaves et al., 1996a). Based on this study, it may not be appropriate to assume that heat stress and exercise are ineffective at inducing PGC1-α expression post-exercise. Further evidence from Slivka et al. (2021) reported that following a single bout of aerobic cycling exercise at 33ºC, untrained individuals had reduced PGC1-α expression compared to participants who completed the exercise at 20ºC (Slivka et al., 2021). Three weeks of exercise in the heat did not increase PGC1-α expression compared to exercise in temperate conditions. Interestingly, $\dot{V}O_{2peak}$ increased in the group that trained at 20ºC only, with no difference in peak power output between groups. The authors concluded that heat stress may limit the effectiveness of aerobic exercise to increase aerobic power and may blunt regular exercise-induced PGC1-α expression following three weeks of heat acclimation.

Another study investigating the effects of heat acclimation in humans on markers of mitochondrial biogenesis (Mang et al., 2020) revealed 10 days of active heat acclimation (*i.e.*, walking at 30-40% $\dot{V}O_2$max twice for 45 min at 42 ºC (interspersed with 10 min rest) increased HSP72 expression but did not enhance markers of mitochondrial biogenesis (CaMK, Tfam & PGC-1α protein expression) nor oxidative protein expression (COX I - IV). The lack of

increased mitochondrial markers may be the relatively low exercise intensity implemented in the present exercise protocol as in temperate environments, 60 min at ~30% peak power has been shown ineffective at inducing increases in PGC-1α (Di Donato et al., 2014).

The optimal strategy for inducing high muscle and core temperatures remains to be elucidated. This lack of consensus makes it difficult to provide practical advice with regard to promoting endurance training adaptation. Future studies should aim to manipulate the time, duration, and modality of heat stress to understand further the optimal approach to optimise training adaptation.

Despite limited evidence in favour of whole-body heat exposure to promote mitochondrial biogenesis, local treatments may provide a suitable alternative. Utilising pulsed shortwave diathermy to increase skeletal muscle temperature by ~4 ºC for 2 hrs, Hafen et al. (2018) reported a positive acute cellular response with HSP27 phosphorylation significantly decreased and increased ERK1/2 and AMPK phosphorylation. Six days of further treatment (2 hrs/day) increased HSP70/HSP90, PGC-1α and mitochondrial complex I and V enzyme expression. Phenotypically, this translated to greater mitochondrial respiratory capacity, isolating the improvements to simultaneous increases in phosphorylation and electron transfer systems instead of improved efficiency. In contrast to Ihsan et al. (2020a), T_{mus} was greater during this study, providing further evidence to support the notion of a critical temperature threshold for optimal adaptation. Hafen et al. (2019) performed a similar experiment where participants underwent single limb immobilisation, whilst the opposite served as a contralateral control, with only one group receiving diathermy. As with their previous work, Hafen et al. (2019) report that, following 16 days of diathermy, HSP 70 and 90 were significantly increased in the treated limb. Notably, local heat treatment prevented the immobilisation-induced loss in

mitochondrial respiratory capacity and maintained mitochondrial respiratory protein expression. As a result, muscle atrophy was mitigated following the diathermy only.

Using an alternative approach to provide local muscle heating, a water-perfused garment (water heated to ~52 ºC) was worn on one randomly selected thigh whilst the other served as a contralateral control for 8 consecutive weeks (90 min, 5 days per week) (Kim et al., 2020b). Whilst the limb exposed to heat treatment improved peak isokinetic torque, no cross-sectional muscle area or mitochondrial content changes were observed. Despite no differences in mitochondrial measures, a similar heat therapy protocol implemented by Hesketh et al. (2019) increased HSP expression and promoted pro-angiogenic responses (endothelial nitric oxide synthase). In summary, collective evidence from the Hafen lab (Hafen et al., 2018; 2019) shows the potential therapeutic benefits of repeated, isolated heat stress on skeletal muscle adaptation, explicitly focusing on mitochondrial adaptations. When administered with endurance exercise, local muscle heat stress may increase signalling responses associated with mitochondrial biogenesis.

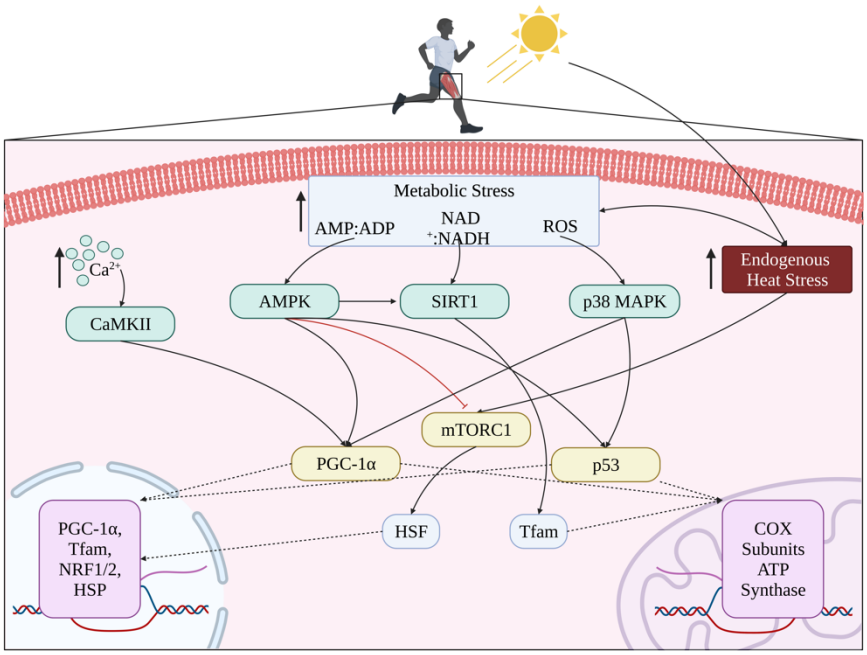

Figure 2.8 – Molecular responses to environmental and local heat stress. Ambient heat stress combined with high ATP turnover and associated heat production during exercise is likely to be a critical exercise/environmental stressor capable of inducing an adaptive response. Increased muscle temperature increases the activation of multiple upstream mediators of PGC-1α and many downstream nuclear and mitochondrial transcription factors encoding for mitochondrial (OXPHOS) and stress proteins (HSP). Created with biorender.com

2.13 Summary

The remarkable ability of skeletal muscle to respond to environmental stressors and undergo major adaptations has been extensively researched over the last 50 years. Understanding the optimal cellular environment to promote training adaptation is paramount for training efficacy and performance. Multiple strategies have been proposed to augment post-training protein activation and transcriptional responses, including training with purposefully reduced muscle glycogen availability. Despite this, metabolic adaptation and performance evidence remains equivocal and only applicable to temperate environmental laboratory conditions.. As such, the chapters within this thesis will investigate the impact of training with low carbohydrate availability on metabolic adaptation and performance to understand the efficacy of such a strategy in both hot and temperate environments. The studies presented

herein will collectively provide novel empirical data examining the effect of carbohydrate periodisation and heat stress on endurance performance and metabolism before employing novel ^{1}H-NMR metabolomics *in vitro* and *in vivo* to characterise the impact of these stressors on the intramuscular and serum metabolomes.

Chapter 3 – General Methodology

This chapter will provide a general methodology and theory of methods used within the thesis, including traditional human physiology, wet-lab, cell culture and metabolomics approaches.

3.1 Location of Testing and Ethical Approval

Home-based exercise interventions (Study 1, Chapter 4) were completed remotely by participants within their own homes, with data collected and reported remotely. The local ethics committee granted ethical approval for this experiment at Liverpool John Moores University (LJMU) (Approval No. 20/SPS/019).

Laboratory exercise tests (Study 2, Chapters 5 & 8) were completed within the Sport and Exercise performance laboratory at the French National Institute of Sport (Institut National du Sport, de l'Expertise et de la Performance [INSEP], Paris, France). Due to high environmental temperatures experienced during summer months, data collection was completed between February and June before resuming in October until December. Ethical approval for this work was granted by the Comité de Protection des Personnes Ouest IV – Nantes (CPP) (N° - 2018-A02544-51). Subsequent processing of the human serum collected during this study was completed at LJMU before metabolomic analysis at The University of Liverpool.

All cell culture procedures (Chapters 6 & 7) were undertaken at LJMU before processing and metabolomics analysis at the University of Liverpool and did not require ethical approval. Exact protocols and procedures will be covered in this chapter.

3.2 Characterisation of Participant Physiology

3.2.1 Study Participants Characteristics

All participants in the current body of work were classified as trained amateur cyclists and triathletes (Tier 2/3) based upon previous characterisation frameworks (Jeukendrup, Craig and Hawley, 2000; De Pauw et al., 2013; Decroix et al., 2016; McKay et al., 2022) (Table 3.1) and had no prior heat acclimation or acclimatisation (i.e. not have travelled to warmer climates in the preceding 3 months). Participants had no history of neurological, skeletal, or metabolic disease and were not under any pharmacological interventions during the studies. Additionally, participants were required to have a minimum of 2 years of cycling or triathlon experience. All participants were instructed to refrain from strenuous exercise and caffeine and alcohol intake in the 24-hrs before any laboratory visit or remote testing battery. All participants provided written informed consent before participating in the study after detailed explanations of procedures and study requirements.

Table 3.1 – Physical and physiological characteristics of study participants.

	Study 1	**Study 2**
	(n = 55)	(n = 23)
Age (years)	32 ± 8	30 ± 6
Height (cm)	177 ± 8	181 ± 8
Weight (kg)	76 ± 18	72.5 ± 7.2
FTP (W)	257 ± 53	238 ± 33 *
FTP ($W \cdot kg^{-1}$)	3.38 ± 0.7	3.28 ± 0.46
MAP (W)	-	332 ± 45
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	4.3 ± 0.8 *	4.7 ± 0.8
$\dot{V}O_{2peak}$ ($mL \cdot kg \cdot min^{-1}$)	56.6 ± 10.5	64.8 ± 11.0
Hours of training ($h \cdot wk^{-1}$)	12 ± 4	11 ± 5

** denotes predicted FTP and $\dot{V}O_2$ peak calculated using* Denham et al. (2020)*. Data reported as mean ± SD.*

3.2.2 Cardio-respiratory Measures

During all laboratory-based exercise tests, participants were fitted with a Polar heart rate monitor (Polar H7, Kempele, Finland) connected to a partner smartphone application for measuring heart rate (Polar flow, Kempele, Finland).

3.2.3 Collection and Analysis of Respiratory Gases During Exercise

To permit the collection of respired gases during exercise (during $\dot{V}O_{2max}$ and sub-maximal substrate utilisation tests), participants were fitted with a Hanns-Rudolph mask allowing breath-by-breath measurements to be recorded throughout exercise using a Quark Cosmed (Cosmed, Rome, Italy) online gas analysis system following calibration with known reference gases.

3.2.4 Characterisation of Substrate utilisation during exercise

Whole-body rates of carbohydrate and fat oxidation ($g \cdot min^{-1}$) were calculated from final 1-min averaged $\dot{V}O_2$ and $\dot{V}CO_2$ values of the intensity of interest (60 and 70% MAP) and nonprotein RER values according to the following equations (Jeukendrup and Wallis, 2005):

$$CHO\ oxidation = 4.120 \times \mathrm{V}CO_2 - 2.962 \times \mathrm{V}O_2$$

$$Fat\ oxidation = 1.695 \times \mathrm{V}O_2 - 1.701 \times \mathrm{V}CO_2$$

Oxidation of 1 g of carbohydrate and fat were assumed to be energetically equivalent to 17.0 KJ and 37.7 KJ respectively with volumes of respired gases expressed in litres per minute ($L \cdot min^{-1}$) throughout.

Energy Expenditure (EE) ($kcal \cdot min^{-1}$) was obtained from the rate of oxygen uptake, using the equation developed by Brouwer (1957) and based on the thermal equivalent of O_2 for nonprotein RER. EE was calculated as follows:

$$EE = \frac{Thermal\ equivalent\ of\ O_2 \times VO_2}{1000}$$

3.2.4 i) Principle of Indirect Calorimetry

Using the measurement of gas exchange in the lungs (whole-body O_2 consumption and CO_2 production), an approximation of substrate oxidation during exercise can be made given that carbohydrate, fat, and protein possess differing chemical compositions and, thus, the amount of O_2 needed and CO_2 produced differs meaning the estimation of substrate utilisation for energy production during exercise can be calculated based on the rates of O_2 consumption and CO_2 production (Table 3.2).

Table 3.2 – Energy and volumes of O_2 required and CO_2 produced in the oxidation of carbohydrate (Glucose & Glycogen), fat, and amino acids. [Taken from Jeukendrup & Wallis (2005)]

	Energy yield (kcal·g)	**O_2 Required (L·g)**	**CO_2 Required (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	104136	0.704	4.85
Amino Acid	4.09	0.9842	0.7931	0.807	4.16

RQ, Respiratory Quotient, the ratio of CO_2 produced relative to O_2 consumed at the tissue level

3.2.5 Assessment of Maximal Oxygen Uptake and Aerobic Power

Before the commencement of Study 2 (Chapter 5), maximal oxygen uptake ($\dot{V}O_{2max}$) and maximal aerobic power (MAP) were assessed during an incremental cycle test performed on an electronically braked cycle ergometer (Excalibur Sport, Lode, Groningen, Netherlands). Participants completed a 10-min warm-up at 100 W and self-selected cadence before commencing the test consisting of 2-min stages with 30 W increments until volitional exhaustion. Breath-by-breath $\dot{V}O_2$ and $\dot{V}CO_2$ data were obtained throughout the exercise using an online gas analysis system (Quark Cosmed, Rome, Italy). MAP (W) was calculated as per Hawley and Noakes (1992), where $W_{completed}$ is the last completed workload, *t* is the duration of increment completed, and PI represents the power increase between stages:

$$MAP = W_{completed} + \left(\frac{t}{120} \times \text{PI}\right)$$

3.3 Performance Tests

3.3.1 Assessment of 20-minute Peak Power Output (PPO) and Estimation of Functional Threshold Power (FTP)

In study 1, participants completed a standardised 15-minute warm-up consisting of 5-min cycling at 100 W, followed by 5-min incremental cycling, increasing by 25 W each minute until 225 W was reached. Two minutes of self-selected recovery was completed before completing three 10-s sprints separated by 50 s recovery at a self-selected intensity. Following the warm-up, participants were asked to commence the 20-min maximal effort, during which they were advised to maintain the highest possible power for the test duration (Figure 3.1). FTP (W) was calculated as 95% of the mean power achieved for 20 min (Allen, 2019). The reliability data for this exercise test is presented in Chapter 4.

Functional Threshold Power (FTP) is defined as the theoretical maximal mean power (MPO) that can be sustained for 60 minutes in a "quasi-steady state" (Allen and Coggan, 2019) and has been used to predict cycling performance in trained cyclists (Hawley and Noakes, 1992; Morgan et al., 2019; Sorensen et al., 2019). Due to the arduous nature of a 60-min effort, it is practically difficult to complete routine 60-min PPO tests as part of a longitudinal athlete monitoring programme, notwithstanding the limited evidence to support the reliability of longer-duration maximal efforts. Alternatively, a shorter 20-min effort is used whereby MPO is scaled by 95% to represent the equivalent 60-min FTP (Allen and Coggan, 2019). The concept of FTP was developed as a practical method to determine the field-based equivalent to the maximal lactate steady state (MLSS) (Allen and Coggan, 2019), an indicator widely used as a measure of the highest sustainable metabolic rate during continuous exercise (Jones et al.,

2019). MacInnis, Thomas and Phillips (2018) have previously investigated the reliability of repeated power tests of 4, 20 and 60 minutes in duration in trained cyclists showing high levels of reliability which were strongly associated with 60-min TT performance. More recent data have supported these observations in laboratory settings across a range of power durations (Lillo-Bevia et al., 2019; McGrath et al., 2019; Borszcz, Tramontin and Costa, 2020). To date, evidence suggests high level between-trial of reliability between PPO in laboratory-based studies utilising online gas analysis and blood lactate measures. Whilst these measures are important for characterising the metabolic demand of exercise, it is at the expense of ecological validity and diverges from the practical benefits afforded by the 20-min FTP test using a pre-programmed training session uploaded to an online exercise platform of the subjects choosing (Zwift or TrainerRoad). In light of this study being completed remotely, all participants completed the test at home; FTP was used to standardise relative exercise intensity for training sessions in place of $\dot{V}O_{2max}$ (Denham et al., 2020).

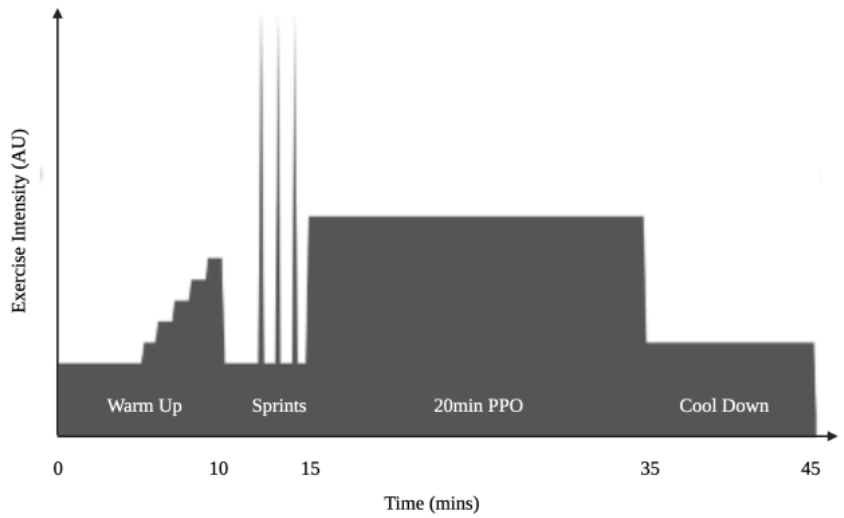

Figure 3.1 – Schematic representation of the prescribed power profile during the 20-minute PPO Test. Initially, participants completed a 15 min warmup of 5 mins at ~50% PPO, followed by a 5 min progressive ramp in intensity before 3 sprints (~80% of perceived max). Participants then completed 20 mins maximal cycling to achieve their highest mean power before an optional 10 min cool down.

3.3.2 Assessment of 5- and 1-min PPO

In study 1, participants completed the same standardised warm-up as before the FTP test before a maximal 1-min cycling effort to achieve the highest mean power possible. Participants then completed a 10-min active recovery at a self-selected intensity before a 5-min maximal effort (Figure 3.2). As with the FTP test, a familiarisation session was prescribed the week before the test to all participants. The tests were repeated 4 and 7 days after the final training session (Post-tests) to allow adequate recovery from the training load. The reliability data for this exercise test is presented in Chapter 4.

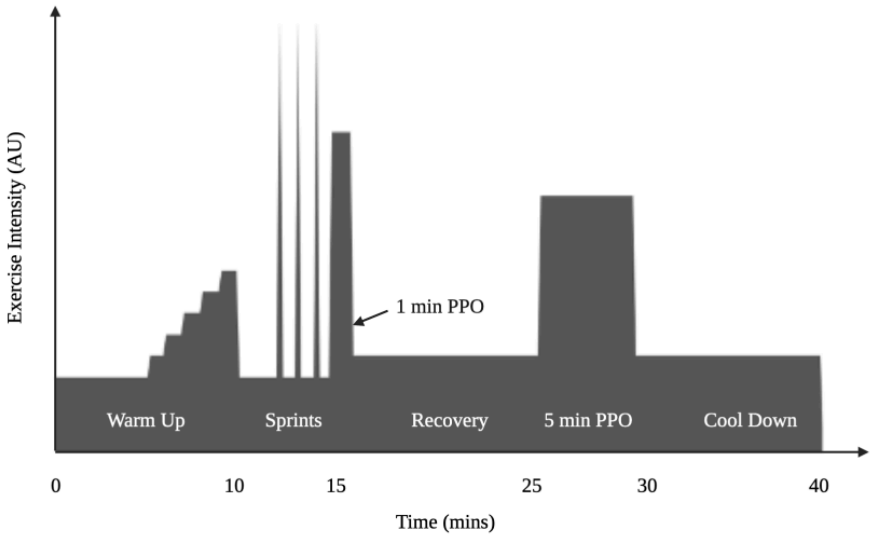

Figure 3.2 – Schematic representation of the prescribed power profile during the 1- & 5-minute PPO Test. Initially, participants completed a 15 min warmup of 5 mins at ~50% PPO, followed by a 5 min progressive ramp in intensity before 3 sprints (~80% of perceived max). Participants then completed a 1 min maximal cycling to achieve their highest mean power before a 10 min recovery period. Participants then completed a 5 min maximal exercise bout before an optional 10 min cool down.

3.3.3 30-minute Intermittent Capacity Test

In study 2, participants completed a 30-min cycling performance test in thermoneutral (20 °C, 50% RH) and hot (35 °C, 50% RH) conditions in a randomised order at the same time

of day on consecutive days. Participants attended the laboratory having consumed a standardised meal at least 2 h before arrival (CHO: 2.0g·kg·BM^{-1}, PRO: 0.3g·kg·BM^{-1}, Fat: 0.3g·kg·BM^{-1}) in a hydrated state. The implementation of a fixed duration exercise test (Time Trial [TT]) with intermittent sprints reflects the demands of Olympic distance triathlon cycling more closely, as typical performance-based tests adhere to constant work (time-trial), duration and power exercise modalities (Hopkins, Schabort and Hawley, 2001), all lacking ecological validity, failing to reflect the power profiles of the cycling discipline of Olympic distance triathlon (Etxebarria et al., 2014).

3.3.3 i) Exercise Protocol

Following a 15-min self-paced warm-up, participants started the test consisting of 6 phases of 4 mins 50 seconds and a 10-second sprint resulting in a cumulative time of 30 mins (Figure 3.3). Participants were instructed to ride as hard as possible for the test to obtain their best mean power (including sprints). The test was conducted on participants' bicycles mounted to a previously validated stationary cycle home trainer (Hammer H2, Cycleops, USA) (Lillo-Bevia and Pallares, 2018). Participants were not provided convective cooling to maximise heat gain during the relatively short exercise bout.

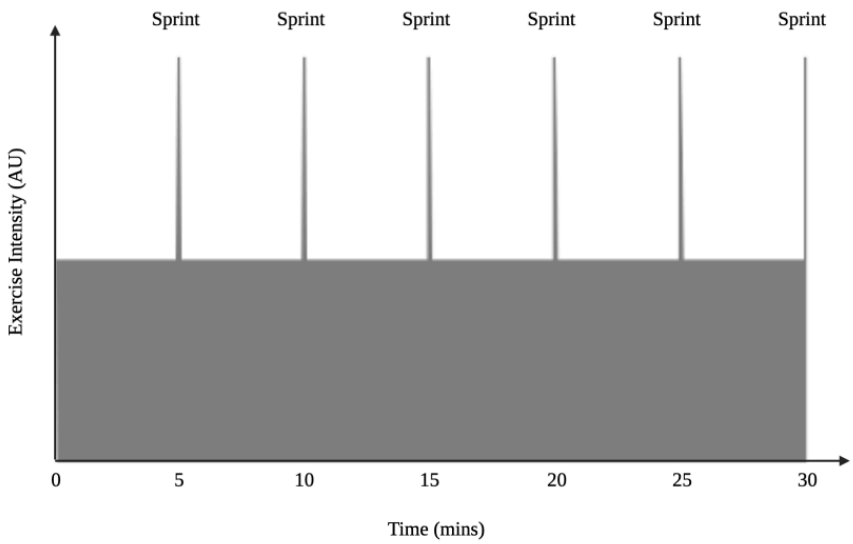

Figure 3.3 – Schematic representation of the 30-minute intermittent capacity test. Each sprint lasted a 10s in duration.

3.3.3 ii) Core & Skin Temperature

Core body temperature (T_{core}) was measured via a suppository approach telemetric pill (E-Celcius, BodyCap, Hérouville Saint-Clair, France) whereby participants self-inserted the pill beyond the anal sphincter approximately 1 hour before exercise as recommended by the national athletic trainers association (Casa et al., 2015); and previously validated against other core body temperature measurement techniques (Casa et al., 2007; Gosselin et al., 2019). Data were recorded at 1-min intervals via a data logger (e-Viewer, BodyCap, Hérouville Saint-Clair, France). To record skin temperature (T_{sk}), wireless thermistors (I-button thermochron, Maxim/Dallas Semiconductor Corp, Dallas, USA) were attached to 5 locations on the body (sternum, upper arm, forearm, quadricep and gastrocnemius). Mean surface temperature (MST) was calculated as per Ramanathan (1964):

$$MST = 0.3\ t_{chest} + 0.3\ t_{arm} + 0.2\ t_{thigh} + 0.2\ t_{gastroc}$$

3.4 Psycho-physiological Measures

3.4.1 Ratings of Perceived Exertion

Laboratory-based exercise tests included participants' ratings of perceived exertion using a 15-point scale (Borg, 1982). Participants were familiarised with the scale during preliminary visits to ensure comprehension of the subjective nature of the measure. During home-based exercise tests and training sessions, participants were asked to rate their perceived session intensity using a 10-point RPE scale (Borg, 2001; Zamuner et al., 2011)

3.4.2 Rating of Thermal Sensation and Comfort

Subjective thermal sensation and comfort measures were assessed using a modified visual scale previously described (Huizenga, Hui and Arens, 2001; Zhang et al., 2004).

3.5 Procurement and Storage of Blood Samples

Blood samples were collected from an in-dwelling cannula in the superficial anti-cubital fossa. Samples were collected in serum separator tubes (SST) and K_2EDTA vacutainers (BD Biosciences, UK). K_2EDTA vacutainers were stored on ice immediately upon collection, whilst SST was stored at room temperature for at least 30 mins before centrifugation at 1500 RCF for 15 mins at 4°C. Following centrifugation, serum and plasma were aliquoted into cryovials and stored at -80°C for later analysis.

3.6 General Cell Culture methodology

Cell culture techniques and associated analytical approaches were implemented throughout Chapters 6 and 7. Detailed protocols will be described below.

3.6.1 Cell Culture

All cell culture procedures were completed in a Class II microbiological safety cabinet (BSC; Kojair, Mänttä-Vilppula, Finland) under aseptic conditions. All cells were sub-cultured and incubated in a HERAcell 150i CO_2 humidified incubator (Thermo Fisher Scientific, Cheshire, UK) at 37°C and 5% CO_2 and were routinely monitored using an inverted light microscope (Olympus, CKX31, Japan). An extraction pump (Charles Austen Pumps Ltd, Surrey, UK) was used to remove waste media and supernatant.

3.6.2 Cell Culture Chemicals and Reagents

All Dulbecco's modified eagle medium (DMEM) and serum, including heat-inactivated horse serum (HS), heat-inactivated new-born calf serum (hiNBCS) and heat-inactivated foetal bovine serum (hiFBS), were purchased from Gibco (Life Technologies, California, USA).

3.6.3 C2C12 Skeletal Muscle Cells

Murine C2C12 skeletal muscle myoblasts (ATCC; Rockville, USA) were passaged to increase yield and stored in liquid nitrogen until required for experimentation. C2C12 cells are

the C12 sub-clone of the C2 parental cell line and are derived from the crushed injured leg of the C3H mouse (Yaffe and Saxel, 1977; Blau et al., 1985). In addition, the C12 sub-clone was selected for its differentiation capability, hence the extensive use of this cell line within *in vitro* research. C2C12 myotubes were used in this thesis to model *in vitro* skeletal muscle during exercise and heat stress.

3.6.4 Resuscitating Cells from Liquid Nitrogen

Previously stored C2C12 myoblasts had been gradually frozen by storing at -80ºC for 24 hrs in 1 mL growth media (GM) (10% foetal bovine serum gold (FBS), 10% new-born calf serum (NBCS) and 5 mL of 1% penicillin-streptomycin) and 10% dimethyl sulfoxide (DMSO) before moving to liquid nitrogen until required. Next, 5 mL of 0.2% gelatin solution was added to T75 flasks and allowed to rest for 10 mins at room temperature; after 10 mins, flasks were placed in an incubator for a further 10 mins at 37ºC. The remaining liquid was removed by aspiration. Then 1 mL of cell suspension (1×10^{-6} per mL) was removed from liquid nitrogen and rapidly thawed before dilution in 14 mL of previously heated (37ºC) GM. Lastly, 15 mL of cell-containing GM was added to the T75 and placed in an incubator at 37ºC for 72 hrs to allow for proliferation until desired confluency.

3.6.5 Passaging and Trypsinising Cells

Following visual inspection to ensure desired confluency (80%), growth media was aspirated from each T75, and subsequently washed 3 times with PBS. Next, 1 mL of Trypsin (0.05%) (Sigma-Aldrich, Poole, UK) was added to T75 before manipulation to ensure coverage of the solution across the flask surface, before incubation for 5 mins at 37ºC. Cells were visually

inspected to ensure non-adherence to the flask, with manual percussion applied to free any remaining fixed cells. Four mL of GM (37ºC) was added to each T75 to neutralise the trypsin resulting in 5 mL total volume. The cell suspension was then transferred to 15 mL falcon tubes for cell counting.

3.6.6 Cell Counting by Trypan Blue Exclusion

A Neubauer haemocytometer was used to count cells, consisting of a glass coverslip resting upon a counting chamber (Figure 3.4). Cells (20 µL) were prepared in a 1:1 suspension in 0.4% trypan blue stain (20 µL) and loaded into the haemocytometer. Cells located in the 4 corners of the grid were counted at 10× magnification. Viable cells were identified as small, round, and visible, while non-viable cells were misshapen, more prominent and had lost membrane integrity, hence positive trypan blue. If a high standard deviation was observed following the initial count, cells were loaded into the opposite side of the haemocytometer, and a different 4 grid was used in the calculation. The mean across 4 or 8 grids was calculated and represented the average number of cells within 0.1mm^3. This value was doubled to account for the dilution factor and multiplied 10^4 to extrapolate the result from 0.1 mm^3 to 1 cm^3 (equation below). The total number of cells within the suspension was calculated by multiplying by the total volume of the cell suspension (mL).

A

$$Cells \cdot mL^{-1} = mean\ cell\ count\ (4-8\ grids)\ \times\ dilution\ factor \times\ 10^4\ cells \cdot mL^{-1}$$

$$Total\ Cells \cdot mL^{-1} =\ Cells \cdot mL^{-1}\ \times\ Cell\ Suspension\ volume\ (mL)$$

B

$$\frac{Cell\ suspension\ required\ (mL) = Desired\ cell\ concentration\ (Cells \cdot mL^{-1})}{Current\ concetration\ (Cells \cdot mL^{-1}) \times\ required\ cell\ suspension\ volume\ (mL)}$$

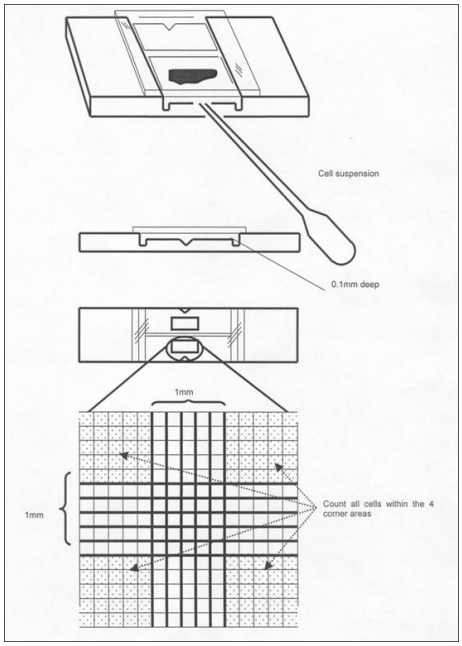

Figure 3.4 – Haemocytometer with coverslip and arrangements of grids with identified counting corners viewed under the microscope.

3.6.7 Differentiation of Cells from Myoblast to Myotubes

Two mL of Cell-GM suspension was used to seed previously gelatinised 6-well plates at ~70,000 cells per well and were allowed to proliferate for 24 hrs until desired 80% confluency before the commencement of differentiation. Once at the desired confluency, growth media was removed, and cells were washed 3 times with PBS. Two mL of differentiation media (DM) (2% horse serum, 1% penicillin-streptomycin) was added per well and topped up by 10% volume until cells were differentiated after ~8 days.

3.6.8 *In Vitro C*ell Stimulation Protocol

Cell monolayer electrical pulse stimulation (EPS) was performed using an in-house built stimulator which was designed to produce biphasic pulses (an initial positive cathodal activation pulse followed by a brief interval and then a second, negative anodal pulse) which

deliver a balanced charge into the cell medium resulting in myotube contraction. Biphasic balanced pulses prevent a net charge injection into stimulated tissue and equipment. Charge balancing is necessary to avoid tissue damage and reduce damage incurred to the electrical equipment delivering the electrical current (Harnack et al., 2004). The electrical stimulation protocol: Twitch at 10 Hz, 10 V, 1 ms pulse train (Figure 3.5)

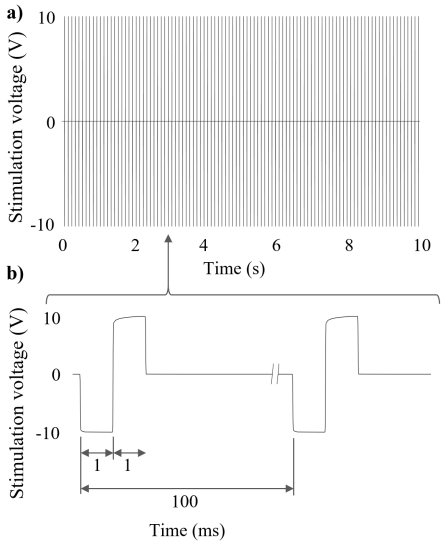

Figure 3.5 – Simplified schematic outlining the EPS protocol used in the twitch stimulation of cell monolayers. (A) 10 Hz pulse frequency delivered to the cells over a 10 s time course. (B) Biphasic 10 V, 1 ms pulses delivered to the tissue with a 1 ms rest between each pulse.

3.6.9 Imaging - Immunofluorescence (IF) Imaging

3.6.9 i) General Principal

Immunocytochemistry (ICC) is used to assess the presence of specific protein(s)/antigen(s) in cells using specific antibodies that bind specifically to the protein/antibody of interest allowing visualisation of the protein under a microscope (Figure 3.6). ICC is a valuable tool for studying the presence of proteins' subcellular localisation. The general ICC/IF protocol involves fixation (maintaining structures within the cell whilst rendering them dead), followed by a blocking step, primary antibody incubation and secondary antibody incubation.

3.6.9 ii) General Protocol

Media was aspirated, and cell monolayers were washed 3 times with PBS. Cells were fixed with 4% paraformaldehyde (PFA) for 10-min at room temperature. After that, PFA was removed, and wells were washed 3 times with PBS. The fixed samples were maintained in PBS at 4˚C until analysis. PBS was aspirated before adding permeabilization buffer (PBS + 0.1% Triton X-100) to each well before 15 mins incubation at room temperature. Permeabilization buffer was removed, and monolayers were washed twice with cool PBS before blocking buffer (10% Goat Serum in PBS) was added and incubated at room temperature for 30 mins. Monolayers were rewashed with PBS, and the primary antibody (MF-20 in BSA, 1:300 dilution) was added to each well in a darkened room. Plates were then foil-wrapped and refrigerated (5°C) overnight.

The following day, the primary antibody (MF-20) was removed, and wells were washed 3 times with cool PBS (~5°C), leaving PBS for 5 mins each wash. Next, the secondary antibody (Alexa-fluor goat anti-mouse 488 in BSA, concentration ratio 1:400) was added, and plates were covered in foil before room temperature incubation for 60 mins. The secondary antibody was then removed, and cell monolayers were washed twice with PBS. Finally, nuclear counterstaining using DAPI in H_2O (concentration ratio 1:100) was added to monolayers and incubated at room temperature in a darkened room covered with foil for 15 mins. DAPI solution was then aspirated, a small volume of PBS was added to wells, and plates were foil-wrapped before being stored in the fridge until imaging.

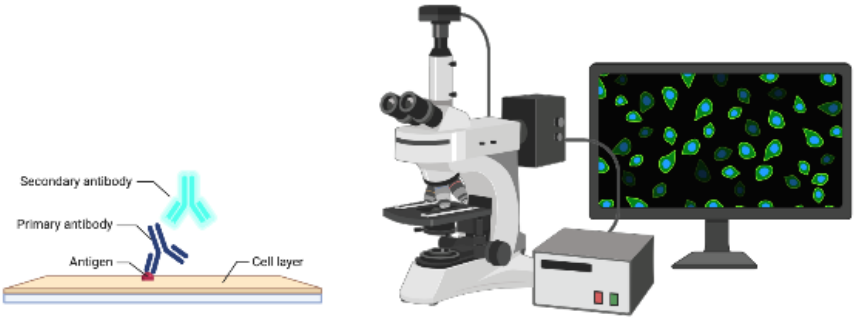

Figure 3.6 – A schematic representation of antigen immunolabeling and fluorescent microscopy, demonstrating the binding of a primary and secondary antibody to an antigen and the visualisation of fluorescence emitting cells through a fluorescent microscope.

All images were captured using a Leica DM116000B Microscope (Leica Biosystems, Wetzlar, Germany) at 10× objective zoom. DAPI was exposed at a wavelength of 490-450nm (blue), and Alexa fluor 488 at 560-520nm (green).

3.6.10 Quantification of mRNA by Polymerase Chain Reaction (PCR)

PCR was used during the optimisation of *in vitro* 'exercise'-like stimulation to confirm the transcriptional response induced by heat stress and electrical pulse stimulation, as previously reported in the literature. Data is presented within Chapter 6.

3.6.10 i) mRNA Extraction

Firstly, TRI reagent (Trizol) (300 µL) was added to each well to extract the mRNA from within myotubes. Using a sterilised plastic cell scraper, cells were homogenised with Trizol in each well before the transfer for Trizol-cell lysate to clean Eppendorf tubes phenol and guanidine thiocyanate that can lyse cells and dissolve all other cell components except her

cell components except for RNA and DNA. As a result, Trizol inhibits the activity of DNAse and RNAse enzymes and maintains RNA integrity.

3.6.10 ii) RNA isolation method

Chloroform (200 µL for every 1mL of Trizol) was added to each cell-lysate sample and shaken vigorously by hand before resting at room temperature for 10 mins before centrifugation at 12,000 RCF for 15 mins at 4°C. Centrifugation induced phase separation into red phenol, middle interphase, and aqueous upper phases. The upper aqueous phase was carefully removed, transferred into a new pre-labelled RNase-free Eppendorf, and mixed with 300 µL of isopropanol. The sample was mixed by inversion and left to stand at room temperature for 10 mins before centrifugation at 12,000 RCF for 10 mins at 4°C. The resultant supernatant was removed and 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 mins at 12,000 RCF at 4°C. The ethanol was removed, and the wash process was repeated. Following the second centrifugation, the ethanol was discarded, and the pellet was left to air dry before resuspension in RNAse and DNAse-free, DEPC water (Thermo Fischer Scientific, UK). Samples were heated in a block heater at 35°C to facilitate resuspension for 10 mins before RNA quality and quantity measurement.

3.6.10 iii) Method of RNA quantification

Using a Nanodrop spectrophotometer (Thermo Fisher Scientific), RNA purity and concentration were assessed by UV spectroscopy. The absorbance of the diluted RNA was measured at known purity ratios of 260/280 and 260/230, where absorbance at 260 nm ($A260$)

provided a specific measurement of nucleic acid concentration and the presence of any contaminants measured at 280 nm ($A280$) for protein and 230 nm ($A230$) for background absorption. Generally, a A_{260}/A_{280} ratio of 1.8 to 2.1 represents pure RNA, with the A260/A230 ratio being higher than that of A_{260}/A_{280}, with values typically ranging from 1.8-2.2 (Figure 3.8). There is no acceptable lower limit for this ratio, with previous research demonstrating no correlation between A_{260}/A_{230} and the qPCR amplification efficiency (Kuang et al., 2018).

Upon quantifying mRNA 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. One µL of diluted sample was pipetted directly onto the measurement pedestal for RNA assessment.

Absorbance at 260 nm was used to determine the RNA concentration, given that an A260 reading of 1.0 is equivalent to 40 $\mu g \cdot ml^{-1}$ of RNA. The Nanodrop software used for the quantification of nucleic acid uses a modified version of the Beer-Lambert equation:

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

Where C is the nucleic acid concentration ($ng \cdot \mu L^{-1}$), A is the absorbance in AU, and ε is the wavelength-dependent extinction coefficient ($ng\text{-}cm \cdot \mu L^{-1}$). For RNA, the wavelength-dependent extinction constant is $40nm\text{-}cm \cdot \mu L^{-1}$. b is the path length in cm.

3.6.10 iv) Polymerase Chain Reaction (PCR)

Following the isolation of mRNA from a biological sample (*i.e.*, C2C12 cell culture), the mRNA is synthesised into complementary DNA (cDNA) through reverse transcription by

reverse transcriptase enzymes and nucleoside triphosphates (dNTPs). The complementary 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 gene by RNA polymerase (Figure 3.7). Finally, the amplified product is made detectible by labelling the cDNA with SYBR-green, fluorescent dye, which increases proportionally with amplification of the cDNA and is detected in real-time following each reaction cycle, allowing for precise semi-quantitative analysis of mRNA content.

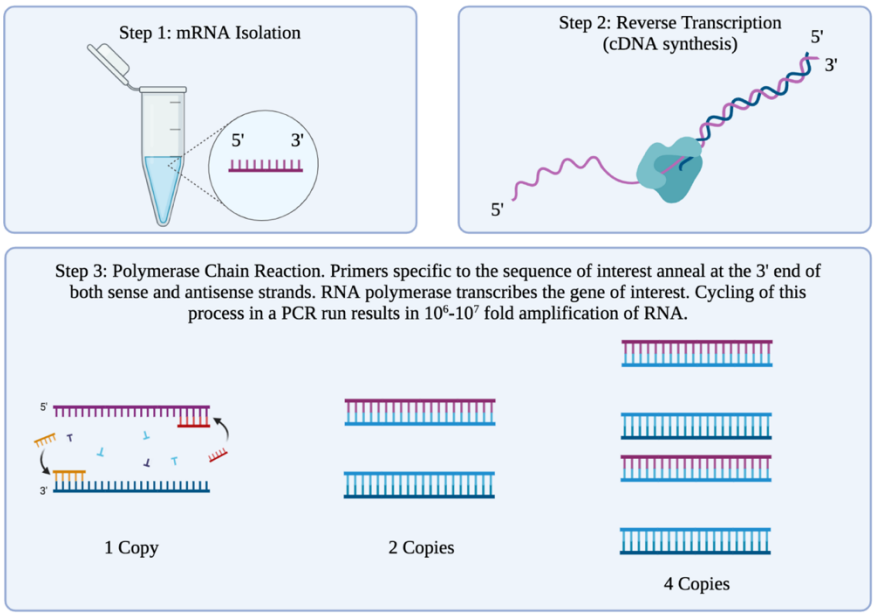

Figure 3.7 – Schematic overview of the critical steps during the real-time quantitative polymerase chain reaction.

For all gene expression data presented within this thesis, purified RNA was diluted to 16.6 ng·µL^{-1} in 3 µL volume, making 70 ng total reactions 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 PCR method allows DNA to be synthesised from a single-stranded RNA template (Step 2 of Figure 3.9). 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 repeated 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 mins, followed by transcriptase inactivation and initial denaturation (95°C for 5 mins). Following the initial denaturation step, complementary 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 denaturation and annealing/extension cycles were repeated for 40 cycles. The SYBR Green mixture contains a fluorescent dye that binds to all double-stranded DNA and emits fluorescence upon binding. With 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.

3.6.10 v) PCR Analysis

PCR efficiency was calculated to determine the increase in the amplicon per cycle, with 100% efficiency demonstrating a perfect doubling of the amplification at each cycle. Melt curve analysis was also performed to assess whether a single, specific product had been amplified and to allow the 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 complement; thus, a unique melt curve will be produced for each specific DNA fragment. All melt curve analyses presented single peaks for each target gene, indicating the amplification of a single product.

Changes in mRNA content were calculated using the comparative Ct ($^{\Delta\Delta}$Ct) equation (Schmittgen and Livak, 2008). 18S ribosomal RNA (18S) and RNA Polymerase II Subunit A (POLR2) were used as the reference genes throughout all experiments and did not change significantly between control and treated cells. mRNA expression for all target genes was calculated relative to the reference gene for the corresponding time point and normalised to a single control sample for each condition, which acted as the calibration condition.

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 calibrator)
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)

3.6.10 vi) Primer Design

Primer sequences were designed using 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).

3.6.11 MTT Cell Viability and Metabolic Activity Assay

This colourimetric assay measures metabolic activity based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye (MTT) into an insoluble, purple-coloured crystal, formazan. The absorbance is quantified at a wavelength of 570nm. Therefore, this assay measures the cell viability/metabolic activity in reductive activity as the enzymatic conversion

of the tetrazolium compound to the insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells.

MTT solution was added to culture media at a ratio of 200 µL for every 2 mL of media and incubated at 37ºC for 3 hrs. Media was then removed via aspiration, and uncovered cells were incubated (37ºC) for 5-10 mins to ensure the plate was dry. Finally, 1 mL of DMSO was added per well and agitated for 2-3 mins on a plate rocker to provide complete coverage. Each plate is added to the plate reader and measured at 570nm.

3.6.12 Flow Cytometry Principle

A high-throughput analytical technique, flow cytometry, is used to characterise biological materials, including whole-cell populations and sub-cellular components, including the organelles (Radcliff and Jaroszeski, 1998). The biofluid sample is drawn through the cytometer and is passed through a known beam of light/laser; the components of the sample cause the light to be scattered, which is measured through an array of fluorescent detectors (Adan et al., 2017) (Figure 3.8).

In the context of this thesis, only cell populations were characterised. To accurately determine the properties of the cell, a cell suspension is drawn through a stream of fluid sheath fluid, which hydrodynamically focuses the cells into a single file as they pass through the laser. Cells passing through this laser interrupt the flow of light, and the cytometer detects two light dynamics, forward scatter (FSC) and side scatters (SSC). Located opposite the laser beam, the FSC detector detects the ‘shadow’ generated as cells pass through the beam and is used to quantify cell size as larger cells generate larger shadows. Sitting perpendicular to the laser, the

SSC detector quantifies the cellular granularity based on the increased scatter induced by more complex cells. Finally, the two components are combined into one plot whereby similarly sized populations are grouped against cells of similar granulation. Based upon the clustering of homogenous populations, live cells will populate one region of the plot, whilst dead or apoptotic cells populate another due to their smaller size.

In addition to FFC and SCC, flow cytometry can also be used to measure fluorescence. This means this technique can quantify a fluorescent signal's emission from labelled probes introduced to the cell population. This drastically enhances flow cytometry's analytical capabilities, allowing the characterisation of multiple aspects of cellular homeostasis. The flow cytometer is equipped with channels that detect specific fluorescent frequencies and vary between devices to characterise these changes. For example, this thesis used the same BD Accuri C6 (BD, New Jersey, USA) with 4 fluorescent channels (FL-1, FL-2, FL-3, FL-4). An overview of the flow cytometry principle can be seen in Figure 3.8.

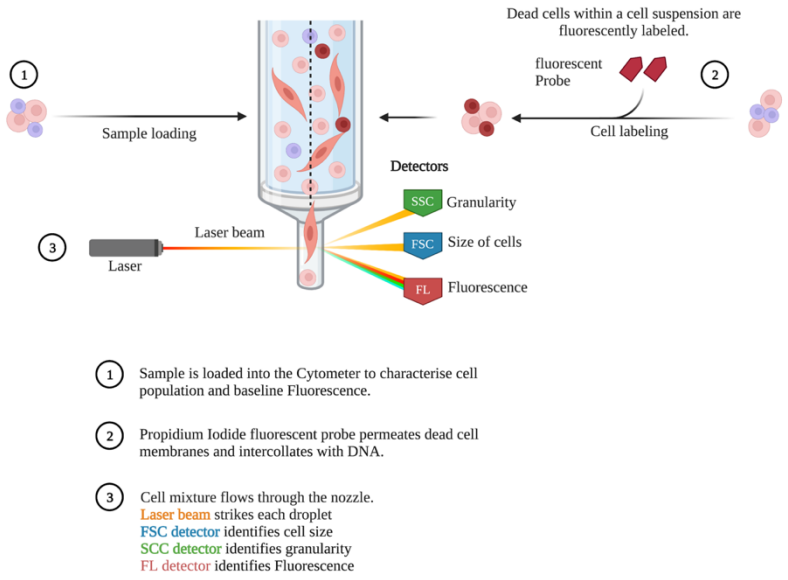

Figure 3.8 – Overview of Flow Cytometry principle including characterisation of myoblast vs myotubes and characterisation of healthy cell population using a fluorescent label.

3.6.12 i) Flow Cytometry Gating

To accurately measure changes in C2C12 fluorescence, control cells were used to generate a background profile whereby the FSC and SSC were gated, and background fluorescence was measured. One ml of cell suspension was passed through the flow cytometer and gated based on their FSC and SSC. Initially, the heterogenous myoblast-myotube C2C12 population was characterised (Figure 3.9). Practically, the size of myotubes poses unique difficulties for flow cytometry, requiring the adjustment of the core size. Once optimised, 2 distinct populations were observed in FSC and SCC due to cellular orientation upon passing the laser, heterogeneous population of myoblasts and tubes or a combination of both factors. Experiments were standardised against a standardised number of events before the quantification of background fluorescence of the C2C12 population across all channels (Figure 3.10) before assessing fluorescent probe uptake.

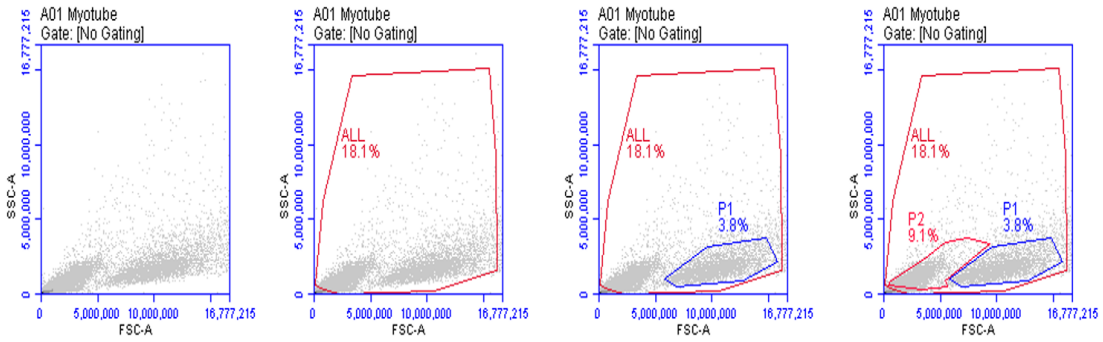

Figure 3.9 – C2C12 gating for myoblasts and myotubes representing 2 population groups P1 and 2, using the BD Accuri C6 Flow cytometer.

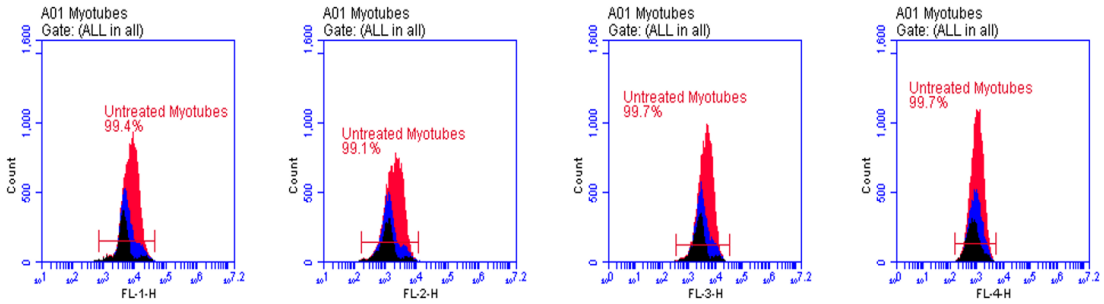

Figure 3.10 – Characterisation of 'normal' background fluorescence generated by C2C12 myotubes.

3.6.12 ii) Propidium Iodide (PI) Assay

Propidium iodide (PI) (Thermo Scientific Inc. Massachusetts, USA) dye exclusion assay was undertaken to characterise the live cell population. PI binds to double-stranded DNA via intercalation between the bases, with little to no sequence preference (Lecoeur, 2002). As PI cannot permeate intact plasma membranes (viable cells), PI is only incorporated into dead/damaged cells resulting in considerably larger fluorescent intensity when dead cells are present (Riccardi and Nicoletti, 2006). The 20-30-fold increase in fluorescence allows the precise characterisation of viable and non-viable cells within a cell suspension mixture (Arndt-Jovin and Jovin, 1989).

For assessment, cell media was initially removed and aliquoted into Eppendorf tubes (to ensure the collection of any damaged cells which had detached) and stored at 37°C. Adherent cells were washed twice with PBS before adding 200 µL trypsin. After a 5-min incubation, trypsin was neutralised by the re-addition of previously collected media. The cell-media suspension was re-aliquoted into Eppendorf tubes before centrifugation at 3000 RCF for 5 mins. The media supernatant was removed, leaving only a cell pellet which was resuspended in 500 µL of fresh differentiation media. PI was added to produce a 1:100 dilution and vortexed

for the 20s before incubation at 37°C for 5 mins. PI fluorescence was analysed via flow cytometry using the FL-3 channel. Negative and positive controls were completed whereby PI-free cells were analysed for fluorescence alongside cells treated with 50% v/v H_2O_2. PI-Free cells had no increase in fluorescence, whilst H_2O_2 increased fluorescence (Figure 3.11). The excitation/emission maximum of the dye is typically 493/636; upon binding to DNA, excitation/emission maxima increased to 535/617 nm.

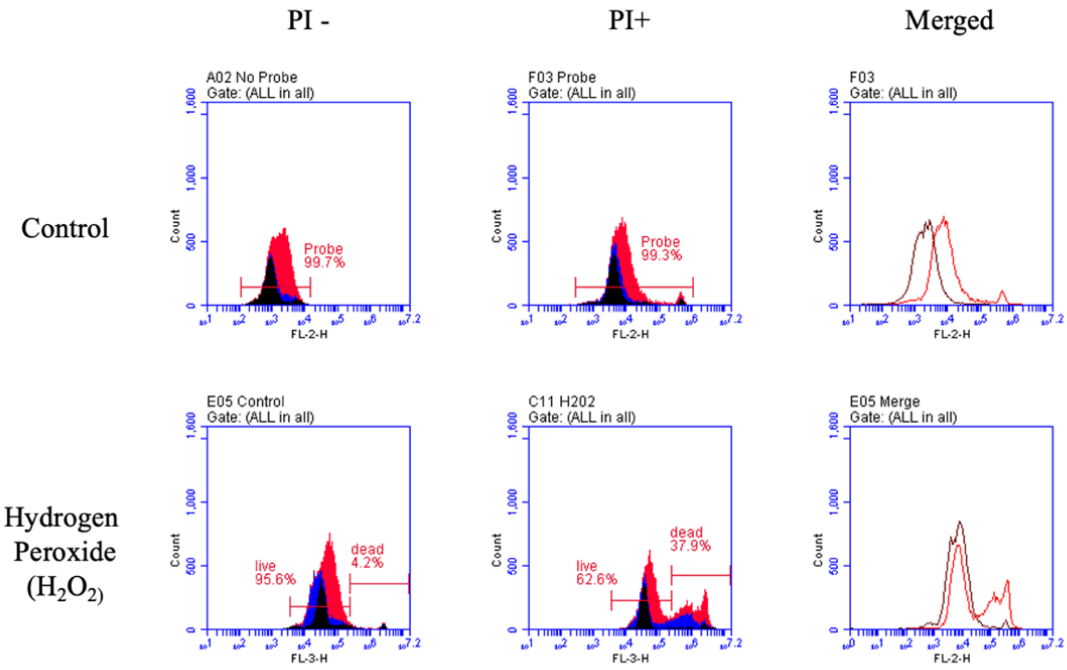

Figure 3.11 – Validation of Propidium Iodide (PI) assay with H_2O_2. Negative (PI-) and positive (PI+) controls were treated with Hydrogen peroxide it induce cell death and subsequent positive signal in the presence of PI.

3.7 Metabolomic Analysis General Principal

3.7.1 Metabolomics in Science

First proposed by Oliver et al. (1998), Metabolomics has been used extensively to determine the phenotype of biofluids and tissues through the analysis of present metabolites. Metabolites represent a classification of a small molecule (<1500 Daltons) and substrates or end products of enzyme-mediated reactions (Dunn et al., 2011), which fundamentally amplify

the changes observed within both the transcript- and proteomes (Raamsdonk et al., 2001). This highlights the potential importance of the biological insights that can be afforded by metabolomic investigations with the conserved nature across mammalian species allowing for metabolite-level data to be translated to human populations.

Metabolomics is an example of the application of analytical chemistry within physiology. Quantifying the abundance, both absolute and relative pre-dates the term ‘metabolomics’; however, the development of the field has coincided with significant advances in spectroscopic measures, including mass spectrometry and chromatography. These developments have allowed samples to be analysed with greater sensitivity, resolution and throughput (Hayton et al., 2017).

3.7.2 Techniques in Metabolomics

Within the field of metabolomics, the two most prevalent analytical techniques are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) (Gowda and Djukovic, 2014). Improvements in the technology and the analytical tools available have allowed the application of these techniques in a high throughput approach, permitting the characterisation of biofluids, a key feature in metabolomics. Furthermore, once data is collected using the equipment of choice, output data can be interrogated by a series of statistical methods. This section will briefly discuss the advantages and disadvantages of NMR and MS for metabolomic investigations (summarised in Table 3.3).

3.7.2 i) Nuclear Magnetic Resonance (NMR)

Typically applied within the field of analytical chemistry for compound quantification, purity and structural elucidation, NMR spectroscopy measures the interaction between atoms and electromagnetic radiation. Although solution and solid-state NMR approaches exist and operate upon the same fundamental physical principles (interaction of atoms and electromagnetic radiation), only solution NMR will be discussed here due to its exclusive use within this body of work and its obvious applicability to biofluid metabolomics.

NMR exploits the intrinsic ability of atoms to behave like magnets when applied within an external electromagnetic field. ^{1}H-NMR works by quantification of the interaction of nuclei with an external magnetic field; at the onset of the application of an electromagnetic pulse, the nuclei undergo 'excitation' and, upon cessation, 'relaxation'. During the 'excitation' phase, nuclei absorb the radio-frequency waves emitted from the magnet, and during the relaxation phase, they emit the stored radio-frequency waves giving rise to radio-frequency waves in the NMR spectrum after Fourier's transformation. In the case of ^{1}H-NMR, the almost 100% natural abundance of ^{1}H and its presence in nearly all biological molecules provides exceptionally high sensitivity and represents a rapid, non-destructive quantitative profiling approach based on a single reference compound (Weljie et al., 2006; Xu et al., 2006).

3.7.2 i) Mass Spectrometry (MS)

Mass spectrometry (MS) represents a significantly more "mainstream" analytical technique and is already commonplace in exercise physiology. MS is highly sensitive in metabolite detection and quantification and involves the interpretation of ionisation patterns of

a particular molecular for identification. Comprised of three parts, an MS requires an ion source, a mass analyser and a detector that measures the mass/charge (m/z) of ions with this information to identify molecules. In the case of metabolomics, the sample is injected into the ioniser, which converts the metabolite into ions, which are transferred to the mass analyser, where ions are separated based on the m/z, which is recorded by the detector. This information is recorded via a computer, and output data is processed and analysed. MS is often used in tandem with chromatography equipment to improve sensitivity, requiring the sample to be in solution, allowing the molecules within to be separated and individually sent into the MS for analysis.

Table 3.3 – Comparison of nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) within metabolomics applications. Benefits are highlighted in **Bold.** [Adapted from Wishart (2019)].

NMR	MS
Non-destructive and sample integrity maintained during analysis means the sample is reusable.	Sample is destroyed during data acquisition.
Robust Equipment and instrumentation. Minimal downtime.	Frail equipment with frequent downtime.
Excellent Reproducibility	Moderate Reproducibility
Simple sample preparation	Complex Sample preparation
No chromatography necessary	Chromatography required
No need for chemical derivatization	Chemical derivatization frequently required
Predictable Spectra	Not frequently predictable spectra
Allows precise determination of chemical structure	Only partial determination of structure
Inherently quantitative	Not inherently quantitative
Easily Automated workflow	Difficult to automate workflow
Poor to moderate sensitivity	**Excellent sensitivity**
Modest Metabolite Coverage	**Extensive metabolite coverage**
Extremely Expensive instrumentation	**Moderately expensive instrumentation**
Large instrument footprint	**Small instrument footprint**
Requires cryogens	**No cryogens necessary**
Expensive maintenance costs	**Moderate maintenance costs**
Small spectral databases	**Large spectral databases**
Few software resources	**Many software resources**

3.8 Metabolomics General Methodology

3.8.1 Metabolomics Workflow

The post-sample preparation workflow employed within the current thesis is shown in Figure 3.15. Briefly, data were collected via ^{1}H-NMR spectroscopy before samples were subject to quality control procedures (QC) to ensure only high-quality spectra were included in subsequent analysis (Dunn et al., 2017). In cases where spectra failed QC, NMR spectroscopy was repeated on respective samples a maximum of 3 times before exclusion. Quality-controlled samples were subsequently normalised and scaled (each process described later in this chapter) before multivariate analysis (Principal Component Analysis [PCA] and Partial Least Squares – Discriminant Analysis [PLS-DA]) and identification of metabolites most important in the variance between groups (VIP) identified (Worley and Powers, 2013). Due to the nature of NMR, metabolites often produce multiple peaks within the same spectra. Representative peak selection was completed by correlation reliability score (CRS) assessment before a representative peak for filtered metabolites was subject to univariate analysis. Metabolite set enrichment analysis (MSEA) was also performed to identify over-represented metabolic pathways before final biological interpretation (Xia and Wishart, 2010).

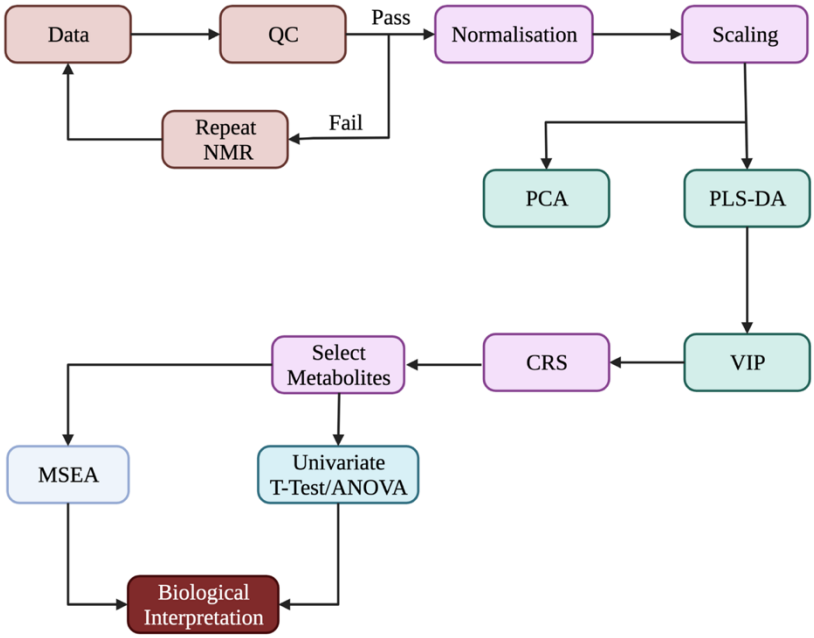

Figure 3.12 – Overview of metabolomics data acquisition and quality control (QC) (Red), spectral/data processing (purple), multivariate analysis (green), univariate analysis (blue), metabolite pathway analysis (MSEA) (grey) and biological interpretation (red/white). PCA, Principal component analysis, PLS-DA, partial least squares discriminant analysis, VIP, variance in the importance of projection, CRS, correlation reliability score.

3.8.2 Sample Preparation for ^{1}H-NMR Acquisition

3.8.2 i) C2C12 Myotubes

Previously Frozen 6-well plates were thawed from -80°C on ice before adding 500µL of ice-cold acetonitrile: H_2O (50:50 *v*/*v*) to each well. Cells were scraped with the base of a pipette tip, and the resultant cell slurry was pipetted into new Eppendorf tubes. Samples remained on ice and were immediately sonicated at 50 kHz, 20% amplitude for 3 × 30s bursts, then vortexed for 20s before centrifugation at 21,500 RCF for 5 mins at 4°C. The supernatant was transferred to fresh Eppendorf tubes before snap-freezing in LN_2. Samples were then lyphilised overnight before resuspension in 200 µL of NMR buffer (100 µM Trimethylsilyl propionate [TSP] [d6 deuterated, Sigma], 99.9 % $^{2}H_2O$ [Sigma], and 100 mM Na_2HPO_4:NaH_2PO_4 pH 7.4 [Thermo-Fisher]). Samples were vortexed for 20 s and centrifuged

at 12,000 RCF for 2 mins at 4°C. One hundred and ninety µL of the centrifuged sample was pipetted into 3 mm outer diameter glass SampleJet NMR tubes (Bruker).

3.8.2 ii) Differentiation Media

Previously stored aliquoted differentiation media was thawed, and 100 µL was diluted at 50% (v/v) with 10% NMR buffer (100 µM Trimethylsilyl propionate [TSP] [d6 deuterated, Sigma], 99.9 % ^2H_2O [Sigma], and 100 mM Na_2HPO_4:NaH_2PO_4 pH 7.4 [Thermo-Fisher]) and vortexed for 20s before centrifugation at 21,500 RCF for 5 mins at 4°C. Next, 190 µL of the centrifuged sample was pipetted into 3 mm outer diameter glass SampleJet NMR tubes (Bruker).

3.8.2 iii) Human Blood Serum

Previously stored aliquoted serum was thawed, and 330 µL was diluted at 50% (v/v) with 10% NMR buffer (^2H_2O with 100 mM sodium phosphate buffer pH 7.4 and 0.1% azide) before centrifugation at 21,500 RCF for 5 mins at 4°C. Next, 600 µL of the centrifuged sample was pipetted into 5mm (outer diameter glass SampleJet NMR tubes (Bruker).

3.8.3 ^{1}H-NMR Acquisition

Spectra were acquired on Bruker 700 MHz Avance IIIHD spectrometer equipped with TCI cryoprobe and chilled autosampler (SampleJet, Ettlingen, Germany). Standard vendor pulse sequences were applied to collect 1D ^{1}H-NMR spectra (cpmg1dpr). A Carr–Purcell–Meiboom–Gill (CPMG) edited pulse sequence was employed to attenuate signals from

techniques within the NMR field; each spectrum was normalised against a reference median spectrum. Using RStudio, data underwent one of two normalisation techniques: 1) total area and 2) probabilistic quotient normalisation (PQN) (Figure 3.13). After normalisation, both total area and PQN produced similar results; however, due to greater robustness, PQN normalisation (Dieterle et al., 2006) was used in all subsequent analyses (Figure 3.15).

Figure 3.13 – Raw and Normalised data using PQN and Total Area methods representative of 68 spectra. (A) Raw data – no normalisation. (B) PQN Normalisation (C)Total Area Normalisation.

3.8.4 Spectral Scaling and Centring

To enable comparisons of metabolites and minimise biological variation confounding the results, scaling of each variable was performed across the entire dataset (Craig et al., 2006). Two scaling methods were used on normalised data: 1) auto (mean centring and scaling by the standard deviation of the bin), and 2) 'Pareto' (mean centring and scaling by the square root of the standard deviation of the bin). Pareto was shown to be the most robust method for subsequent statistical analysis as it did not appreciably increase the amount of noise in the spectra to the same degree as auto scaling (Figure 3.14).

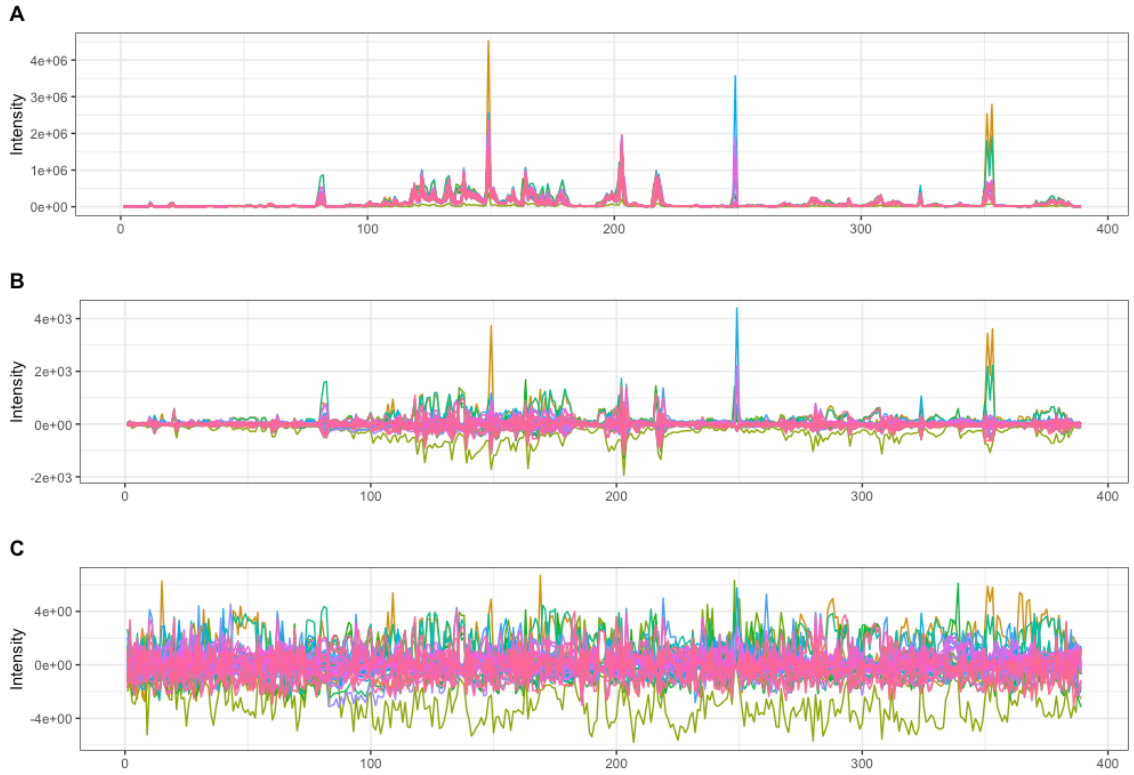

Figure 3.14 – Comparison of data scaling methods. (A) Raw data – no scaling. (B) 'Pareto' scaled data. (C) Total-area scaled data.

Figure 3.15 – Illustration of data processed via Pareto scaling and PQN. (A) Raw data. (B) Pareto scaled, PQN data.

3.8.5 Statistical Analysis of Metabolomics Data

3.8.5 i) Principal Component Analysis (PCA)

PCA is an orthogonal data transformation that returns unobserved (latent) variables (principal components [PC]). Each PC is the linear combination of the original variable, so the first PC explains the most variance in the data. The second component explains the most variance unexplained by the first PC and is orthogonal and uncorrelated to the first. Subsequent PCs follow the same procedure. This transformation results in a dataset where uncorrelated PCs replace the original variables. In this new dataset of PCs, typically, only the first few PCs are required to explain much of the variance in the dataset, thus reducing the high number of starting variables with minimal information loss (Worley and Powers, 2013).

PCA was preferred in the present thesis as a data exploration tool to reveal any observed and hidden structures in the data. Each point represents a sample on a PCA scores plot and can give information about the (dis)similarities between samples. This is elucidated from the distances between points on the scores plot. Furthermore, the observed structures in the PCs can be linked to the original variables (metabolites) by observing the associated loadings plot. However, selecting metabolites of interest in such datasets is often more nuanced.

3.8.5 ii) Partial Least Squares Discriminant Analysis (PLS-DA)

Partial least squares - discriminant analysis (PLS-DA) is a variation of partial least square (PLS) regression. PLS is a supervised statistical model applied to multivariate datasets to generate predictive models between two matrices (Worley and Powers, 2013). A PLS model requires a matrix of input data (predictors) and a second matrix (response) where the model's output is recorded. PLS projects both matrices (predictors and response) into two new matrices

where the covariance between the two is minimised (Westerhuis et al., 2008). PLS and PCA are similar because both create latent (unobserved) variables by using projections into new spaces. However, while PCA is an unsupervised method that projects maximum variance in latent variables called PCs, PLS is supervised (information on sample groupings retained) and projects predicted and observable variables using a linear regression model in latent variables called variates or components. In addition, PLS-DA variation uses a nominal vector as the response, which allows the building of models for classification problems. PLS models are particularly suitable for multicollinear data in predictors such as NMR data, where a single metabolite can be represented by multiple peaks depending on its molecular structure. This thesis used PLS-DA to build predictive models between experimental groups. PLS-DA model performance was assessed by using a 5-fold cross-validation over 50 repetitions. Classification errors were used to determine the optimal model complexity parameter (number of components). From the refined model, representative metabolites could be selected to reveal those driving the differences between experimental groups.

3.8.5 iii) Variable Importance of the Projection (VIP)

To extract biologically relevant information, Bin (variable) selection from a statistical model is crucial in metabolomics studies. PLS-DA and its derivative methods are designed to transform the data and make predictions. However, variable (bin) selection is not integrated into the model-building process. Variable importance of the projection (VIP) scores is a method that is often preferred with datasets with multicollinearity. VIP scores are the sum of squares of PLS weights (calculated during PLS-DA model building) which also explain variance in PLS variates. This method is designed for use on multivariate datasets with a correlation between variables and a larger number of variables than samples. Once VIP scores are

calculated, a cut-off threshold must be defined to include or exclude variables. VIP scores are calculated as the average of all VIP scores squared (Akarachantachote, 2014). Hence, a lower threshold of 1 was used to select variables with above-average influence in the PLS-DA model. Due to the nature of NMR-derived data, metabolites with multiple signals will present multiple entries in VIP scores. Therefore, additional steps were undertaken to select the most representative bin per metabolite to progress through the analysis pipeline.

3.8.5 iv) Correlation Reliability Score (CRS)

Depending upon their molecular structure, some metabolites may have multiple NMR signals. To address the problem of selecting an appropriate representative bin for a specific metabolite, a correlation reliability score (CRS) method was employed (Grosman, 2020). Multiple signals from a single metabolite should theoretically yield a high correlation score. However, some areas of the NMR spectra are populated by peaks belonging to numerous metabolites. Therefore, some bins may be more representative markers for a metabolite than others. Given this challenge, bins of the same metabolite were correlated and scored to determine their reliability in reporting on the assigned metabolite. The CRS score is determined using the following algorithm:

1. Calculate the Pearson correlation matrix for all the identified bins per metabolite.
2. For each unique metabolite extract, individual bin correlation values.
3. Calculate the mean for each bin of the unique metabolite.
4. Multiply each score by 100 to present the percentage.

To separate candidate representative bins from non-candidate bins, previously calculated CRS scores were used to generate a passing score in the following manner:

1. Exclude all bins with a 100% CRS (single peak metabolites)
2. Calculate median and standard deviation with the remaining scores.
3. CRSpass = median - standard deviation (SD)

4. A CRS above the threshold represents a high correlation of the bin to the rest of the signals of the same metabolite.

Finally, the highest CRS scores of non-overlapping bins (where applicable) were selected so that only representative metabolites were used for univariate and pathway analyses.

3.8.5 v) Univariate Analysis

Depending on the study design, univariate analyses were performed using Welch's t-test or two-way analysis of variance (ANOVA) to compare the means of selected metabolites. To account for type-I errors arising from multiple hypothesis testing, *P*-values were corrected via Benjamini & Hochberg (BH) (Benjamini and Hochberg, 1995) method unless otherwise stated. When ANOVA was used, Post hoc analysis was performed to establish the group(s) responsible for any differences identified by the ANOVA.

3.8.5 vi) Metabolite Set Enrichment Analysis (MSEA) and Interpretation

Upon selecting metabolites through PLS-DA modelling, a qualitative metabolic set enrichment analysis (MSEA) was used based on Fisher's exact test (Xia and Wishart, 2010). MSEA provides a probability measure for a set of metabolites' likelihood of representing a pathway in a system. Given both the qualitative nature of this analysis and metabolomics showing a 'metabolic snapshot', it is impossible to annotate a pathway as being increased/decreased or up-regulated/down-regulated. MSEA's sole purpose is to provide possible leads on pathways to be explored and discussed further, considering complimentary data and/or further research. This thesis performed pathway analysis using metabolite sets from the KEGG database. Identified metabolite names were used to calculate the probability of

individual pathways via a one-sided Fisher's exact test. The resulting *P*-values were adjusted for Type I errors with BH adjustment, and pathways with *P*-values less than 0.05 were presented as significant and discussed further.

Chapter 4 - Remote Prescription of 3-Weeks "Sleep Low" Training and Nutritional Intervention Improves Functional Threshold Power in Trained Cyclists and Triathletes.

This work is published work in PLoS One (2021).

Bennett S, Tiollier E, Brocherie F, Owens DJ, Morton JP, Louis J (2021) Three weeks of a home-based "sleep low-train low" intervention improves functional threshold power in trained cyclists: A feasibility study. PLoS ONE 16(12): e0260959.

https://doi.org/10.1371/journal.pone.0260959

4.1 Introduction

Muscle glycogen can mediate cell signalling pathways associated with endurance training adaptation (Philp, Hargreaves and Baar, 2012), inducing an augmented muscle transcriptional response when exercise is completed under conditions of reduced muscle glycogen availability (Pilegaard et al., 2002; Bartlett et al., 2013; Psilander et al., 2013; Lane et al., 2015; Stocks et al., 2019). Indeed, compared with loaded glycogen stores, training with reduced muscle glycogen has been shown to increase AMPK activity due to decreased AMPK-glycogen binding (Polekhina et al., 2005; McBride et al., 2009). AMPK, therefore, acts as a cellular energy sensor, up-regulating PGC-1α activity and expression (Canto and Auwerx, 2009; Philp et al., 2011; Philp, Hargreaves and Baar, 2012), a transcriptional co-activator often touted as the master regulator of mitochondrial biogenesis (Wu et al., 1999; Lin et al., 2002; Lin, Handschin and Spiegelman, 2005), a key hallmark of endurance training adaptation (Holloszy, 1967; Holloszy et al., 1970; Holloszy and Coyle, 1984). Concomitant with these adaptations, the increased adipose tissue lipolysis and increased circulating fatty acids for energy supply during exercise with low glycogen availability up-regulates peroxisome proliferator-activated receptor (PPARδ) transcription factor (Pilegaard et al., 2005), increasing the expression of proteins involved in lipid metabolism. Such metabolic adaptation may improve performance during prolonged sub-maximal steady-state exercise via sparing glycogen stores for later use (Stellingwerff et al., 2007; Hearris et al., 2018). Accordingly, over the last decade, various exercise-dietary carbohydrate periodisation strategies (*i.e.*, twice-a-day training, fasted training, withholding carbohydrate intake between exercise sessions) to train with low muscle glycogen (coined as “train low”) have been tested in athletes (2008a; Yeo et al., 2008b; Hulston et al., 2010; Psilander et al., 2013; Lane et al., 2015; Marquet et al., 2016a;

Gejl et al., 2017; Riis et al., 2019; Burke et al., 2020). However, despite growing evidence of the molecular adaptation triggered by such "train low" strategies, the translation to improved physical performance remains limited (Yeo et al., 2008b; Morton et al., 2009; Hulston et al., 2010; Gejl et al., 2017).

In nutrition and exercise prescription for athletes, an amalgamation of multiple "train low" strategies appears optimal as it can be tailored to individual requirements throughout a training cycle (Stellingwerf, 2012; Stellingwerff, Morton and Burke, 2019). A popular example of this approach is the so-called "sleep low-train low" strategy, which includes three different training-nutrition interventions: high-intensity training (HIT) in the evening to deplete glycogen stores, followed by low carbohydrate availability overnight (i.e., sleeping low), and low-intensity training (LIT) the following morning under conditions of low muscle glycogen/carbohydrate availability. The "sleep low-train low" model seems particularly adapted to athletic populations because the timing of exercise and carbohydrate restriction minimises waking hours and maximises the duration under low carbohydrate conditions, potentially maximising the adaptive response (Pilegaard et al., 2005; Hawley, 2013). Marquet et al. (2016a) advanced the "sleep low-train low" model to reflect closer "real world training practices" as carbohydrate availability is periodised to suit specific exercise session demands. In a cohort of trained triathletes, these researchers observed that a 3-week "sleep low-train low" intervention improved body composition (-1.1% fat mass), 10-km running performance (2.9 % decrease in time) and submaximal cycling efficiency (+12.5%) when compared to training in conditions of consistently high carbohydrate availability. Implementing a similar "sleep low-train low" design over 4 weeks, Riis et al. (2019) reported no superior effects on endurance performance in cyclists compared to a control condition. Whilst both studies provided comprehensive pre-and post-intervention performance data, each failed to report the sessional

impact (i.e., ability to complete the desired workload) of training chronically with periodised carbohydrate availability. Given that reduced carbohydrate availability negatively impairs exercise capacity and may impact session quality (Lane et al., 2015; Impey et al., 2016; Hearris et al., 2019), it is essential to characterise further the daily training response associated with low carbohydrate availability.

Laboratory-based training studies are often limited by logistical constraints and burdening participants who must report daily to the research facility. Additionally, some sessions are often completed away from the laboratory environment, thus reducing the opportunity for data collection by the research team. Within this context, home-based studies that allow participants to train at home and collect their data present an alternative to researchers. Several commercially available online training platforms are available to athletes and coaches (TrainingPeaks, Golden Cheetah, Strava), allowing athletes to record and upload exercise data and provide general and specific subjective feedback for each training session, ultimately allowing the coach to monitor the training programme effectiveness without meeting the subject in person. The interest in this training monitoring solution has grown significantly among athletes and coaches in 2020 (likely due to the worldwide COVID-19 pandemic), with a 400% increase in virtual sessions uploaded to the TrainingPeaks (TrainingPeaks, LLC. CO, USA) platform in May 2020 compared to 2019.

The present study aimed to assess the feasibility of undertaking a 3-week home-based "sleep low-train low" programme and measure its effects on cycling performance in trained athletes. The current chapter hypothesises that the battery of home-based performance tests will be reliable, and performance will be improved in the Sleep Low (SL) group compared to the control group in line with current sleep low literature.

4.2 Methods

4.2.1 Participants and Ethical Approval

Following an online social media recruitment drive, seventy-one trained cyclists and triathletes initially volunteered to participate in the present study. A priori sample size estimation indicated a minimum sample size of 21 participants was required to detect a small to moderate effect (0.25) for mean power during performance tests with 80% statistical power using the G*Power (v 3.1) software. Ten participants withdrew, citing changes in work, lockdown conditions and illness. Six participants were excluded from the study for failing to adhere to the training programme as prescribed (100% session completion was required). Consequently, fifty-five (47 males and 8 females) trained cyclists and triathletes were considered for the analysis. Participants were classified as trained based on training status and modelled $\dot{V}O_{2max}$ PPO based upon a linear regression model of FTP and P_{max} (Denham et al., 2020) against previous characterisation frameworks (Jeukendrup, Craig and Hawley, 2000; De Pauw et al., 2013; Decroix et al., 2016). Participants were matched based on sex, age, and functional threshold power (FTP) before being randomly assigned to either sleep low (SL, n = 28) or control (CON, n = 27) groups using a simple random allocation approach (Kim and Shin, 2014). All participants self-reported that they were in good health, had a minimum of 2 years' experience in either cycling or triathlon and had a minimum of 10 h of weekly training volume in the year preceding the study. Participants' characteristics are summarised in Table 4.1. The experimental protocol was approved by the Liverpool John Moores University research ethics committee (Ethics number 20/SPS/019) and performed in line with the declaration of Helsinki. After comprehensive written and verbal (online) explanations of the study, participants gave their informed consent for participation.

Table 4.1 – Participants' characteristics in the Sleep low and control Groups. 12:3 male to female ratio in each group. (mean ± SD).

	Sleep low (SL)	**Control (CON)**
	(n = 28)	(n = 27)
Age (years)	32 ± 8	32 ± 8
Height (cm)	177 ± 6	177 ± 9
Weight (kg)	75 ± 18	77 ± 19
FTP (W)	255 ± 53	258 ± 52
Hours of training ($h \cdot wk^{-1}$)	12 ± 3	11 ± 4

FTP, Functional Threshold Power

4.2.2 Experimental Design

In progressing towards greater real-world application, all exercise and dietary interventions were prescribed and monitored remotely using a commercially available online training platform (TrainingPeaks Inc, CO, USA). Performance measures were typical of field-based tests employed by coaches in practice. During government-enforced confinement in 2020, a remote prescription training study was implemented utilising a matched pairs design. Following an initial familiarisation, both groups completed two pre-and post-performance tests, with pre-tests completed twice in the same week to assess test battery reliability. Pre- and post-tests were separated by an identical 3-week exercise intervention, which consisted of an evening HIT session, followed by a LIT session the next morning, three times per week. Both groups had the same daily carbohydrate intake (~ 6 $g \cdot kg\ BM^{-1} \cdot d^{-1}$) but timed differently between groups to achieve low carbohydrate availability around specific training sessions in SL and normal carbohydrate availability in CON. This cycle was repeated three times during four consecutive days before participants could resume their usual dietary intake as recorded before the study (described later in "*4.3.4 i) Nutritional Protocol*").

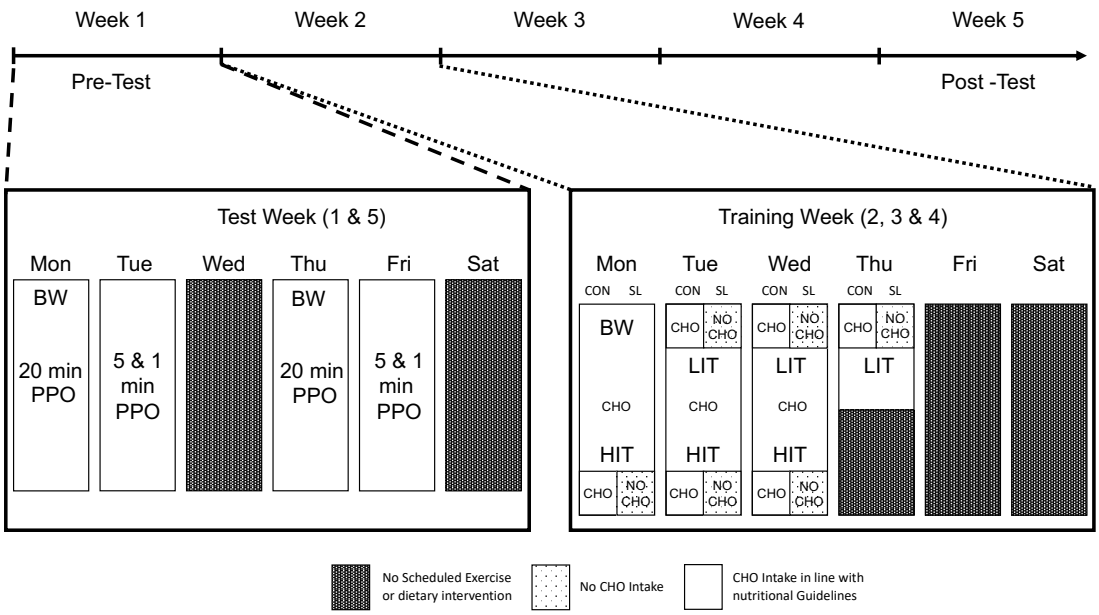

Figure 4.1– Timeline and schematic overview of the study. CHO refers to carbohydrate.

4.2.3 Performance Tests

To mirror the reality of online coaching and training practices, standard field/home-based cycling tests were used to assess performance (*i.e.*, 20-min, 5-min and 1-min peak power output [PPO]) (Allen, 2019) via online software (TrainingPeaks, CO, USA). Participants were asked to complete the exercise tests at the same time each day, having consumed the same food and fluid intake, abstained from alcohol, caffeine, and vigorous exercise for 24 hrs prior. A standardised meal (CHO: 2.0g·kg·BM, PRO: 0.3g·kg·BM, fat: 0.3g·kg·BM) 2 hrs before the test and water consumption was allowed *ad libitum* during exercise tests. Upon completion, participants uploaded power data to the online software to be analysed later.

4.2.3 i) Assessment of Functional Threshold Power (FTP)

The protocol to assess functional threshold power (FTP) has already been described in detail (Chapter 3.3.1). Briefly, all participants completed a standardised warm-up followed by

a maximal 20-min self-paced capacity test, during which they were advised to maintain the highest possible power for the test duration. FTP (W) was calculated as 95% of the mean power achieved for 20 min (Allen, 2019). In light of this study being completed remotely, all participants completed the test at home and FTP was used to standardise relative exercise intensity for training sessions in place of $\dot{V}O_{2max}$ (Denham et al., 2020).

4.2.3 ii) Assessment of 1-min and 5-min Peak Power Output (PPO)

The protocol employed to assess 5-min and 1-min peak power output has already been described in detail (Chapter 3.3.2). Participants completed the same standardised warm-up as before the FTP test, after which they were asked to complete the 1-min maximal cycling effort, aiming to achieve the highest mean power possible. Next, participants completed a 10-min active recovery at a self-selected intensity and then the 5-min maximal effort. As with the FTP test, a familiarisation session was prescribed the week before the test to all participants for an attempt at maximal efforts before the pre-test. The tests were repeated 4 and 7 days after the final training session (Post-tests) to allow adequate recovery from the training load.

4.2.4 Carbohydrate Periodisation & Training Programme

4.2.4 i) Nutritional Protocol

During the familiarisation week, participants recorded dietary intake using a nutrition analysis application (MyFitnessPal inc, CA, USA). Participants weighed their food before preparation or cooking and input the food and quantities into the nutrition application daily. Macronutrient intake was automatically synchronised to the online training platform. This familiarised the nutritional monitoring used during the study, with the final 4 days used to calculate baseline dietary intake. Participants followed prescribed meal plans that consisted of

the same total daily macronutrient intake, albeit the timing of carbohydrate intake was altered to manipulate carbohydrate availability for specific exercise sessions. Total daily carbohydrate was the same for each group (6 g·kg $BM^{-1}·d^{-1}$), but the intake was distributed differently throughout the day to achieve either low (SL) or normal (CON) carbohydrate availability around specific training sessions (Table 4.2). Across the 4 days per week that the "sleep low-train low" model was implemented, carbohydrate consumption was prohibited between completing HIT and LIT sessions in SL. No carbohydrate was consumed during any training session; to maintain satiety, participants were allowed to consume high-protein food sources such as lean meat or whey protein powder in line with daily macronutrient intake. A high carbohydrate diet was consumed upon completion of LIT sessions to replenish muscle glycogen before the following HIT session. For CON, carbohydrate availability was maintained throughout the day, including the post-HIT session and before the completion of the LIT session. All participants were instructed to consume 2 $g·kg^{-1}$ $BM·d^{-1}$ of protein evenly distributed across the day to maintain muscle protein synthesis. To ensure that the meal plan was followed, nutritional intake was monitored across the 4 consecutive days of exercise-nutrition intervention.

4.2.4 ii) Training Protocol

Following performance tests, all participants completed a 3-week online-supervised training programme. Throughout three consecutive weeks, participants followed identical exercise programmes (Table 4.2), but each group followed a different nutritional intervention (subsequently described in detail). The training programme consisted of six sessions over 4 days, including HIT sessions in the evenings and LIT the following mornings. Training intensity was standardised across all participants and prescribed relative to FTP. Low-intensity

training sessions consisted of 60 min of cycling at 75% FTP (mean ± SD for all participants: 192 ± 41 W), whilst HIT sessions consisted of 6 × 5 min cycling at 105% FTP (269 ± 58 W) interspersed with 5-min recovery at 55% FTP (141 ± 30 W). Eight 5-min bouts at high intensity with 60 s recovery (Stepto et al., 2001) have shown to be effective at significantly reducing muscle glycogen content (~50%) and have previously been implemented in a "sleep low-train low" intervention (Marquet et al., 2016a). All sessions were structured on the online training platform and could be exported and completed on third-party applications in ergometer mode on the participants' home trainers. Additional LIT exercise was permitted on the other 3 days of each week but was limited to 1-1.5 h per day for a total weekly training volume of 10-15 h, and data for these sessions were monitored. Participants performed all the exercise sessions at home, using their equipment and recording their data. Power output was available for all participants via a power meter or smart trainer, and heart rate (HR) was recorded by those who had a thoracic HR belt (n = 48; SL = 25, CON = 23) which were either Polar (Polar Electro Oy, Kempele, Finland) (n = 21) or Garmin (Garmin International, Kansas City, MO) (n = 27) HR monitors. Reported Heart rate data is a percentage of predicted maximal HR calculated as Tanaka (2001) described. Participants reported their power meter brand and model to the research team and were instructed to complete a factory calibration for power meters and spin-down calibration for home trainers at the start of each week of the study. Characteristics of power meters (including brand, location of the strain gauge, and data accuracy) utilised by participants are reported in Table 4.3.

Immediately post-exercise, participants uploaded data files to the online platform and rated their overall perceived exertion for the session within 15 mins of exercise completion. For ease of understanding for participants, a BORG CR-10 scale (described in detail in Chapter 3.4.1) (Zamuner et al., 2011) was used in line with the in-house RPE scale in the online

platform, supplemented with an electronic version of the Borg CR-10 scale, including written cues for the level of exercise (Borg, 2001). All participants' training sessions were prescribed at the start of the study and overseen by a central coach account where data could be exported for analysis.

Table 4.2 – Overview of prescribed exercise (in bold) and carbohydrate intake ($g \cdot kg^{-1} \cdot BM^{-1}$) during 1-week intervention for Sleep low and control groups.

TIME	DAY 1		DAY 2		DAY 3		DAY 4		DAY 5-7	
GROUP	CON	SL	CON	SL	CON	SL	CON	SL	CON	SL
MORNING (BEFORE 10AM)	Breakfast (2 g.kg^{-1})		Breakfast (2 g.kg^{-1}) **LIT**	**LIT** (Fasted) Breakfast (2 g.kg^{-1})	Breakfast (2 g.kg^{-1}) **LIT**	**LIT** (Fasted) Breakfast (2 g.kg^{-1})	Breakfast (2 g.kg^{-1}) **LIT**	**LIT** (Fasted) Breakfast (2 g.kg^{-1})		
MIDDAY	Lunch (1.5 g.kg^{-1})	Lunch (2 g.kg^{-1})	Lunch (1.5 g.kg^{-1})	Lunch (2 g.kg^{-1})	Lunch (1.5 g.kg^{-1})	Lunch (2 g.kg^{-1})	Usual Diet		1 free **LIT** session per day	
AFTERNOON (BEFORE 5PM)	Snack (0.5 g.kg^{-1})	Snack (2 g.kg^{-1})	Snack (0.5 g.kg^{-1})	Snack (2 g.kg^{-1})	Snack (0.5 g.kg^{-1})	Snack (2 g.kg^{-1})			Usual diet	
EVENING (AFTER 5PM AND BEFORE 9PM)	**HIT**									
	Dinner (2 g.kg^{-1})	Dinner (0 g.kg^{-1})	Dinner (2 g.kg^{-1})	Dinner (0 g.kg^{-1})	Dinner (2 g.kg^{-1})	Dinner (0 g.kg^{-1})				

HIT, high intensity training session; LIT, low intensity training session.

Table 4.3 – Distribution of power meter utilisation by participants and literature to support validity and reliability of each device.

Power meter brand	Model	Count	Strain Gauge location	Claimed Accuracy*	Literature for Validity and Reliability
Wahoo	Kickr Kickr Core	10 6	Home Trainer	1.50%	(Hoon, 2016)
TACX	Neo Pro Neo Lite	8 5	Home Trainer	1%	NA
Power tap	P1	6	Pedal	1.50%	(Miller, 2015; Czajkowski, 2016; Bouillod et al., 2017; Maier et al., 2017; Pallares and Lillo-Bevia, 2018; Schneeweiss, 2018)
Stages		6	Crank	1.50%	(Hurst, 2015; Miller, 2015; Bouillod et al., 2017; Maier et al., 2017; Costa, 2019)
Assioma		4	Pedal	1%	(Montalvo-Perez et al., 2021)
Wattbike	Pro	4	Home Trainer	2%	(Wainwright, Cooke and O'Hara, 2017)
Quarq		4	Crank	1.50%	(Miller, 2015; Maier et al., 2017)
Garmin Vector	V2	2	Pedal	1%	(Novak, 2016; Bouillod et al., 2017; Maier et al., 2017)

** Manufacturer's claimed power meter accuracy.*

4.2.5 Statistical Analysis

All data were assessed for normality via the Shapiro-wilk test (Shapiro and Wilk, 1965). Differences between repeated trials were identified using within-participants t-tests for each measure (20-min, 5-min, 1-min PPO tests [mean power and HR] and FTP) with comparisons of reliability completed by calculating the mean difference, effect size (Cohen's *d*), coefficient of variation (CV), typical error of the mean (TEM) and intraclass correlation (ICC) using a spreadsheet provided by Hopkins (Hopkins, 2015). Simple linear regressions were fitted for paired data, including test and retest, and the coefficient of determination (r^2) was calculated. Linear regressions were performed between 20-min, 5-min, and 1-min PPO tests and calculated FTP.

Between-group comparison via independent t-test to investigate the effect of the intervention between experimental groups (SL vs. CON). A repeated measure two-way (group × time) ANOVA was used to assess the effects of the dietary strategy (SL vs. CON) and time (pre vs. post, and week 1 vs. week 2 vs. week 3) on performance outcomes (20-min, 5-min, 1-min PPO tests) and training responses (HR, RPE). Bonferroni multiple comparisons tests were performed when a significant effect was identified. Degrees of freedom were adjusted using the Greenhouse-Geisser correction when violations of sphericity were present. Cohen's *d* coefficient for effect size was calculated and referenced against benchmarks suggested by Cohen (Cohen, 1988a), where *d* is considered small, medium, and large for 0.2, 0.5 and 0.8 values, respectively. All data are presented as mean ± SD unless otherwise stated. The level of significance was set at $P < 0.05$. Data analysis was completed using Graphpad Prism v.9 (Graphpad Software, CA, USA).

4.3 Results

4.3.1 Test-Retest Reliability

To understand the reproducibility of each performance test used during the study, participants completed repeated testing sessions 3 days apart. The coefficient of variation (CV) was calculated between tests and mean, and median CVs were calculated for all participants during each test (n=55). Based on the current results, all CV results are below 5% and would therefore be considered acceptable (Campbell, 2010). Reliability data for power variables and HR are reported in Table 4.4. There was no significant difference between test and retest for mean power during 20-min ($t(108) = 0.51$, $P = 0.61$), 5-min ($t(108) = 0.50$, $P = 0.62$) and 1-min tests ($t(108) = 0.64$, $P = 0.53$), estimates of FTP ($t(108) = 0.52$, $P = 0.60$). HR was not significantly different between test and retest in 20-min ($t(94) = 0.68$, $P = 0.49$), 5-min ($t(92) = 0.95$, $P = 0.34$) and 1-min PPO tests ($t(92) = 0.78$, $P = 0.45$). All reliability data and statistical analysis is summarised in Table 4.4.

Table 4.4 – Mean power output, heart rate data across first and second re-tests, and reliability statistics between re-tests.

Measures	Test 1[a]	Test 2[a]	Mean[a]	Mean Diff (W)[a]	Mean Diff (%)[a]	*d*	CV[a]	TEM (W)[b]	TEM (CV, %)[b]	ICC[b]	r^2	Bias (W)[c]
20 min MPO												
Power (W)	266 ± 55	271 ± 55	269 ± 55	4.18 ± 5.92	1.66 ± 2.42	0.08	1.52 ± 1.45	4.2 (3.6-5)	1.7 (1.5-2.1)	0.994 (0.991-0.996)	0.995	4.182 (-7.414 – 15.78)
Heart Rate (Beats·min^{-1})	170 ± 10	171 ± 11	170 ± 10	-0.11 ± 2.27	-0.08 ± 1.43	0.01	0.85 ± 0.54	1.7 (1.5-2.1)	1 (0.9-1.2)	0.977 (0.963-0.986)	0.974	- 1.458 (-5.272 – 2.355)
5 min MPO												
Power (W)	310 ± 64	314 ± 65	312 ± 64	3.91 ± 7.4	1.23 ± 2.41	0.06	1.4 ± 1.31	5.2 (4.5-6.2)	1.7 (1.5-2)	0.994 (0.991-0.996)	0.994	3.909 (-10.60 – 18.42)
Heart Rate (Beats·min^{-1})	171 ± 10	169 ± 11	170 ± 10	1.76 ± 3.18	1.21 ± 2.02	0.17	1.04 ± 1.38	2.4 (2-2.9)	1.5 (1.3-2.3)	0.944 (0.91-0.965)	0.944	-2.064 (-8.633 – 4.506)
1 min MPO												
Power (W)	441 ± 123	448 ± 127	445 ± 125	7.11 ± 22.31	1.42 ± 4.61	0.06	2.36 ± 2.53	15.8 (13.7-18.8)	3.4 (2.9-4)	0.989 (0.982-0.993)	0.988	7.109 (-36.63 – 50.85)
Heart Rate (Beats·min^{-1})	164 ± 13	164 ± 13	164 ± 12	-0.24 ± 4.02	-0.19 ± 2.61	0.02	1.48 ± 1.08	3.1 (2.6-3.7)	1.9 (1.6-2.3)	0.944 (0.91-0.965)	0.943	-2.021 (-9.585 – 5.542)
FTP												
Power (W)	253 ± 52	257 ± 52	255 ± 52	3.97 ± 5.62	1.66 ± 2.42	0.08	1.52 ± 1.45	4 (3.4-4.7)	1.7 (1.5-2.1)	0.994 (0.991-0.996)	0.995	-3.973 (-14.99 – 7.044)

[a] Data reported as mean ± standard deviation
[b] Values in parentheses represent 95% Confidence intervals
[c] Values in parenthesis represent 95% limits of agreement
MPO, Mean power output; FTP, Functional Threshold Power; CP, Critical Power; d, effect size; TEM, Typical error of the mean; CV, coefficient of variation; ICC, intraclass correlations; r^2, Pearson's correlation coefficient

4.3.2 Training Response

The total weekly training volume w for each group was 12.9 ± 0.7 h and 13.4 ± 1.2 h in SL and CON, respectively ($t(53) = 1.90$, $P = 0.06$ in line with the instruction to maintain typical weekly training hours and represented only a small increase from habitual training loads. SL completed an additional 1h of exercise per week ($F(1, 106) = 2.97$, $P > 0.05$), and CON completed an additional 0.8 hrs ($P > 0.05$).

Mean absolute power output across all LIT (SL: 192 ± 40W; CON: 194 ± 39W) and HIT (SL: 269 ± 56W; CON: 271 ± 54W) sessions were not different over time ($F(2.9, 153.8) = 1.32$, $P = 0.26$, $d = 0.0006$) or between groups ($F(1, 53) = 0.64$, $P = 0.43$, $d = 0.01$). However, power output normalised as a percentage of FTP (Figure 4.2A) resulted in systematically lower power output in SL than CON ($F(1, 53) = 10.82$, $P = 0.0018$, $d = 0.13$). SL power output was lower in sessions 1 (SL: 72 ± 4 % *vs*. CON: 76 ± 3%, $P \leq 0.01$, $d = 1.08$), 2 (SL: 72 ± 5% *vs*. CON: 75 ± 2%, $P = 0.01$, $d = 0.93$), 3 (SL: 72 ± 4% *vs*. CON: 75 ± 2%, $P = 0.01$, $d = 0.92$) and 9 (SL: 72 ± 4% *vs*. CON: 75 ± 3%, $P = 0.01$, $d = 0.95$). There was no between-group difference ($F(1, 477) = 2.06$, $P = 0.15$, $d = 0.004$) in PPO (expressed as % FTP) during high-intensity intervals of HIT sessions, nor significant effect of time across the intervention ($F(8,477) = 1.08$, $P = 0.38$, $d = 0.02$) (Figure 4.2B).

Mean HR similarly decreases during the intervention ($F(5.27, 237.4) = 3.4$, $P = 0.0047$, $d = 0.01$) in SL (session 1: 144 ± 10; session 9: 139 ± 9 to beats.min^{-1}, $d = 1.12$) and CON (session 1: 145 ± 14; session 9: 142 ± 14 beats.min^{-1}, $d = 1.05$) with no difference between groups ($F(1, 45) = 0.08$, $P = 0.78$, $d = 0.02$) (Figure 4.2C). No time ($F(4.515, 203.2) = 1.52$, P

= 0.19, d = 0.003) or group (F(1, 45) = 1.27, P = 0.27, d = 0.02) differences in mean HR were observed for each HIT session (Figure 4.2D).

RPE was significantly higher in the SL compared to CON during LIT sessions (F(1, 53) = 29.11, P < 0.0001, d = 0.16). Differences were observed between groups for sessions 1 (SL: 7.2 ± 0.8 *vs*. CON: 5.8 ± 1.5, P < 0.01, d = 1.29), 2 (SL: 7.4 ± 1 *vs*. CON: 6.2 ± 1.1, P < 0.01, d = 1.18), 3 (SL: 7.3 ± 1.0 *vs*. CON: 6.2 ± 1.1, P < 0.01, d = 0.89), 6 (SL: 6.8 ± 0.8 *vs*. CON: 6.2 ± 1.1, P = 0.04, d = 0.69), and 9 (SL: 7.0 ± 1.2 *vs*. CON: 5.8 ± 1.1, P < 0.01, d = 1.06). There was no effect of time (F(5.89, 312.2) = 1.22, P = 0.30 d = 0.01) on RPE during LIT sessions (Figure 4.2E). During HIT, RPE was significantly higher in the SL than CON F(1, 53) = 16.98, P = 0.0001, d = 0.06). Differences between groups were present for sessions 3 (SL: 7.2 ± 0.8 *vs*. CON: 5.9 ± 1.5, P < 0.01, d = 1.16), 6 (SL: 8.5 ± 0.8 *vs*. CON: 6.4 ± 1.3, P < 0.01, d = 2.04) and 9 (SL: 8.6 ± 0.8 *vs*. CON: 7.5 ± 0.9, P < 0.01, d = 1.31). RPE decreased (P < 0.01) over HIT sessions in week 1 in both groups (Figure 4.2F). RPE during HIT sessions then increased (all P < 0.01) in SL in week 2 (from 8.1 ± 0.5 to 8.5 ± 0.6 au., d = 0.43) and week 3 (from 7.9 ± 0.7 to 8.6 ± 0.8 au., d = 0.58) whilst CON decreased RPE (all P < 0.01) in a stepwise manner in week 1 (d = 1.67), week 2 (d = 1.17) in week 2, and week 3 (d = 0.46). A significant group × time interaction effect (F(8, 424) = 13.71, P < 0.0001, d = 0.1) and an effect of time (F(5.74, 304.4) = 29.46, P < 0.0001, d = 0.23) were observed.

Body mass decreased from Pre- to Post-intervention in SL (Pre: 74.7 ± 10.7; Post: 73.6 ± 10.5 kg, P < 0.01, d = 0.11), whereas no significant change was recorded in CON (Pre: 74.1 ± 9.5; Post: 74.1 ± 9.5 kg). There was a significant group × time interaction effect (F(1, 54) = 32.49, P < 0.0001, d = 0.0007) observed alongside a significant effect of time (F(1, 54) = 34.78, P < 0.0001, d = 0.00007).

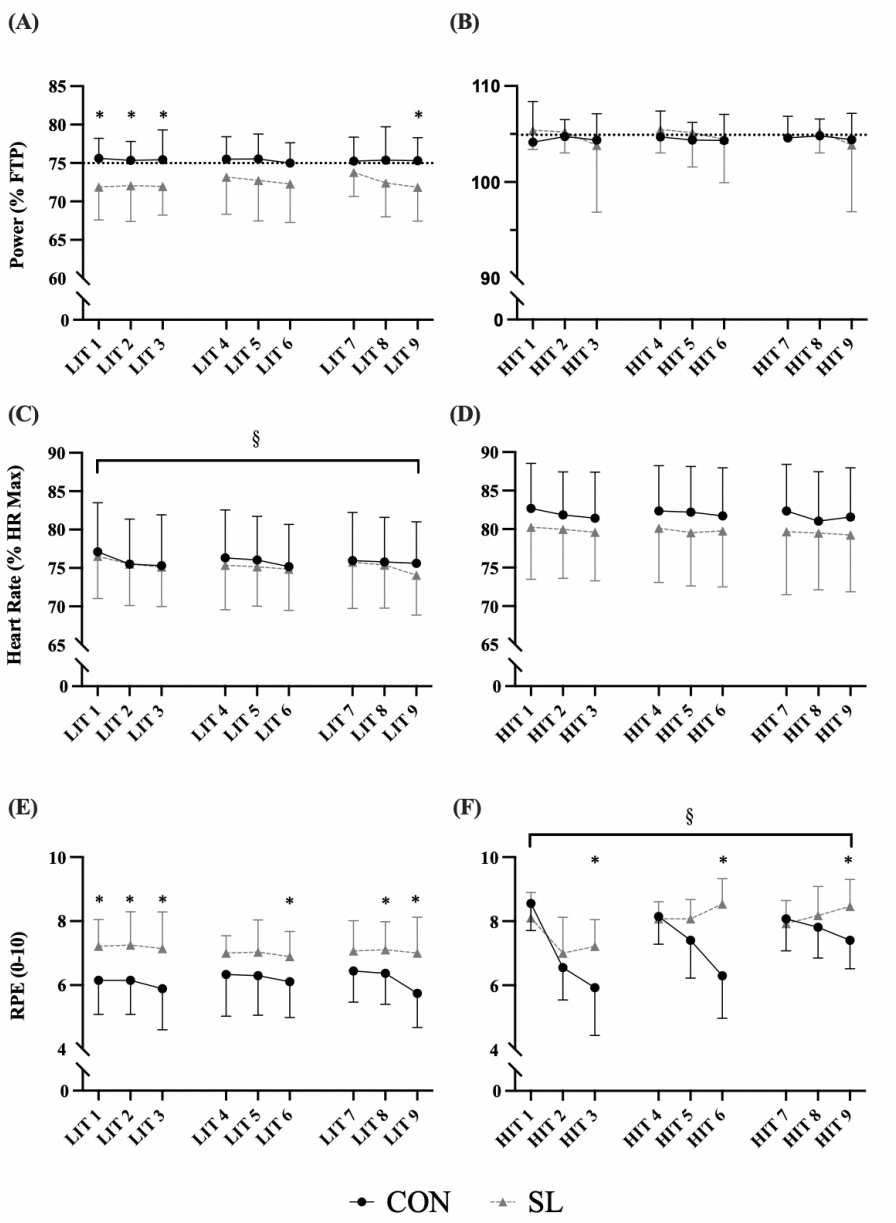

Figure 4.2 – Training data recorded during each low- and high-intensity training session (LIT and HIT, respectively). (A) relative power output with the dotted line representing the 75% FTP target intensity for LIT sessions; (B) relative power output with a dotted line representing 105% FTP target intensity for HIT sessions; (C) Heart rate for LIT sessions; (D) Heart rate for HIT sessions; (E) RPE for LIT sessions; (F) RPE for HIT sessions. Grey triangles and black circles represent mean responses for sleep low (SL) and control (CON), respectively. All data are presented as mean ± SD. * denotes significant between-group difference, § denotes a significant time effect. $P < 0.05$ for all significant differences.

4.3.3 Performance Outcomes

4.3.3 i) 20-min Peak Power Output (PPO) and Functional Threshold Power (FTP)

Following the intervention, 20-min PPO was increased in both groups (SL: +4.0 ± 3.2%, CON: +1.2 ± 3.5%) ($F(1,53) = 30$, $P < 0.0001$, $d = 0.01$). A significant interaction effect was observed ($F(1,53) = 9.7$, $P = 0.003$, $d = 0.01$) with SL increasing to a greater extent (Pre: 271 ± 56; Post: 282 ± 59 W, $P < 0.01$, $d = 0.18$) compared to CON (Pre: 272 ± 54; Post: 275 ± 54 W, $P = 0.19$ $d = 0.05$) (Figure 4.3A). FTP was enhanced by 5.5 ± 2.6% (Pre: 3.43 ± 0.61; Post: 3.61 ± 0.63 W·kg^{-1}, $P < 0.01$, $d = 0.34$) in SL, whereas it was only enhanced by 1.3 ± 3.6% (from 3.49 ± 0.82 to 3.46 ± 0.8 W·kg^{-1}, $P = 0.10$, $d = 0.05$) in CON (figure 4.3B). The HR response during the 20-min PPO test was not altered following the intervention ($F(1, 46) = 1.8$, $P = 0.19$, $d = 0.034$) both in SL (Pre: 171 ± 9; Post: 171 ± 9 beats·min^{-1}) and CON (Pre:171 ± 12; Post: 169 ± 14 beats·min^{-1}).

4.3.3 ii) 5-min Peak Power Output (PPO)

Both groups increased their 5-min PPO following the intervention by +2.3 ± 2.7% and +2.6 ± 4.9% in SL (Pre: 319 ± 69; Post: 327 ± 74 W, $P < 0.01$, $d = 0.10$) and CON (Pre: 311 ± 60; Post: 319 ± 62 W, $P < 0.01$, $d = 0.13$), respectively ($F(1, 53) = 24.24$, $P < 0.01$, $d = 0.03$) with no significant difference between groups ($F(1, 53) = 0.21$, $P = 0.65$, $d = 0.04$) (Figure 4.3C). Mean HR during the 5-min PPO test was unchanged following the intervention ($F(1, 45) = 0.25$, $P = 0.61$, $d = 0.001$) with no difference between SL (Pre: 170 ±11; Post: 170 ± 10 beats·min^{-1}) and CON (Pre: 171 ± 11; Post: 171 ± 11 beats·min^{-1}) ($F(1,45) = 0.0002$, $P = 0.99$, $d = 0.0003$)

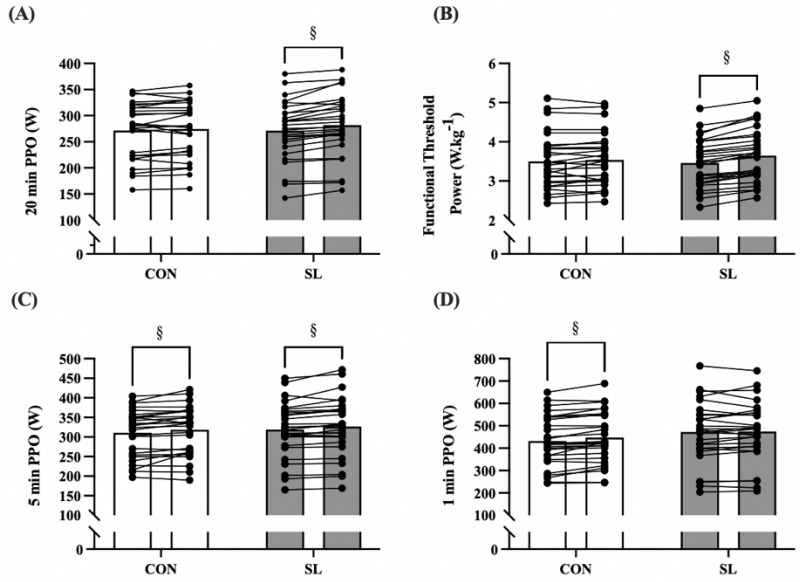

Figure 4.3 – Mean power output recorded during the performance tests performed before (Pre) and after (Post) intervention. (A) mean power output (W) during the 20-min PPO test; (B) mean functional threshold power ($w \cdot kg^{-1}$); (C) mean power output (W) during the 5-min PPO test; (D) Mean power output (W) during the 1-min PPO test. Bars represent means for Sleep low (SL) and control (CON) groups with individual changes represented by connected dots. § denotes a significant difference between Pre and Post. $P < 0.05$ for all significant differences.

4.3.3 iii) 1-min Peak Power Output (PPO)

There was a significant main effect of time ($F(1, 53) = 10.06$, $P = 0.003$, $d = 0.01$) and interaction effect ($F(1, 53) = 5.78$, $P = 0.02$, $d = 0.007$) on 1-min PPO with CON increasing by 3.9 ± 4.9% in CON following the intervention (Pre: 431.7 ± 115.0; Post: 447 ± 116 W, $P < 0.01$, $d = 0.13$), whereas 1-min PPO was unchanged in SL (Pre: 473 ± 136; Post: 475 ± 133 W, $P = 0.31$, $d = 0.01$) (Figure 4.3D). Mean HR during the 1-min PPO test was unchanged following the intervention ($F(1, 45) = 0.7806$, $P = 0.38$, $d = 0.006$) or between SL (Pre: 166 ± 12; Post: 166 ± 12 $beats \cdot min^{-1}$) and CON (Pre: 163 ± 14; Post: 164 ± 14 $beats \cdot min^{-1}$) ($F(1,45) = 0.56$, $P = 0.46$, $d = 0.01$).

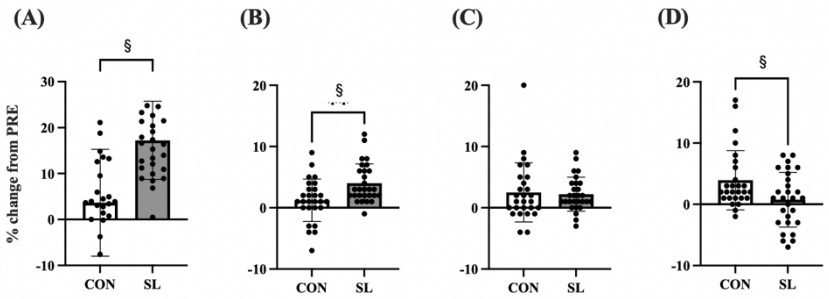

Figure 4.4 – Percentage change from PRE to POST intervention during each performance test. (A) percentage change for FTP. (B) percentage for 20-min PPO. (C) percentage change for 5-min PPO. (D) Percentage change for 1-min PPO. Bars represent means for Sleep low (SL) and control (CON) groups with individual changes represented by connected dots. § denotes a significant difference between Pre and Post. $P < 0.05$ for all significant differences.

4.3.4 Nutritional Intake

Mean energy intake increased significantly during training compared to baseline ($F(1, 53) = 10.06$, $P = 0.003$, $d = 0.04$), in both groups ($F(1, 53) = 0.4602$, $P = 0.5$, $d = 0.006$). Bonferroni post-hoc comparison tests revealed SL significantly increased energy intake (+300 ± 402 kcal·d^{-1}, $P = 0.004$, $d = 0.93$), whereas it was not significant in CON (+151 ± 498 kcal·d^{-1}, $P = 0.41$, $d = 0.59$). Carbohydrate intake increased during training compared to baseline ($F(1,53) = 104.3$, $P < 0.0001$, $d = 0.52$) in both groups ($F(1,53) = 1.64$, $P = 0.21$, $d = 0.006$) with a concomitant reduction in fat intake ($F(1,53) = 181.3$, $P < 0.0001$, $d = 0.57$) in both groups ($F(1,53) = 0.10$, $P = 0.32$, $d = 0.005$). Protein intake was significantly increased from baseline ($F(1,53) = 7.02$, $P = 0.01$, $d = 0.05$) in both groups ($F(1,53) = 2.86$, $P = 0.10$, $d = 0.02$). There was no significant between-group difference for energy and/or macronutrient intake (Table 4.5).

Table 4.5 – Mean daily energy and macronutrient intake for sleep low (SL) and control (CON) groups before the training programme (baseline) and during the training-nutrition intervention.

		Energy (kcal·d^{-1})	**CHO** (g·kg^{-1}·d^{-1})	**Fat** (g·kg^{-1}·d^{-1})	**Protein** (g·kg^{-1}·d^{-1})
CON	**Baseline**	2621 ± 485	3.5 ± 0.7	1.7 ± 0.3	1.7 ± 0.3
	Training	2772 ± 496	5.1 ± 0.9§	1.1 ± 0.1§	1.8 ± 0.3
SL	**Baseline**	2616 ± 495	3.2 ± 0.6	1.7 ± 0.3	1.7 ± 0.2
	Training	2905 ± 426§	5.3 ± 0.7§	1.1 ± 0.3§	1.9 ± 0.2§

§ denotes a significant difference from baseline, with P < 0.05. CHO refers to carbohydrate

4.4 Discussion

The over-arching objective of the present study was to translate the current "Sleep Low-Train low" literature into "real-world" application. To do this, a remote 3-week home-based exercise and nutrition intervention was implemented in endurance-trained. Utilising a commercially available online coaching platform (TrainingPeaks LLC), the day-to-day training responses of participants adhering to a "sleep low-train low" model (SL) or control (CON) strategies were characterised. Despite consistently impaired exercise intensity and higher RPE during LIT sessions, SL improved their 20-min PPO, 5-min PPO, and FTP (normalised to BM), whereas CON only improved 5-min and 1-min PPO. Overall, results support the reliability of home-based exercise testing and provide evidence to show that periodised carbohydrate intake improves aerobic exercise performance.

A 4.0%, 2.3% and 5.5% improvement in 20-min PPO, 5-min PPO, and FTP (w·kg^{-1}) were reported, in SL, whereas CON only improved 5-min PPO and 1-min PPO by 2.6% and 3.9%. Utilising a similar exercise-nutrition intervention, Marquet et al. (2016a) reported improved 10-km running time (-2.9%) and increased supra-maximal cycling (150% maximal aerobic power) time to exhaustion (+11%) in triathletes, showing improvements during both aerobic and anaerobic type exercise. More recently, endurance-trained individuals subject to

four weeks of "sleep low" also improved their 30-min cycling time trial performance (+19% compared to +14% in the control group). However, the between-group difference was insignificant (Riis et al., 2019) due to the small sample size (n = 13) compared to the present study (n = 55). Additionally, differences in training load between studies as training intensities were prescribed either according to maximal HR or power output. Albeit not recorded in the present study, an improved locomotion efficiency has previously been associated with improvements in endurance performance following the "sleep low-train low" model, thus allowing athletes to sustain higher exercise intensities during physical tests (Marquet et al., 2016a; 2016b). Given the correlation between FTP and performance during mass start cycling events, a greater improvement in FTP would be considered a desirable performance outcome for cyclists and triathletes (Sorensen et al., 2019). The intensity and duration of a 20-min effort mean the predominant energy substrates are endogenous carbohydrate stores, specifically skeletal muscle glycogen (Romijn et al., 1993; van Loon et al., 2001). Whilst increased resting muscle glycogen content is frequently observed following chronic low carbohydrate training (Hargreaves, 1997; Hansen et al., 2005; Yeo et al., 2008b; Bartlett et al., 2013; Lane et al., 2015), the duration of the FTP test disallows muscle glycogen depletion as a limiting factor to performance (Areta and Hopkins, 2018). Alternatively, augmented muscle oxidative capacity is a potential mechanism for increased 20-min PPO via improved mitochondrial function and/or biogenesis (Ghiarone et al., 2019; Andrade-Souza et al., 2020). It is well established within the "train-low" literature that periodically completing exercise with low muscle glycogen availability augments post-exercise cell signalling responses characterised by increased AMPK (Wojtaszewski et al., 2003; Steinberg et al., 2006; Yeo et al., 2010; Lane et al., 2015), acetyl-CoA carboxylase (ACC) activity (Lane et al., 2015) and p53 signalling (Bartlett et al., 2013). An augmented molecular response to exercise under conditions of low muscle glycogen leads to elevated PGC-1α mRNA expression (Wojtaszewski et al., 2003;

Pilegaard et al., 2005; Bartlett et al., 2013; Psilander et al., 2013; Lane et al., 2015), and nuclear translocation (Jager et al., 2007). Furthermore, the chronic application of "train-low" has shown an amplified adaptive response with increased markers of mitochondrial adaptation such as citrate synthase, OXPHOS subunit COX IV (Yeo et al., 2008b), 3-hydroxyacyl-CoA dehydrogenase (β HAD) (Hansen et al., 2005; Hulston et al., 2010; Ghiarone et al., 2019), and succinate dehydrogenase (SDH) (Morton et al., 2009) activities suggestive of improved mitochondrial efficiency (Ghiarone et al., 2019). Utilising an alternative carbohydrate periodisation strategy (twice daily), Cochran et al. (2015) showed improved performance following two weeks of training with low carbohydrate availability, despite no changes in mitochondrial protein content, speculating that improved performance may be due to changes in efficiency, which can occur independently of changes in content (Starritt, Angus and Hargreaves, 1999). Considering the acute molecular responses to training with low carbohydrate availability and the improvements in mitochondrial efficiency following the chronic application of "train-low", it is justified to speculate that improvements in muscle oxidative capacity may lead to increased performance following a period of low carbohydrate training. However, the placebo effect of the "sleep low-train low" model cannot be excluded and may also explain part of the performance improvement (Halson and Martin, 2013).

The lack of improvement to the 1-min PPO test in SL contrasts with evidence from previous studies (Lindsay et al., 1996; Marquet et al., 2016a) and may be explained by several factors. Firstly, the lack of supervision during this supra-maximal testing session could have led to a sub-optimal execution (as reflected by a higher CV than the 5-min and 20-min tests) as it is not often performed by endurance athletes (Halson and Martin, 2013). Secondly, a mechanistic rationale exists, whereby pyruvate dehydrogenase kinase 4 (PDK4) expression is increased following a "train low" period, leading to alterations in mitochondrial carbohydrate

flux. PDK4 is an exercise-sensitive gene which encodes the protein PDK (Pilegaard and Neufer, 2004), and mRNA expression is increased following a single bout of "sleep low-train low" (Pilegaard et al., 2002; Bartlett et al., 2013; Psilander et al., 2013; Lane et al., 2015). Crucially, PDK inhibits pyruvate dehydrogenase (PDH), a critical enzyme complex in the regulation of substrate metabolism during exercise. Therefore, it remains to be reasoned that decreased PDH activity would impair carbohydrate flux into the mitochondria, increasing lactate production and limiting performance at supra-maximal exercise intensities (Howlett et al., 1998b). This rationale has been used to justify impaired high-intensity sprint performance following the use of low carbohydrate, high fat (LCHF) nutritional interventions (Burke and Kiens, 2006; Havemann et al., 2006; Stellingwerff et al., 2006) and may be similarly responsible for impaired 1-min PPO in SL. Contrastingly, a previous laboratory-based study utilising the same study design has shown improvements in supra-maximal cycling performance following as little as 1 week (Marquet et al., 2016b) and 3 weeks of sleep low-train low (Marquet et al., 2016a). This highlights the importance of further research to gain greater insight into the performance effect of periodically training with low carbohydrate availability, meaning coaches and nutritionists must consider when to implement low carbohydrate training during an athlete's training programme (Stellingwerf, 2012).

To address the aims of this chapter, a 3-week home-based "sleep low-train low" model where participants monitored their training responses, performances, and dietary intake was conducted. This was possible with the participants using their cycling power meter connected to an online training platform. Due to the expansion of power meters through reduced cost and improvements in their reproducibility (Bouillod et al., 2017), the implementation of power-based training prescription has become increasingly popular among cyclists over the last several years. Using this approach, coaches can consult, analyse, and monitor various

physiological (HR, power, pace/speed, energy expenditure) and perceptual (RPE, overall feeling and wellness) training metrics for multiple athletes simultaneously. Additionally, this approach allowed us to recruit a large number of participants (55 in this study compared to 21 and 13 in Marquet et al. (2016a) and Riis et al. (2019), who used a similar study design, albeit laboratory-based, respectively), and analyse day-to-day responses to the "sleep low-train low" intervention for the first time. Moreover, since this study was conducted during the worldwide COVID-19 pandemic (from April to June 2020), participants were subject to strict National lockdowns where the prescribed training sessions constituted their daily physical activity. Therefore, the data presented in this chapter represents a novel dataset, whereby the reliability of repeated exercise tests in participants' homes using a range of power meters without direct supervision was assessed. The data revealed high test-retest reliability across 20-, 5- and 1-min PPO tests with low coefficients of variation and typical error of measurement (TEM) within 2%, in line with laboratory-based assessments of peak power output (MacInnis, Thomas and Phillips, 2018; Lillo-Bevia et al., 2019; McGrath et al., 2019; Borszcz, Tramontin and Costa, 2020). This reliability data, taken alongside the high ecological validity of the present study, reaffirms the validity of the data presented and the sensitivity of the current testing battery in detecting differences between time points and groups.

Understanding the daily impact of training with reduced carbohydrate availability is essential for coaches, sport scientists and nutritionists alike. Hulston et al. (2010) and Yeo et al. (2008b) have previously reported the effects of training with low carbohydrate availability on HIT session power output across a "train low" programme. The former reported reduced power output across all HIT sessions, whilst Yeo et al. (2008b) reported reduced power output across the first week in the carbohydrate group, trending towards similar exercise intensity between groups after 3 weeks of intervention. In these two studies, participants completed LIT

sessions with high carbohydrate, with subsequent HIT sessions with low carbohydrate availability, resulting in less work done under low carbohydrate conditions and compromised training quality. As with Marquet et al. (2016a) and in line with the fuel for the work required paradigm (Impey et al., 2018), the present study aimed to align carbohydrate intake with exercise demand with the purpose of augmenting training adaptation during fasted LIT sessions, extending the period of low carbohydrate availability overnight and providing high carbohydrate availability during HIT session to ensure session completion as prescribed. Whilst there was no difference between groups in mean HR, the data is clear when considering exercise intensity and the perceptual effect of training with low carbohydrate availability. Despite being asked to maintain 75% FTP during the LIT sessions, SL participants were consistently below target power. Typically, endurance exercise undertaken with critically low muscle glycogen concentrations (< 200 mmol kg dw-1) results in impaired exercise capacity due to lacking muscle energy substrates and impaired contractile capacity via compromised calcium regulation (Duhamel, Perco and Green, 2006; Ortenblad et al., 2011; Gejl et al., 2014). Practically, the provision of a carbohydrate-free sweet placebo drink could partially restore exercise capacity during a "sleep low-train low" approach (Waterworth et al., 2020). Progressing this further, providing exogenous carbohydrate ingestion during exercise under low carbohydrate conditions does not impair fat oxidation during exercise (Podlogar, Free and Wallis, 2020; Fell et al., 2021) and, therefore, providing a moderate dose of carbohydrates may serve to improve exercise capacity by preventing hypoglycaemia during exercise and recovery. However, in this study, since mean HR during LIT sessions were similar between SL and CON, the physiological demand of the exercise was likely similar between groups despite a reduction in power output in the SL group. Moreover, RPE during LIT sessions was consistently higher in SL compared to CON, which could contribute to better fatigue resistance, amplifying the metabolic benefits of the session. By periodising carbohydrates effectively, exercise intensity

was successfully restored during HIT sessions, allowing both groups to perform the sessions as prescribed. This was evident by the lack of difference between SL and CON across all intervals and sessions for power output, HR and RPE. Performance data presented here confirm that the strategic periodisation of carbohydrates around training sessions, with HIT sessions performed with high carbohydrate availability and LIT sessions performed with low carbohydrate availability, may confer an advantage to endurance athletes. A high carbohydrate availability for HIT sessions allows the maintenance of higher training intensity. However, it remains to be understood whether completing HIT exercise with deliberately low muscle glycogen alters the metabolic milieu within the muscle, even at the expense of absolute workload.

Concerning the nutritional intervention, participants in both groups increased their carbohydrate intake during the "sleep low-train low" intervention (5.2 and 5.1 $g \cdot kg^{-1} \cdot d^{-1}$ in SL and CON, respectively) compared to their habitual diet (3.1 and 3.4 $g \cdot kg^{-1} \cdot d^{-1}$ in SL and CON, respectively). Despite this increase in carbohydrate intake, all participants failed to reach the prescribed 6 $g \cdot kg^{-1} \cdot d^{-1}$, a similar finding to Marquet et al. (2016a), where participants' carbohydrate intake was 5.4 and 5.6 $g \cdot kg^{-1} \cdot d^{-1}$ in SL and CON, respectively. A likely reason subjects consumed below the desired carbohydrate intake is da result of the ‘food first’ approach and the omission of any high carbohydrate drink or gel supplements as is often provided in acute carbohydrate periodisation studies to maximise refuelling between sessions (Hearris et al., 2019). This provides valuable, practical evidence that athletes may struggle to achieve the high carbohydrate intake required between sessions to replenish muscle glycogen stores without the provision of high carbohydrate supplements.. Furthermore, in SL, carbohydrate ingestion was only permitted in the hours between LIT and HIT sessions, providing a relatively small window within which to consume carbohydrates. Despite

consuming below the recommended carbohydrate intake, SL sufficiently restored carbohydrates to maintain the required exercise intensity during the HIT session (105% FTP), as inferred by the lack of difference between SL and CON for power output and HR. This result also suggests that a higher daily carbohydrate intake ($\geq 6\ g \cdot kg^{-1} \cdot d^{-1}$) may be unnecessary during a "sleep low-train low" bout in endurance athletes. Moreover, it cannot be excluded that the underconsumption of carbohydrates throughout the study may be due to underreporting nutritional intake, an issue well known when collecting food diaries in athletic populations (Burke et al., 2001), and/or the use of a nutrition app as a tool to record dietary intake may also lead to underestimation of macronutrient intake (Teixeira et al., 2018). Despite this, there is no reason to believe the likelihood of underreporting would be greater in either group; therefore, comparisons between groups remain valid. To mitigate this risk, all participants were provided with comprehensive, written dietary guidelines with macronutrient compositions for all meals and snacks and volumes and quantities of all drinks/foods required to achieve the desired dietary intakes.

Using technology to facilitate exercise monitoring, prescription, and testing, the present study has highlighted the potential for data collection away from traditional laboratory-based testing and supervision. The tests selected were familiar to athletes and coaches and, therefore, directly transferable to practice through the alignment of common testing methodologies and the lack of need for expensive laboratory equipment. Despite this, the best approach for research-centric interventions may be combining online training and nutritional prescription with laboratory-based testing protocols to ensure optimal reliability and subject safety during extreme exercise tests. Exercise can be completed in a semi-supervised manner using online coaching platforms, allowing researchers to prioritise pre- and post-testing athletes in the laboratory and alleviating strain on laboratory resources and space. Similarly, the requirement

for participants to attend the laboratory for testing sessions only, instead of multiple times weekly to complete a training programme, may facilitate greater participant numbers in chronic exercise training interventions. Despite this, a potential limitation of remote data collection is ensuring that standardisation and quality control are maintained across all participants. In addition, clear instructions must be given on the necessary protocols to ensure robust data collection. In the present study, all participants were given the exact same instructions but were encouraged to contact the researcher if they needed clarification or assistance with understanding the written instructions. Furthermore, to ensure that only correctly collected data was used in the study, only participants with 100% compliance rates across all tests and training sessions were included in the analysis. Accordingly, in future protocols, all training sessions and tests should be visually inspected (i.e., review power files) to ensure that the protocols are followed correctly.

4.4.1 Practical Implications

The practical implications of the present study are twofold. Firstly, home-based performance testing represents a highly reliable strategy for athletes and coaches to monitor longitudinal training programme efficacy. With appropriate standardisation of diet, exercise and physical activity prior to performance tests, coaches and athletes can expect ~2% variation between repeated trials. Any greater difference between repeated trials can be attributed to variables outside of the inherent variability of these tests. Secondly, the characterisation of exercise capacity and performance outcomes following a period of training with low-carbohydrate availability highlight that performance outcomes are still improved despite reductions in exercise intensity. Coaches and athletes should be acutely aware that training with low endo- and exogenous carbohydrate availability impairs exercise capacity, but exercise

intensity can be restored on the same day providing adequate carbohydrate is provided between exercise bouts.

4.4.2 Conclusion

Despite reductions in relative training intensity, data within this chapter demonstrates that three weeks of "sleep low-train low" is effective in improving functional threshold power and 5-min PPO in trained cyclists and triathletes, with no benefit to high-intensity exercise performance (1-min PPO), compared to "normal" carbohydrate availability. A major novelty of this study is the use of remote exercise and nutrition prescription and data collection during a home-based intervention (completed throughout the COVID-19 pandemic). This data supports the feasibility of implementing a "sleep-low, train-low" intervention outside typical laboratory-controlled conditions.

Chapter 5 - Two Weeks of Combined "Sleep Low - Train low" Carbohydrate Periodisation and Heat Acclimation on Performance and Adaptation

Aspects of this work were presented at "A La Recherche au Service de la Performance", organised by the Institut National du Sport, de l'Expertise et de la Performance (INSEP), Paris, France, in April 2019

and by

Oral Communication at the 25th Annual European Congress of Sport Science (ECSS) (Online)

and by

E-Poster at the 27th Annual European Congress of Sport Science (ECSS), Seville, Spain.

5.1 Introduction

Champion endurance athletes are characterised by their ability to sustain high substrate utilisation rates at high relative exercise intensities (80-90% $\dot{V}O_{2max}$) without incurring increased blood lactate concentration (Jones, 1998; Lucia et al., 2002; Larsen, 2003). This high "lactate threshold" is attributed to high muscle oxidative capacity, owing to increased mitochondrial protein expression and respiratory chain capacity (Lundby and Jacobs, 2016) across the TCA cycle, β-oxidation and electron transport chain (Holloszy et al., 1977). Crucially, this improved aerobic metabolic capacity reduced carbohydrate: fat oxidation (Brooks and Mercier, 1994) and lactate production (Messonnier et al., 2013), leading to reduced cellular homeostatic disturbance during exercise (Holloszy et al., 1977). The blunted intramuscular perturbation results in impaired adaptive responses highlighted by attenuated signalling protein activity following exercise in highly-trained individuals (Yu et al., 2003). Multiple proposed strategies currently exist to increase acute "metabolic stress" and maximise chronic exercise adaptation.

To maximise cellular homeostatic disturbance and enhance subsequent exercise adaptive responses, athletes and coaches may surmise that combining multiple strategies may confer greater adaptive and performance outcomes. Data presented within the previous chapter reported that periodically training with low carbohydrate availability via the SL-TL model improved aerobic exercise capacity following 3 weeks of exercise-nutrition intervention in line with previous literature (Marquet et al., 2016a; 2016b; Salokannel, Hakulinen and Ahtiainen, 2021). This data contrasts with others who implemented SL-TL and reported no additional benefit to performance compared to consistently high carbohydrate intake (Gejl et al., 2017; Riis et al., 2019) with a recent meta-analysis questioning the efficacy of training with low

carbohydrate intake on performance (Gejl and Nybo, 2021). Understanding the effectiveness of combining multiple strategies to maximise endurance performance has yet to be investigated, such as combining carbohydrate periodisation and heat acclimation to enhance exercise capacity in hot and temperate environments. The appropriateness of implementing the SL-TL model in elevated environmental temperatures remains to be determined.

Muscle glycogen is a critically important energy substrate during exercise (Bergstrom and Hultman, 1966; 1967; Bergstrom et al., 1967), essential for maintaining exercise capacity (Karlsson, Nordesjo and Saltin, 1974) and is easily manipulated through diet and exercise (Hultman and Bergstrom, 1967). Commencing exercise with low muscle glycogen (< 200 mmol·kg dw^{-1}) has shown a greater muscular transcriptional response (Pilegaard et al., 2002; Bartlett et al., 2013; Psilander et al., 2013; Lane et al., 2015; Stocks et al., 2019) and increased intramuscular signalling activity. AMP-activated protein kinase (AMPK) activity is significantly increased (Wojtaszewski et al., 2003; Yeo et al., 2010), likely as a result of decreased Glycogen-AMPK binding (Polekhina et al., 2005; McBride et al., 2009) leading to the downstream activation of PGC-1α (Canto and Auwerx, 2009; Philp et al., 2011; Philp, Hargreaves and Baar, 2012), a transcriptional co-activator often touted as the master regulator of mitochondrial biogenesis (Wu et al., 1999; Lin et al., 2002; Lin, Handschin and Spiegelman, 2005), a key hallmark of endurance training adaptation (Holloszy, 1967; Holloszy et al., 1970; Holloszy and Coyle, 1984). The increase in fat oxidation observed under conditions of low muscle glycogen (Maunder et al., 2021a) simultaneously up-regulates peroxisome proliferator-activated receptor (PPARδ) transcription factor (Pilegaard et al., 2005), increasing the expression of proteins implicated in lipid metabolism (Maunder et al., 2021a). The last decade has given rise to multiple exercise-nutrition strategies intended to reduce muscle glycogen around specific training bouts (coined “train low”) in athletes (2008a; Yeo et al., 2008b;

Hulston et al., 2010; Psilander et al., 2013; Lane et al., 2015; Marquet et al., 2016a; Gejl et al., 2017; Riis et al., 2019; Burke et al., 2020). However, despite growing evidence of an augmented molecular adaptation triggered by "train low" strategies, the translation to improved physical performance is limited (Yeo et al., 2008b; Morton et al., 2009; Hulston et al., 2010; Gejl et al., 2017).

"Training camps" completed in hot environments are commonplace amongst endurance-sport athletes (Maunder et al., 2019) and are intended to increase training stimulus and subsequent adaptation (Hawley et al., 2018). Initially, exercise undertaken in high ambient temperatures results in impaired exercise capacity (Febbraio et al., 1996b; Galloway and Maughan, 1997; Gonzalez-Alonso, Calbet and Nielsen, 1999; Parkin et al., 1999) with repeated exposures to exogenous heat stress during endurance exercise (active heat acclimation) translating to robust increases in exercise capacity in the heat (Racinais et al., 2014; 2015b; 2015c; Periard, Racinais and Sawka, 2015) through multiple integrated physiological mechanisms. Alongside the phenotypic adaptations, endurance exercise performed in hot conditions may lead to augmented adaptive response at the skeletal muscle level, with numerous factors implicated in mitochondrial biogenesis increased during exercise in the heat. *In vitro,* acutely heated myotubes and rat skeletal muscle increased phosphorylation of AMPK and downstream targets, increased mitochondrial transcription factor activation and elevated heat shock protein transcriptional response (hspa1a & hspa1b mRNA) (Moon et al., 2003; Liu and Brooks, 2012; Koshinaka et al., 2013; Goto et al., 2015). Heat-acclimated myotubes robustly increased PGC-1α protein expression and markers of mitochondrial biogenesis (OXPHOS subunit complexes I-IV, ATP synthase and mtDNA copy number) (Liu and Brooks, 2012). *In vivo,* mitochondrial adaptation was greatest in exercise and heat compared to either condition alone in rat muscle (Tamura et al., 2014). Data from human exercise studies remain

equivocal and rarely applicable to trained populations and contrary to cell and animal evidence, PGC-1α expression was suppressed following 3-h recovery in the heat (33ºC) in moderately trained individuals (Slivka et al., 2012), impairing improvements in $\dot{V}O_{2peak}$ following 3 weeks of RPE clamped heat acclimation (Slivka et al., 2021). In contrast, Maunder et al. (2021c) reported increased mitochondrial protein activity (citrate synthase), corresponding to improved performance in temperate conditions when exercise is matched relative to cardiovascular demand during heat acclimation in endurance-trained individuals (Maunder et al., 2021b).

To maximise training adaptation, athletes and coaches may combine multiple strategies to amplify cellular stress, theoretically provoking a heightened intramuscular signalling response to increasing training adaptation. Whilst exercise completed under low muscle glycogen availability augments the adaptive response to exercise, the translation to improved performance is limited. Conversely, heat acclimation induces robust increases in performance in hot conditions, with emerging evidence suggestive of beneficial effects on skeletal muscle adaptation and mitochondrial biogenesis. Given the knowledge that each intervention will benefit performance and adaptation individually, coaches may conclude that combining these approaches confer a greater adaptive response and improve performance. Despite this, there is no scientific evidence investigating the roles of combined heat acclimation and training with low carbohydrate availability.

The present study aimed to determine whether a 2-week heat acclimation protocol whereby carbohydrate availability is periodised to match exercise demands (utilising the SL-TL model) would benefit performance in hot and temperate conditions. It is hypothesised that carbohydrate periodisation alone would benefit exercise performance in the temperate

condition, whilst heat acclimation and SL-TL would improve performance in hot and temperate conditions, respectively.

5.2 Methods

5.2.1 Participants and Ethical Approval

Twenty-four trained male triathletes (29 ± 7 years, 72.9 ± 6 kg) with a mean peak oxygen consumption ($\dot{V}O_{2peak}$) and Maximal aerobic power (MAP) of 62.3 ± 6.6 $ml \cdot kg^{-1}$ min^{-1} and 330 ± 40 W, respectively, were recruited for the study. Participant characteristics are reported in Table 5.1. A priori sample size estimation indicated a minimum sample size of 21 participants was required to detect a small to moderate effect (0.25) for mean power during performance tests with 80% statistical power using the G*Power (v 3.1) software. The effect size used for this calculation was based on moderate effect sizes in performance outcomes following 3 weeks of SL-TL intervention (Marquet et al., 2016a). Participants were free of musculoskeletal or neurological disease and were not receiving any pharmacological treatment during the study. All participants were provided written informed consent, and all procedures conformed to the standards of the Declaration of Helsinki 2008. Ethical approval was granted by the Comité de Protection des Personnes Ouest IV – Nantes (CPP) (No - 2018-A02544-51). One person in the CON group withdrew, citing personal reasons reducing *n* to 7.

Table 5.1 – Detailed participant characteristics for each group. All data is presented as mean ± standard deviation.

	Control (CON)	**Sleep Low (SL)**	**Sleep Low + Heat Acclimation (SL_{Heat})**
N	7	8	8
Age (years)	34 ± 7	28 ± 5	28 ± 7
Height (cm)	182 ± 7	180 ± 7.0	181 ± 4
Weight (kg)	71.8 ± 6.2	73.1 ± 7.9	72.7 ± 3.6
Lean mass (kg)	59.4 ± 5.5	59.7 ± 5.3	59.0 ± 2.3
Fat mass (kg)	9.5 ± 4.3	10.7 ± 4.4	10.9 ± 3.3
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	4.7 ± 0.6	4.9 ± 0.9	4.8 ± 0.6
MAP (W)	343 ± 49	323 ± 31	330 ± 42
Experience in triathlon (years)	6 ± 4	5 ± 2	7 ± 4
Habitual training load ($hr \cdot week^{-1}$)	11 ± 6	10 ± 3	11 ± 5

MAP, Maximal Aerobic Power

5.2.2 Experimental Design

In a matched, independent groups, repeated measures design, participants were matched based on age and maximal aerobic power before being randomly allocated (Kim and Shin, 2014) to one of three groups and completing a 2-week exercise-nutritional intervention. Participants were prescribed identical exercise training protocols, but nutritional and environmental conditions varied between groups. One group completed all sessions in a thermoneutral environment (20°C, 50% Relative Humidity [RH]) with "normal" carbohydrate availability for all sessions (CON). The other groups completed the training programme with periodised carbohydrate intake to reduce carbohydrate availability for specific sessions (SL-TL) in thermoneutral (SL; 21°C, 50% RH) or hot conditions (SL_{Heat}; 35°C, 50% RH). Having completed a familiarisation of all tests, participants were subject to a physiological testing battery in the week prior to training intervention (pre), immediately post-intervention (post [~3 days following the completion of the intervention]) and 2 weeks post (Post+1, [~10 days following the completion of the intervention]) intervention. To account for season heat

acclimation, all data collection were halted during the summer months (June-September). A schematic overview of the study is shown in Figure 5.1

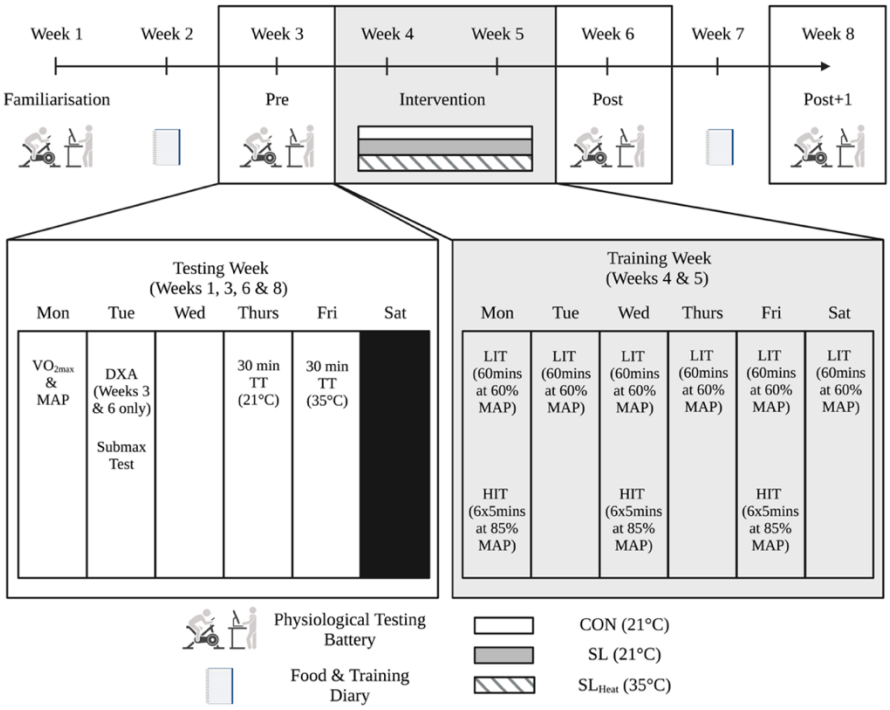

Figure 5.1 – Schematic overview of study timeline with weekly testing and training schedules. During test weeks, Body mass (BW) was measured before and after each exercise test, with tests performed at similar times each day. Prior to each testing session, participants consumed a standardised meal. During training weeks, LIT sessions were completed daily, with HIT exercise on alternating days. In SL and SL_{Heat}, the following LIT sessions were completed fasted, whilst CON consumed a carbohydrate rich breakfast beforehand.

5.2.3 Performance Tests

5.2.3 i) Assessment of Maximal Oxygen Uptake ($\dot{V}O_{2max}$) and Maximal Aerobic Power (MAP)

Briefly, $\dot{V}O_{2max}$ and MAP were assessed during an incremental cycle test performed on an electronically braked cycle ergometer (Excalibur Sport, Lode, Groningen, Netherlands) (Described in detail in Chapter 3.2.5). Participants completed a standardised warm-up before commencing the test consisting of 2-min stages with 30 W increments until volitional

exhaustion with respired gases collected throughout. MAP (W) was calculated per Hawley and Noakes (1992).

5.2.3 ii) Sub-maximal Cycling Test

The general principle and protocol used during the sub-maximal cycling test and characterisation are described in Chapter 3.2.4. Briefly, following the consumption of a standardised meal, participants were fitted with a facemask before completing a standardised warm-up, followed by 2 successive 6-min stages at 60% and 70% of previously calculated MAP. Breath-by-breath data were recorded throughout the exercise (Quark Cosmed, Rome, Italy). Whole-body rates of carbohydrate and FAT oxidation ($g \cdot min^{-1}$) were calculated from final 1-min averaged $\dot{V}O_2$ and $\dot{V}CO_2$ values of the intensity of interest and nonprotein RER values according to the following equations (Jeukendrup and Wallis, 2005). Energy Expenditure (EE) ($kcal \cdot min^{-1}$) was obtained from the rate of oxygen uptake using the equations developed by Brouwer (1957) and based on the thermal equivalent of O_2 for nonprotein RER.

5.2.3 iii) 30-minute Intermittent Capacity Test

Previously described in detail in Chapter 3.3.3, the 30-min intermittent capacity test was completed at the same time of day in temperate (21°C, 50% RH) or Hot (35°C, 50% RH) conditions in a randomised order.

Exercise Protocol

Following a 15-min self-paced warm-up, participants started the test consisting of 6 phases of 4 mins 50 s separated by a 10 s sprint resulting in a cumulative time of 30 mins.

Participants were instructed to ride as hard as possible for the test to obtain their best mean power (including sprints). The test was conducted on participants' bicycles mounted to a previously validated stationary cycle home trainer (Hammer H2, Cycleops, USA) (Lillo-Bevia and Pallares, 2018).

Core and Skin Temperature

Core body temperature (T_{core}) was measured as previously described in section 3.3.3ii via a telemetric pill (E-Celcius, BodyCap, Hérouville Saint-Clair, France) inserted ~7-10 cm rectally with data recorded at 1-min intervals via a data logger (e-Viewer, BodyCap, Hérouville Saint-Clair, France). Skin temperature (T_{sk}) was recorded using wireless thermistors (I-button thermochron, Maxim/Dallas Semiconductor Corp, Dallas, USA) were attached to 5 locations on the body (sternum, upper arm, forearm, quadricep and gastrocnemius). The mean surface temperature was calculated as per Ramanathan (1964).

Perceptual Measures

Fifty seconds prior to each sprint interval, participants were required to report their perceived exertion (Borg, 2001), thermal comfort and thermal sensation using a colour-coded, audio-visual scale (Huizenga, Hui and Arens, 2001). Perceptual measures used have been described in detail in Chapter 3.4.1 and Chapter 3.4.2.

Blood Lactate

Prior to, and every 10 min of the exercise test (0, 10, 20, 30 min), a 3 μL earlobe prick sample of blood lactate ([La]) was collected and analysed using a Lactate Pro 2 Device (Arkay, Kyoto, Japan).

5.2.4 Carbohydrate Periodisation & Training Programme

5.2.4 i) Nutritional Intervention

During week 2, participants recorded dietary intake, including all food and liquid, in a written food diary to inform of the subject's habitual dietary intake. In addition, participants weighed their food before preparation and noted food quantities and cooking directions in the diary. The food diary was completed throughout the 2-week exercise-nutrition-environmental intervention. Mean macronutrient intake of the middle 5 days (first and last days excluded) was used to calculate baseline and experimental dietary intake.

Dietary guidelines were provided to participants to alter carbohydrate availability for specific sessions during the week ("Train low"). All participants were provided with standardised dietary instructions and asked to adhere to the prescribed menus. Total daily carbohydrate intake was the same ($6g \cdot kg \cdot day^{-1}$) throughout the study, but the intake timing varied to achieve either high or low carbohydrate availability before specific training sessions. SL and SL_{Heat} groups were barred from consuming carbohydrates following each HIT session (no carbohydrates at dinner) until after the fasted LIT session the following morning. Participants were required to eat carbohydrates ($6g \cdot kg^{-1}$) post-LIT session before the next HIT session. To ensure muscle protein synthesis was maintained and improve overnight satiety, all participants consumed a high protein, low carbohydrate gel (SIS WHEY 20, Science in Sport, Lancashire, UK) each night before bed. Participants were allowed direct contact with the research team to ask dietary questions.

5.2.4 i) Training Programme

All participants completed identical 2-week training programmes in their assigned environmental conditions. Participants completed 1 hr at 60% MAP daily for six consecutive days with interval sessions consisting of 6x 5mins at 85% MAP with 5 mins recovery (Adapted from Marquet *et al.,* 2016a) on alternate evenings. Participants consumed water *ad libitum* only during the training sessions. All sessions were recorded on participants' sports watches (Garmin, Polar or Suunto devices) and uploaded to an online training monitoring platform (Playsharp, France).

Table 5.2 – Overview of prescribed exercise (in bold) and carbohydrate intake ($g.kg^{-1}$) during the intervention for CON, SL and SL_{Heat} groups.

	DAY 1 & 8		**DAY 2 & 9**		**DAY 3 & 10**		**DAY 4 & 11**		**DAY 5 & 12**		**DAY 6 & 13**	
GROUP	**CON**	**SL & SL_{Heat}**	**CON**	**SL & SL_{Heat}**	**CON**	**SL & SL_{Heat}**	**CON**	**SL & SL_{Heat}**	**CON**	**SL & SL_{Heat}**	**CON**	**SL & SL_{Heat}**
MORNING (BEFORE 10:00)	Breakfast (2g)		Breakfast (2g)	**LIT** (Fasted)	Breakfast (2g)		Breakfast (2g)	**LIT** (Fasted)	Breakfast (2g)		Breakfast (2g)	**LIT** (Fasted)
	LIT		**LIT**	Breakfast (2g)	**LIT**		**LIT**	Breakfast (2g)	**LIT**		**LIT**	Breakfast (2g)
MIDDAY	Lunch (1.5 g)	Lunch (2g)	Lunch (1.5 g)		Lunch (1.5 g)	Lunch (2g)	Lunch (1.5 g)		Lunch (1.5 g)	Lunch (2g)	Normal Diet	
AFTERNOON (BEFORE 17:00)	Snack (0.5g)	Snack (2g)	Snack (0.5g)		Snack (0.5g)	Snack (2g)	Snack (0.5g)		Snack (0.5g)	Snack (2g)		
	HIT				**HIT**				**HIT**			
EVENING (AFTER 17:00 AND BEFORE 21:00)	Dinner (2 g)	Dinner (0 g)	Dinner (2 g)		Dinner (2 g)	Dinner (0 g)	Dinner (2 g)		Dinner (2 g)	Dinner (0 g)		

CON, control group; SL, sleep low group; SL_{Heat}, sleep low in heat group; LIT, low intensity training; HIT, high intensity training

5.2.5 Dual X-ray Absorptiometry (DXA)

Following an overnight fast, participants reported to the radiology department, where height and weight were measured using a combined stadiometer and scales (SECA) before a whole-body DXA scan. Participants lay supine on the scanner bed, positioned with their arms at their sides, with the palmer surface of the hand orientated to the lateral aspect of the thigh. Once the participants were in position, they remained still for the scan (~3 min). DXA scans were completed during the pre-test and post-test weeks, with all anthropometric and DXA analyses performed by a trained radiographer.

5.2.6 Statistical Analysis

Firstly, data were assessed for normality via the Shapiro-Wilk test (Shapiro and Wilk, 1965). Then , repeated measures two-way (group × time) ANOVA was conducted to assess the effects of group (CON vs. SL vs. SL_{Heat}) and time (Pre vs. Post vs. Post+1) on performance outcomes (MAP, 30-min TT) and metabolic responses (substrate utilisation). Greenhouse-Geisser correction when violations of sphericity were present. Statistical significance was $P < 0.05$ for all tests. In addition, a two-way mixed effect ANOVA was used to assess the effect of group (CON vs SL vs SL_{Heat}) and time (Days 1 to 13) on training responses (MPO, HR, and perceptual measures). Post-hoc Bonferroni, multiple comparison tests, were performed when significant effects were present. Shapiro-Wilk's test systematically checked normal distribution. Effect sizes were calculated using partial eta squared (η^2_p) and with values of 0.01, 0.06, and over 0.14 considered small, medium, and large, respectively (Cohen, 1988b). Data analysis was completed using Graphpad Prism v.9 (Graphpad Software, CA, USA). All data reported as mean ± SD.

5.3 Results

5.3.1 Maximal Oxygen Uptake ($\dot{V}O_{2Max}$) and Maximal Aerobic Power (MAP)

Maximal oxygen uptake ($\dot{V}O_{2max}$) was not different between groups ($F(2, 20) = 1.919$, $P = 0.17$, $d = 0.14$) nor over time($F(2, 20) = 1.919$, $P = 0.17$, $d = 0.14$) nor over time ($F(1.880, 37.61) = 2.169$, $P = 0.13$, $d = 0.01$). Maximal aerobic power (MAP) was not different between groups ($F(2, 20) = 0.05971$, $P = 0.94$, $d = 0.005$) however did increase over time ($F(1.693, 33.86) = 6.861$, $P < 0.01$, $d = 0.05$). CON increased from pre (326 ± 36 W) to post (343 ± 49 W) before decreasing to 335 ± 44 W at post+1. Similarly, SL increased from pre (315 ± 36 W) to post (345 ± 39 W) before decreasing at Post+1 (334 ± 41 W). SL_{Heat} increased from pre to post and again from post to post+1, starting at 323 ± 35 W and increasing to 334 ± 49 W before increasing further to 338 ± 45 W at post +1.

5.3.1 Sub-maximal Substrate Utilisation Test

At 60% MAP (208 ± 24 W), the mean heart rate (HR) was significantly changed over time ($F(1.776, 35.52) = 4.495$, $P = 0.02$, $d = 0.06$). Mean HR decreased from 145 ± 12 to 133 ± 12 and 130 ± 15 beats·min^{-1} at pre, to post ($P < 0.01$) to post+1 ($P < 0.01$), respectively, in the CON group. Likewise, HR decreased in SL from pre (144 ± 9 beats·min^{-1}) to post+1 (130 ± 5 beats·min^{-1}) ($P < 0.01$) and post (142 ± 8 beats·min^{-1}) to post+1 ($P < 0.01$). There was no significant difference in mean HR between time points in the SL_{Heat} group, although an increase in HR was observed as such a significant group × time interaction effect was present ($F(1.776, 35.52) = 4.495$, $P = 0.02$, $d = 0.10$).

Respiratory exchange ratio (RER) decreased in the SL group only with no difference in CON or SL_{Heat} with a significant interaction effect ($F(4, 40) = 2.626$, $P < 0.05$, $d = 0.11$).

This was maintained at the substrate level with carbohydrate oxidation significantly reduced in the SL group from pre to post ($P = 0.04$) and maintained at post+1 ($P = 0.02$) with no difference in CON or SL_{Heat} groups with a significant interaction observed ($F(4, 40) = 2.664$, $P < 0.05$, $d = 0.10$). The decrease in RER and carbohydrate oxidation was accompanied by an increase in fat oxidation over time ($F(1.640, 32.79) = 3.862$, $P = 0.04$, $d = 0.06$) and a significant interaction effect (F (4, 40) = 2.731, $P = 0.04$, $d = 0.09$). Fat oxidation significantly decreased in CON from post to post+1 ($P = 0.04$) only, whilst decreased fat oxidation rates were observed from pre to post ($P = 0.007$) and pre to post+1 ($P = 0.03$) in the SL group. The relative contribution of each macronutrient (carbohydrate and Fat) to whole-body energy expenditure is presented in Figure 5.2.

At 70% MAP (254 ± 32 W), mean HR was significantly reduced over time in both CON and SL groups (F (1.760, 35.20) = 8.158, $P = 0.002$, $d = 0.07$) with no change in HR for SL_{Heat} resulting in a significant interaction effect (F (4, 40) = 3.643, $P = 0.01$, $d = 0.06$). CON reduced mean HR from 160 ± 11 beats·min^{-1} at pre to 150 ± 8 beats·min^{-1} post and 148 ± 12 beats·min^{-1} at post+1. Between pre-to-post ($P = 0.03$) and pre-to-post+1 ($P < 0.01$), significant differences were observed. SL decreased mean HR from pre (157 ± 12 beats·min^{-1}) to post+1 (145 ± 7 beats·min^{-1}) ($P = 0.02$) and from post (157 ± 7 beats·min^{-1}) to post+1 ($P < 0.01$).

RER was significantly impacted by time (F (1.751, 35.02) = 9.704, $P = 0.0007$, $d = 0.10$); however, responses varied depending on the experimental group (F(4, 40) = 5.403, $P = 0.001$, $d = 0.11$). CON significantly reduced RER at Pre (from 0.93 ± 0.04) to post (0.90 ± 0.03) ($P = 0.04$) before returning to pre-intervention values at post+1 (0.94 ± 0.03) ($P = 0.02$). SL decreased RER from pre (0.93 ± 0.04) to post (0.89 ± 0.04) ($P = 0.01$) and post+1 (0.88 ± 0.04) ($P < 0.01$). No difference was observed in the SL_{Heat} group for RER.

In line with changes in RER, carbohydrate oxidation was significantly changed over time (F(1.636, 32.72) = 9.454, P = 0.001, d = 0.08) with divergent responses between groups leading to a significant interaction effect (F (4, 40) = 4.093, P = 0.007, d = 0.07). CON decreased carbohydrate oxidation from pre (3.35 ± 0.75 g·min^{-1}) to post (2.9 ± 0.61 g·min^{-1}) (P < 0.01) but returned to baseline at post+1 (3.37 ± 0.54 g·min^{-1}) (P = 0.02). SL decreased from Pre (3.38 ± 0.77 g·min^{-1}) to Post+1 (2.5 ± 0.65 g·min^{-1}) (P = 0.02) with no differences in the SL_{Heat} group.

Concomitant to alterations in carbohydrate metabolism, fat oxidation was altered over time (F(1.892, 37.84) = 8.469, P = 0.001, d = 0.09) with differing responses within groups (F(4, 40) = 4.329, P = 0.005, d = 0.09). CON increased Fat oxidation from pre (0.39 ± 0.20 g·min^{-1}) to post (0.54 ± 0.14 g·min^{-1}), albeit this did not reach significance (P = 0.07) before a significant reduction in fat oxidation from post to post+1 (0.34 ± 0.2 g·min^{-1}) (P = 0.04). Following the intervention, SL increased fat oxidation from 0.36 ± 0.22 g·min^{-1} to 0.67 ± 0.27 g·min^{-1} immediately post (P = 0.01) and maintained this increase at Post+1 (0.67 ± 0.20) (P < 0.01). Complete physiological and substrate utilisation data from the sub-maximal substrate utilisation test can be found in Table 5.3.

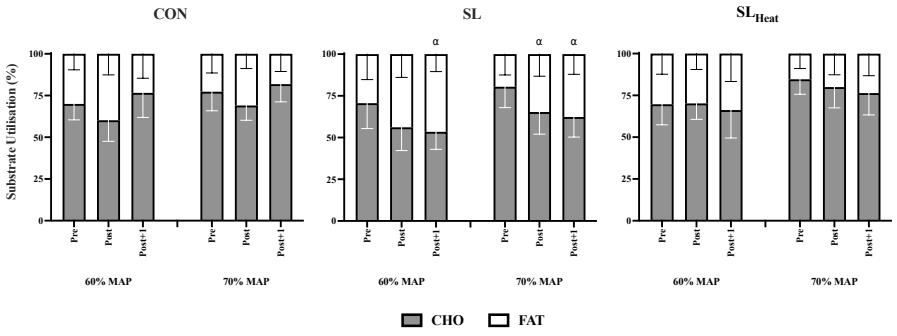

Figure 5.2 – Relative macronutrient contribution to total energy expenditure at 60 and 70% MAP. CHO represents carbohydrate. Pairwise comparisons "*α*" denotes significant difference from Pre with threshold set to P < 0.05.

Table 5.3 – Physiological and substrate utilisation metrics collected during the sub-maximal cycling test. Data present as mean ± SD.

		60% MAP			ANOVA (η^2_p)			70% MAP			ANOVA (η^2_p)		
		PRE	**POST**	**POST+1**	**Group**	**Time**	**G x T**	**PRE**	**POST**	**POST+1**	**Group**	**Time**	**G x T**
HR (beats·min^{-1})	CON	145 ± 12	133 ± 12 $^{\alpha}$	130 ± 15$^{\beta}$	0.021 (0.06)	ns	0.007 (0.11)	160 ± 11	150 ± 8$^{\alpha}$	148 ± 12$^{\beta\gamma}$	0.002 (0.07)	ns	0.008 (0.06)
	SL	144 ± 9	142 ± 8	130 ± 5$^{\beta\gamma}$				157 ± 12	157 ± 7	145 ± 7			
	SL_{Heat}	135 ± 11	141 ± 16	140 ± 16				154 ± 12	153 ± 16	154 ± 15			
VO_2 (mL·min^{-1}·kg^{-1})	CON	39.20 ± 4.11	37.40 ± 5.5	38.24 ± 5	ns	ns	ns	50.23 ± 4.97	49.16 ± 4.28	47.85 ± 6.11$^{\beta}$	0.02 (0.03)	ns	ns
	SL	38.23 ± 4.14	37.87 ± 4.36	34.90 ± 3.8 $^{\gamma}$				47.54 ± 4.83	49.37 ± 3.25	47.01 ± 5.33$^{\gamma}$			
	SL_{Heat}	34.51 ± 3.34	37.83 ± 4.16	35.82 ± 6.01				45.66 ± 3.97	46.71 ± 4.9	45.59 ± 5.69			
Respiratory Exchange Ratio (RER)	CON	0.91 ± 0.03	0.87 ± 0.04	0.93 ± 0.05	ns	ns	0.049 (0.11)	0.93 ± 0.04	0.89 ± 0.04	0.95 ± 0.04	ns	0.0007 (0.10)	0.001 (0.11)
	SL	0.90 ± 0.05	0.86 ± 0.06	0.86 ± 0.04				0.92 ± 0.06	0.90 ± 0.06	0.88 ± 0.05			
	SL_{Heat}	0.90 ± 0.04	0.93 ± 0.06	0.89 ± 0.05				0.95 ± 0.03	0.96 ± 0.07	0.92 ± 0.07			
CHO Oxidation (g·min^{-1})	CON	2.59 ± 0.48	2.18 ± 0.67	2.73 ± 0.48 ^{a}	ns	ns	0.046 (0.10)	3.35 ± 0.75	2.75 ± 0.7$^{\alpha}$	3.47 ± 0.59 $^{\gamma a}$	ns	0.001 (0.08)	0.007 (0.07)
	SL	2.54 ± 0.74	2.16 ± 0.82$^{\alpha}$	1.95 ± 0.54 $^{\beta a}$				3.28 ± 1.06	2.96 ± 0.88	2.57 ± 0.74 $^{\beta a}$			
	SL_{Heat}	2.34 ± 0.53	2.78 ± 0.55	2.35 ± 0.81				3.44 ± 0.41	3.63 ± 0.84	3.11 ± 1.15			
Fat Oxidation (g·min^{-1})	CON	0.47 ± 0.15	0.62 ± 0.2	0.34 ± 0.24 $^{\gamma a}$	ns	0.038 (0.06)	0.042 (0.09)	0.39 ± 0.2	0.60 ± 0.26	0.26 ± 0.25 $^{\gamma a}$	ns	0.001 (0.09)	0.005 (0.09)
	SL	0.52 ± 0.28	0.71 ± 0.32$^{\alpha}$	0.68 ± 0.21$^{\beta a}$				0.40 ± 0.31	0.58 ± 0.39 $^{\alpha}$	0.65 ± 0.26 $^{\beta a}$			
	SL_{Heat}	0.47 ± 0.18	0.38 ± 0.32	0.52 ± 0.25				0.29 ± 0.18	0.34 ± 0.28	0.47 ± 0.29			

α Significantly different from Pre to Post
β Significantly different from Pre to Post+1
γ Significantly different from Post to Post+1
a Significantly different between CON and SL
b Significantly different between CON and SL_{Heat}
c Significantly different between SL and SL_{Heat}

5.3.2 30-min Intermittent Capacity Test – Temperate Condition (20ºC)

Mean power output during the 30-min intermittent capacity test was significantly different following the intervention ($F(1.515, 30.30) = 14.65$, $P = 0.0001$, $d = 0.02$) with no difference between groups ($F(2, 20) = 0.2491$, $P = 0.78$, $d = 0.02$). Bonferroni post-hoc analysis revealed a significant increase in mean power output in SL group from Pre (222 ± 97 W) to Post (239 ± 106 W) ($P < 0.01$) and Post+1 (240 ± 108 W) ($P = 0.02$). There were no difference in CON or SL_{Heat} groups (Figure 5.3A). Mean HR was reduced following the intervention ($F(1.697, 35.64) = 9.653$, $P = 0.0008$, $d = 0.06$) with no difference between groups ($F(2, 21) = 0.1426$, $P = 0.87$, $d = 0.01$). Mean HR significantly reduced from PRE (165 ± 10 beats·min-1) to Post+1 (158 ± 11 beats·min-1) ($P = 0.02$) in SL group only. No differences were observed between time points in either CON or SL_{Heat} groups (Figure 5.3C). Core body temperature (T_{core}) was reduced following the intervention with a significant effect of time ($F(1.477, 29.54) = 4.849$, $P = 0.02$, $d = 0.10$). Mean T_{core} decreased in SL_{Heat} from pre (37.9 ± 0.3 ºC) to post (37.6 ± 0.2 ºC) and post+1 (37.5 ± 0.2 ºC) ($P = 0.04$ and $P < 0.01$ respectively. No differences were observed between time points in either CON or SL groups (Figure 5.3E). There were no differences in mean T_{sk} between groups or time points. Mean blood lactate was not different following the intervention ($F(1.807, 36.15) = 2.106$, $P = 0.14$, $d = 0.02$) or between groups ($F(2, 20) = 0.8114$, $P = 0.45$, $d = 0.06$).

5.3.3 30-min Intermittent Capacity Test – Hot Condition (35ºC)

Mean power output was significantly impacted following the intervention ($F(1.927, 38.53) = 5.380$, $P = 0.009$, $d = 0.01$) with no difference between groups ($F(2, 20) = 0.001$, $P = 0.99$, d

= 0.0001). Post-hoc analysis revealed a significant increase in mean power from pre (220 ± 26 W) to post+1 (233 ± 31 W) in the SL_{Heat} group ($P < 0.01$), with no differences observed in any other group or timepoint (Figure 5.3B). Similarly, a significant main effect of time was observed for mean heart rate ($F(1.907, 38.14) = 6.030$, $P = 0.006$, $d = 0.04$) with no effect for group ($F(2, 20) = 0.1562$, $P = 0.86$, $d = 0.01$). SL reduced mean heart rate from Pre (166 ± 9 beats·min^{-1}) to post (158 ± 12 beats·min^{-1}) and post+1 (159 ± 10 beats·min^{-1}) ($P = 0.04$ and $P = 0.03$, respectively). No differences were identified between time points in either CON or SL_{Heat} groups (Figure 5.3D). There was a significant main effect of time ($F(1.978, 39.56) = 3.410$, $P = 0.04$, $d = 0.03$) and significant interaction (group*time) ($F(4, 40) = 3.211$, $P = 0.02$, $d = 0.06$) on mean T_{core} during the 30-min test in hot conditions. Bonferroni post-hoc analysis revealed a significant decrease in mean T_{core} from pre (37.8 ± 0.3ºC) to post (37.6 ± 0.1ºC) ($P = 0.04$) before returning to baseline at post+1 (37.9 ± 0.2ºC) ($P = 0.04$) (Figure 5.3F). There were no differences in T_{sk} between groups or time points. Mean blood lactate was not different following the intervention ($F(1.779, 35.58) = 2.330$, $P = 0.12$, $d = 0.03$) or between groups ($F(2, 20) = 1.608$, $P = 0.23$, $d = 0.1$).

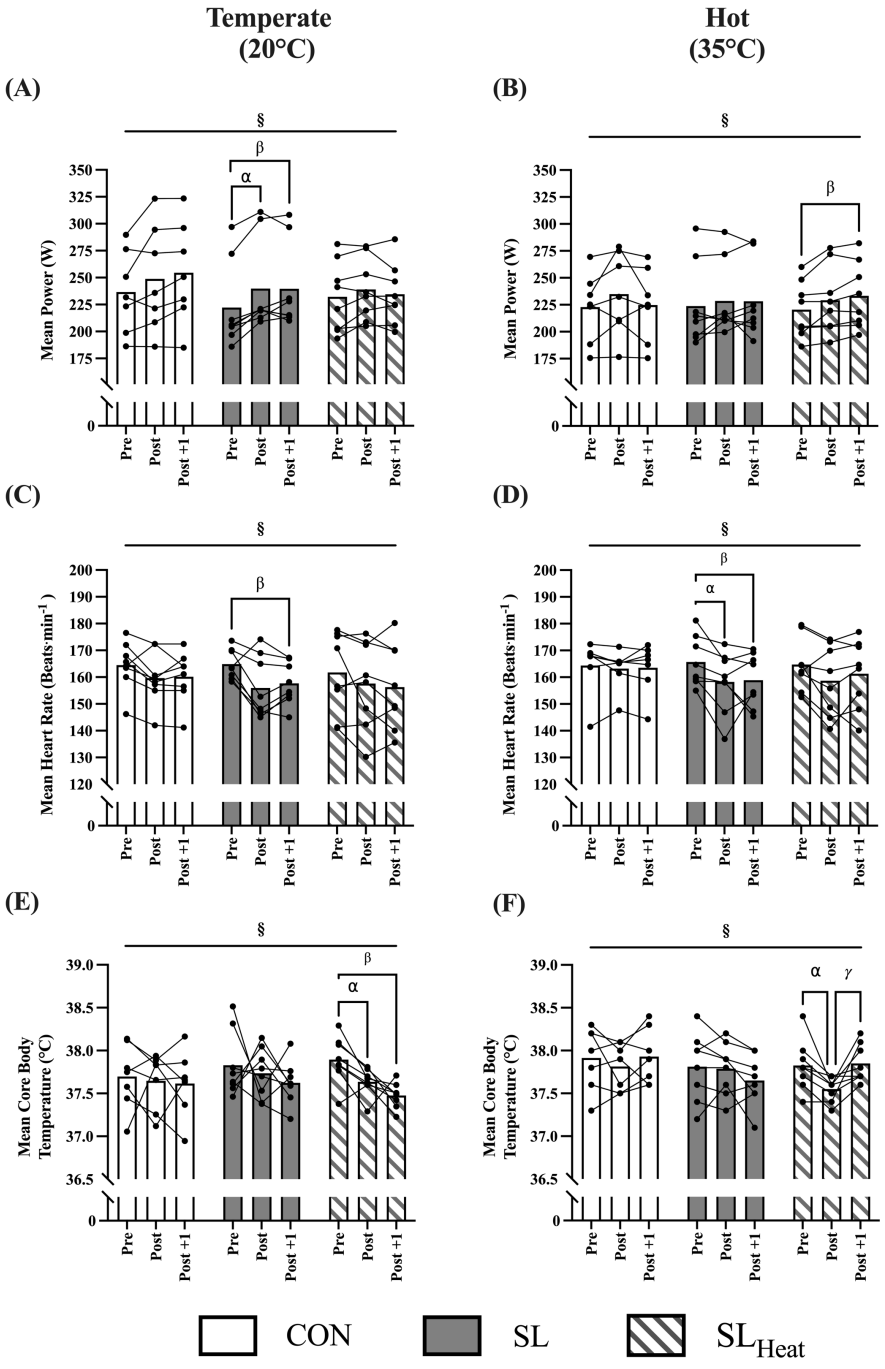

Figure 5.3 – Summary metrics during 30-min capacity tests in Temperate and Hot conditions across Pre, Post and Post+1 timepoints. (A) Mean power output for each group in temperate conditions (B) Mean power output for each group in hot conditions (C) Mean heart rate for each group in temperate conditions (D) Mean heart rate for each group in hot conditions (E) Mean core body temperature for each group in temperate conditions (F) Mean core body temperature for each group in hot conditions All data presented in grouped mean bar charts spanning all timepoints (pre, post, post+1) with individual data represented by points and individual changes between time points represented by connecting line. Significant effect of time is represented by § with threshold set to $P < 0.05$. Differences following post-hoc analysis are represented by; "α" a significant difference from pre-to post, "β" a significant difference from pre to post+1 and "γ" a significant difference between post and post+1.

5.3.4 Training Response

5.3.4 i) Physiological Measures

Relative mean power (% MAP) during LIT exercise significantly differed over time. (F(2.688, 53.75) = 4.658, P = 0.008, d = 0.10) and between groups (F(2, 20) = 3.599, P < 0.05, d = 0.1). Mean power output was consistent across the intervention in the CON group (~60% MAP) in line with the prescribed exercise intensity. Power output was significantly reduced in the SL group during the first two low- carbohydrate sessions (days 2 and 4) (P = 0.007 & P = 0.003, respectively) before returning to prescribed exercise intensity across the remaining sessions irrespective of carbohydrate availability. The SL_{Heat} group reduced power output throughout the training intervention, with exercise intensity further reduced on low carbohydrate days (2, 3, 6, 9 and 10) (Figure 5.4B). During HIT exercise, mean relative power output (% MAP) was significantly different over time (F(2.543, 50.86) = 8.835, P = 0.0002, d = 0.03) and between groups (F(2, 20) = 5.090, P = 0.02, d = 0.30). Power output increased for SL_{Heat} towards mean power observed in CON and SL groups (Figure 5.5B). Mean HR decreased throughout the training intervention (F(5.537, 110.7) = 4.082, P = 0.001, d = 0.03) with no difference between groups (F(2, 20) = 2.723, P = 0.09, d = 0.17) during LIT exercise (Figure 5.4C). Mean HR (% maximal HR) was significantly higher in the SL_{Heat} group compared to CON and SL groups throughout the 2-week intervention during HIT exercise (F(2, 20) = 18.83, P < 0.0001, d = 0.60) (Figure 5.5C).

5.3.4 ii) Perceptual Measures

During the LIT exercise, RPE was significantly different between groups (F(2, 20) = 11.58, P = 0.0005, d = 0.27) with differing responses (G ×T = F(22, 220) = 5.378, P < 0.0001, d = 0.11) over time (F(4.552, 91.04) = 17.66, P < 0.0001, d = 0.18) (Figure 5.4D). There was

a statistically significant interaction effect for thermal comfort during LIT exercise ($F(22, 220) = 3.798$, $P < 0.0001$, $d = 0.12$) with a significant main effect for group ($F(2, 20) = 18.00$, $P < 0.0001$, $d = 0.32$) and time ($F(5.108, 102.2) = 5.970$, $P < 0.0001$, $d = 0.09$) (Figure 5.4E). Group ($F(2, 20) = 10.46$, $P = 0.0008$, $d = 0.30$) had a significant main effect on thermal sensation during the exercise also; however, there was no effect of time ($F(5.048, 101.0) = 1.616$, $P = 0.16$, $d = 0.03$) (Figure 5.4F).

During the HIT exercise, there was a significant interaction effect for mean RPE ($F(10, 100) = 2.743$, $P = 0.005$, $d = 0.14$). Principally, RPE is consistent across the intervention for the CON group, whilst opposing trends are present for SL and SL_{Heat}, with each increasing and decreasing, respectively (Figure 5.5D). There was no main effect of time ($F(3.739, 74.79) = 0.2207$, $P = 0.92$, $d = 0.006$) or group ($F(2, 20) = 1.919$, $P = 0.1729$, $d = 0.06$) present.

5.3.1 Dietary Intake

Mean energy intake increased significantly during training compared to baseline ($F(1.363, 27.27) = 83.10$, $P < 0.0001$, $d = 0.49$) for all groups ($F(2, 20) = 0.02171$, $P = 0.98$, $d = 0.0008$) with significant increases in carbohydrate from baseline ($F(1.639, 32.78) = 170.1$, $P < 0.0001$, $d = 0.81$) across each group ($F(2, 20) = 0.6556$, $P = 0.53$, $d = 0.005$). Protein intake was significantly increased during training from baseline ($F(1.837, 36.74) = 25.62$, $P < 0.0001$, $d = 0.38$) with a significant reduction in fat intake ($F(1.661, 33.22) = 22.65$, $P < 0.0001$, $d = 0.41$) with no difference between groups for protein ($F(2, 20) = 1.672$, $P = 0.21$, $d = 0.03$) or fat intake ($F(2, 20) = 1.720$, $P = 0.20$, $d = 0.03$). Mean daily energy and macronutrient intake are reported in Table 5.4.

Table 5.4 – Mean daily energy and macronutrient intake for control (CON), sleep low (SL) and sleep low and heat (SL_{Heat}) groups before the training programme (baseline) and during the training-nutrition-environmental intervention.

		Energy (kcal·d^{-1})	**CHO** (g·kg^{-1}·d^{-1})	**Fat** (g·kg^{-1}·d^{-1})	**Protein** (g·kg^{-1}·d^{-1})
CON	**Baseline**	2280 ± 231	3.5 ± 0.4	1.2 ± 0.1	1.8 ± 0.2
	Training	2890 ± 265*	5.8 ± 0.1*	1.0 ± 0	2.0 ± 0
SL	**Baseline**	2339 ± 436	3.4 ± 0.6	1.3 ± 0.2	1.5± 0.1
	Training	2919 ± 306*	5.6 ± 0.5§	1.1 ± 0.1	1.9 ± 0.2*
SL_{Heat}	**Baseline**	2208 ± 265	3.2 ± 0.5	1.2 ± 0.0	1.5 ± 0.1
	Training	2922 ± 295*	5.6 ± 0.4*	1.0 ± 0.1	1.9 ± 0.2*

** Denotes a significant difference from baseline, with P < 0.05. CHO represents carbohydrate*

5.3.1 Body Composition

There was a significant main effect of time on mean body mass ($F(1, 19) = 8.164$, $P = 0.01$, $d = 0.002$) with no significant effect of group ($F(2,19) = 0.08455$, $P = 0.92$, $d = 0.009$). SL significantly decreased body mass by 1.3% from 73.1 kg to 72.2 kg from pre-to-post, respectively ($P = 0.03$). There was no change in mean body mass in SL_{Heat} (72.7 kg to 72.1 kg [-1%]) or CON groups (71.4 kg to 71.4 kg [-0.1%]). In line with changes in body mass, there was a significant main effect of time on fat mass ($F(1, 19) = 25.09$, $P < 0.0001$, $d = 0.009$) with a significant interaction effect ($F(2, 19) = 9.317$, $P = 0.0015$, $d = 0.007$). Although post-hoc analysis failed to identify significant differences between groups or time points, reductions in fat mass were observed in SL and SL_{Heat} groups. There was no difference in fat-free mass following the intervention ($F(1, 19) = 0.7196$, $P = 0.41$, $d = 0.001$) or between groups ($F(2, 19) = 0.3923$, $P = 0.68$, $d = 0.04$).

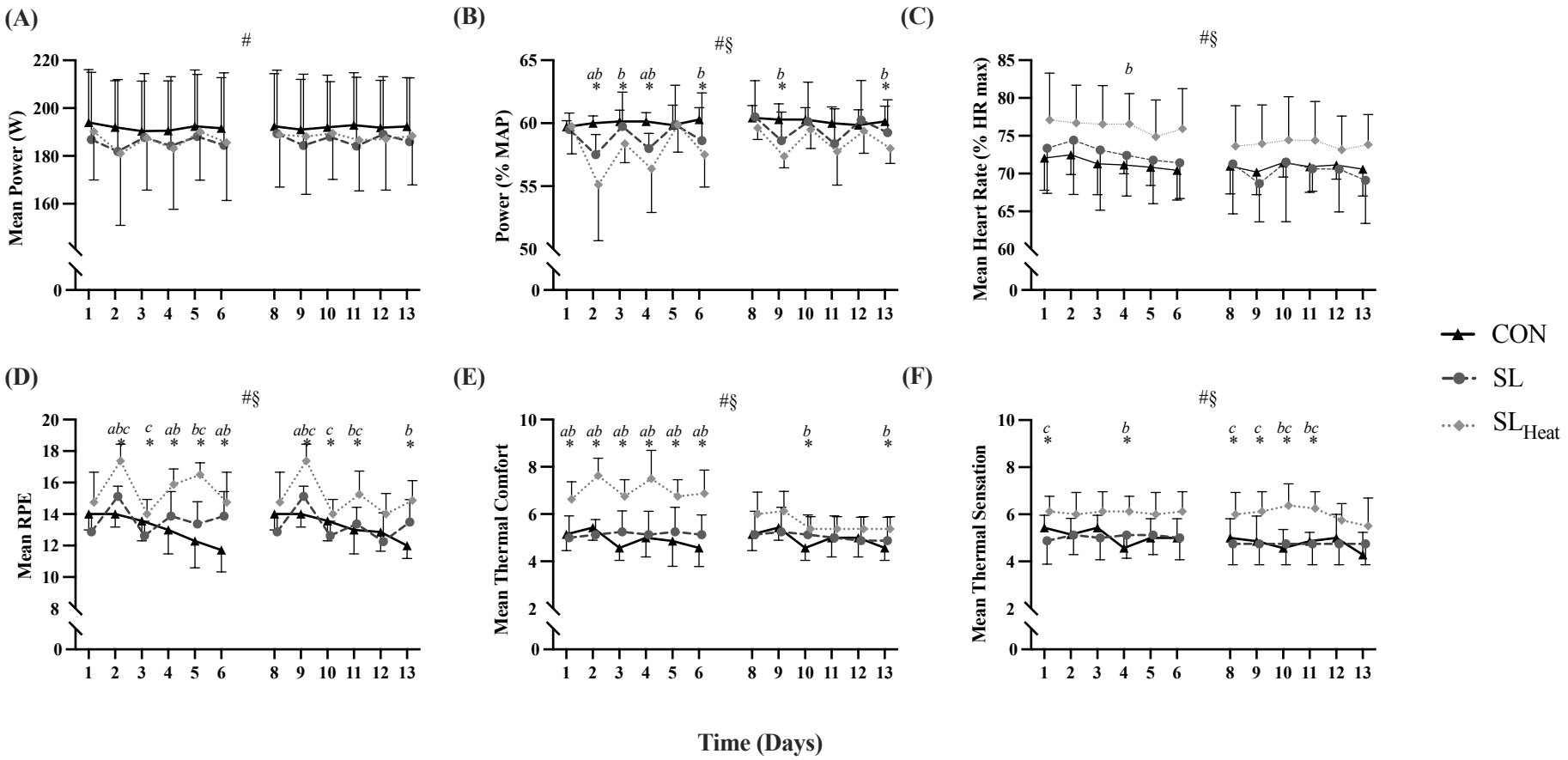

Figure 5.4 – Training Data for daily low intensity training (LIT) (A) Mean power output (W), (B) Relative Mean power output (% MAP), participants were asked to maintain 60% for the hour, (C) Mean heart rate (beats·min-1), (D) Mean RPE, (E) Mean Thermal Comfort, (F) Mean Thermal Sensation. ANOVA outputs represented as - Main Group effect represented by '#'and Time effect by '§'. Post Hoc analysis represented as significantly different by a *. Pairwise comparisons "a" denotes significant difference between CON and SL "b" denotes a significant difference between CON and SL_{Heat} "c" denotes a significant difference between SL and SL_{Heat}. A significance level of $P < 0.05$ was used.

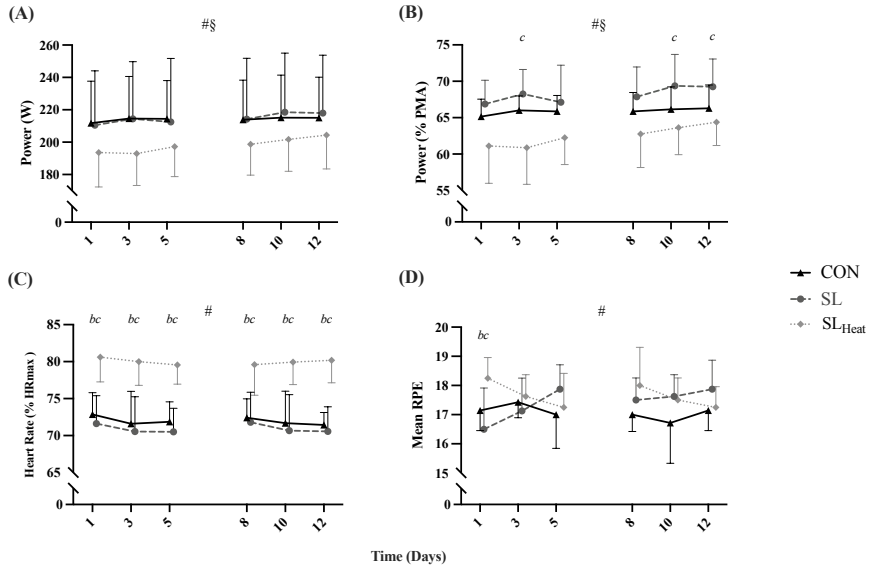

Figure 5.5 – Training data for high intensity training (HIT) (A) Mean power output (W), (B) Relative mean power output (% MAP), (C) Mean heart rate (beats·min^{-1}), (D) Mean RPE. ANOVA outputs represented as - Main Group effect represented by '#'and Time effect by '§'. Post Hoc analysis represented as significantly different by a *. Pairwise comparisons "*a*" denotes significant difference between CON and SL "*b*" denotes a significant difference between CON and SL_{Heat} "*c*" denotes a significant difference between SL and SL_{Heat}. A significance level of $P < 0.05$ was used.

5.4 Discussion

The primary aim of this study was to determine the efficacy of periodising carbohydrate intake during two weeks of heat acclimation provided a performance benefit in hot (~35ºC) and temperate (~20ºC) conditions. To achieve this, a traditional heat acclimation protocol (Periard, Racinais and Sawka, 2015) was adapted and combined with a "sleep-low, train-low" carbohydrate periodisation approach (Marquet et al., 2016a), whereby carbohydrate availability was manipulated before and after specific sessions via exercise and nutrition. HIT sessions were completed on alternating evenings to achieve low muscle glycogen concentration, which was maintained overnight by withholding carbohydrate intake. A fasted LIT session was completed the following morning. Strategically reducing carbohydrate availability for specific exercise bouts in temperate conditions induced a shift towards greater fat oxidation for a given

workload and improved cycling performance. By comparison, no metabolic or performance benefits were observed in either SL_{Heat} or control groups. Furthermore, there were no improvements in performance across SL or CON in hot conditions. Despite hallmarks of heat acclimation (decreased core body temperature, reduced blood lactate and improved perception of heat stress), performance was not enhanced in SL_{Heat} immediately following 2 weeks of heat acclimation but did improve following a short period of recovery.

Implementing an active heat acclimation strategy to improve exercise performance in the heat is ubiquitously positive (Tyler et al., 2016; Benjamin et al., 2019) and attributed to multiple integrated physiological and psychological factors (Periard, Racinais and Sawka, 2015). Of the results presented here, the lack of performance improvement in SL_{Heat} following 2 weeks of heat acclimation with periodised carbohydrate intake is concerning. The lack of immediate performance improvement may be due to reduced exercise training intensity in the heat, leading to reduced mechanical work rates during training (Drust et al., 2005; Boynton et al., 2019), a response which is further compounded with the addition of low carbohydrate availability (Pitsiladis and Maughan, 1999). The implementation of a constant work rate regimen (daily exposures at 60% MAP) may represent a suboptimal strategy compared to progressive overload approaches such as controlled hyperthermia (to a core temperate of 38.5ºC) (Taylor, 2000; 2014). The latter strategy would allow for continual adaptation due to the forcing function (i.e. metabolic heat production) being continually increased through the manipulation of internal thermal load (as work capacity increases over time) (Taylor, 2000). Similarly, a controlled HR approach may have been preferred, as an initial decrease in power output would be diminished as heat acclimation occurs (Febbraio et al., 1994a) whilst maintaining comparable physiological loads between hot and temperate conditions (Maunder et al., 2021b). Furthermore, the constant work rate approach (fixed power output) used in this

study induces a state of physiological habituation as tolerance to heat stimulus increases over time (Taylor, 2014), the influence of which progressively reduces as adaptations develop (Eichna et al., 1950; Fox et al., 1963; Rowell et al., 1967).

Superimposing additional stressors during exercise, although potentially positive for endurance adaptation and performance, likely constituted a substantial period of intensified training load for participants subject to periodically training with low carbohydrate availability and heat stress. It has previously been reported that exercise alone in hot conditions may suppress appetite (Jeukendrup et al., 1992; Hedelin et al., 2000; Halson et al., 2002), elevate stress hormones and heart rate during exercise (Hargreaves et al., 1996a) collectively negatively impacting exercise adherence (Sylta, Tonnessen and Seiler, 2014) and inducing maladaptation (Reeve et al., 2019). Greatly increasing training load increases acute fatigue and impairs short-term exercise performance characterised by reduced $\dot{V}O_{2max}$, PPO and HR (Jeukendrup et al., 1992; Hedelin et al., 2000; Halson et al., 2002). Following 2 weeks of exercise in hot conditions with periodised carbohydrate availability, exercise capacity and performance were impaired. Crucially, increasing training intensity, volume or combining multiple physiological stressors may result in short-term impairments in exercise performance with performance restored or improved following a period of adequate recovery, i.e., overreaching (Jeukendrup et al., 1992; Halson et al., 2002). To account for acutely impaired performance, participants repeated the testing battery 1 week after the completion of the study, allowing for a period of recovery. Performance was improved in the previously unchanged SL_{Heat} group in line with previous literature characterising the time course of performance in response to a period of intensified exercise training (Halson et al., 2002). The evidence presented here highlights the need to manage training load and accompanying recovery effectively to ensure athletes are prepared appropriately for competition. Furthermore, the

results presented here do not disqualify this strategy from elite athlete populations with typically higher training loads who may be better able to tolerate additional stressors during training. Further research should be conducted in elite population groups with athlete case studies potentially insightful for using carbohydrate periodisation in the heat.

When completing carbohydrate periodisation only, data reported within this thesis reveals improved performance and metabolic adaptations, with altered substrate utilisation and enhanced time-trial performance. Previous applications of "sleep low – train low" have completed consecutive bouts of low carbohydrate training across 3 days, with exercise and nutrition used to manipulate carbohydrate availability for specific sessions (Marquet et al., 2016a; 2016b; Riis et al., 2019). To amalgamate a heat acclimation protocol and the SL-TL strategy, a novel exercise-nutrition strategy was implemented to permit the manipulation of carbohydrate availability across a heat acclimation protocol with HIT sessions completed on alternate days to deplete muscle glycogen. The following morning session was completed in a low carbohydrate state, meaning that only half of the low-intensity sessions were conducted with low carbohydrate availability. Despite the reduced proportion of sessions conducted with low carbohydrate, there was a 6.7% increase in mean power for the 30 min capacity test in temperate conditions following 2 weeks of SL-TL and maintained following 2 weeks of recovery. Typically, sleep low strategies have been implemented for between 3 and 5 weeks, with the present study being the shortest duration to provide a positive performance response. Evidence of improved performance following a period of Sleep-low remains equivocal, with studies reporting decreased 10km running time (-2.9%), increased supra-maximal time to exhaustion (+11%) (Marquet et al., 2016a) and increased FTP (+5.5%) (Bennett et al., 2021) following 3 weeks of nutrition and exercise intervention. These performance benefits have been disputed within the literature, with no additional benefit of SL being observed over high

carbohydrate diets in several studies (Riis et al., 2019; Burke et al., 2020). A recent meta-analysis assessing the performance benefits of carbohydrate periodisation indicated no benefit to training with periodically low carbohydrate availability. The present study highlights the necessity for further research to understand potential benefits and optimise training and carbohydrate periodisation strategies (Gejl and Nybo, 2021).

Whilst fat oxidation is shown to be increased acutely when training with low carbohydrate availability (Lane et al., 2015; Maunder et al., 2021a), limited evidence exists showing long-term improvements following the chronic SL-TL intervention (Marquet et al., 2016a; 2016b; Salokannel, Hakulinen and Ahtiainen, 2021), with some literature suggesting despite acute increases in fat oxidation, this is not maintained following 3 weeks of sleep low (Riis et al., 2019). In contrast to these studies, data presented here provide evidence that strategically training with low carbohydrate availability decreases HR, RER, and subsequent carbohydrate: fat substrate utilisation at a fixed exercise intensity following 2-weeks of exercise nutrition intervention and sustained for a further week. Compared to previous literature, the sustained metabolic adaptation observed in this study may be due to the altered training regimen implemented here. For trained individuals, completing 3 LIT sessions per traditional SL-TL approach likely constitutes a sub-optimal training load, resulting in blunted adaptation across the intervention period. Future SL-TL interventions should consider altering training prescription to reflect the habitual training practices of athletes more closely.

Interestingly, a difference was observed across all measures during the substrate utilisation test despite completing the same exercise-nutrition intervention in the SL_{Heat} group compared to SL, suggesting a deleterious effect of heat acclimation on substrate utilisation associated with SL-TL. The increase in fat oxidation during exercise with low carbohydrate

availability contradicts the increase in carbohydrate utilisation observed during exercise in the heat, including increased muscle glycogenolysis and carbohydrate oxidation (Febbraio et al., 1994a; 1996a; Hargreaves et al., 1996a; Febbraio, 2001). Whilst heat stress and low carbohydrate availability are likely to increase specific molecular pathways associated with endurance training adaptation, such as AMPK activation and PGC-1α expression, a reduction in fat oxidation associated with exercise in the heat is likely to negatively impact PPAR signalling and impair adaptation associated with fat oxidation.

Alongside a comprehensive battery of performance tests, the present chapter characterises the training responses during exercise in hot and temperate conditions with periodised carbohydrate availability in line with the fuel for the work required paradigm (Impey et al., 2016). As with previous sleep low literature, exercise intensity was reduced during exercise with low carbohydrate availability (Yeo et al., 2008b; Hulston et al., 2010) but restored following the first week of exercise (Hulston et al., 2010; Bennett et al., 2021). During the first week of training, SL_{Heat} exercise intensity was impaired, which was restored in the second week. During training sessions with low carbohydrate availability, exercise intensity is impaired to a greater extent in the heat than in temperate conditions. Together, training and performance data highlight the importance of ensuring adequate carbohydrate provision during exercise in the heat. Traditional nutritional guidelines for exercise in the heat have advised increased carbohydrate intake to account for increased carbohydrate metabolism (Burke, 2001) and to ensure glycogen resynthesis between exercise bouts.

Unfortunately, the omission of a heat acclimation group makes it difficult to draw conclusive statements regarding the role of each stressor on performance. Including a heat acclimation group that completed all sessions with “normal” carbohydrate availability (in line

with CON) would have allowed for assessing the present heat acclimation protocol's impact on exercise performance and identifying the potential differences between groups. Nevertheless, SL_{Heat} showed key characteristics of heat acclimation throughout the intervention, including increased work rate, decreased HR, improved thermal tolerance, reduced core body temperature and blood lactate during the 30-minute trials. Based on the current literature, a lack of improvement during exercise in the heat is unprecedented and undoubtedly raises concerns when considering nutritional intervention during training in the heat.

It is increasingly common for endurance athletes to travel to warm-weather training camps (Maunder et al., 2019) to increase training stimulus and maximise adaptive response (Hawley et al., 2018). Given the potential for nutritional manipulation to amplify the training response (Hawley and Morton, 2014), it would not be implausible for athletes to combine multiple stressors to induce greater adaptation. Tentative evidence exists to suggest increases in mitochondrial adaptation in response to heat acclimation in humans (Maunder et al., 2021c) with supporting *in vivo* (Tamura et al., 2014) and *in vitro* (Liu and Brooks, 2012; Tamura and Hatta, 2017; Patton et al., 2018) mechanistic data suggesting skeletal muscle signalling responses associated with endurance adaptation are augmented following heat stress. Comparatively, human studies have shown increased post-exercise signalling responses following exercise with reduced carbohydrate availability (Pilegaard et al., 2000; 2002; 2005; Morton et al., 2009; Bartlett et al., 2013); however, combining heat acclimation and low carbohydrate availability during specific sessions does not benefit performance. The lack of invasive measures precludes us from investigating the molecular responses following exercise bouts in heat stress or low carbohydrate availability, meaning the mechanisms by which changes in performance occur are still unknown, nor whether the associated signalling

responses are augmented or reduced following combined exercise, carbohydrate manipulation and heat stress.

5.4.1 Practical Implications

With major sporting competitions regularly held in hot environments and extreme weather increasingly commonplace, endurance athletes are increasingly required to consider the impact of heat stress on performance. Currently, nutritional guidelines during exercise and heat stress recommend increased carbohydrate intake to account for increased utilisation during exercise (Burke, 2001). The present study has highlighted that periodically training with low carbohydrate availability in hot environmental conditions is not conducive to performance or metabolic adaptation in trained triathletes. Together with the data presented here, the importance of managing training load during exercise and heat stress is evident. Superimposing additional physiological stress, including reducing carbohydrate availability, during exercise in hot conditions leads to impaired exercise capacity and symptoms of overreaching. Practically, manipulation of carbohydrate availability during exercise should be avoided until the short-term physiological demands of heat stress are reduced with specific attention paid to managing athlete training load.

These data support the concept of ‘fuelling for the work required’ whereby carbohydrate availability is strategically manipulated per the specific exercise demands. Crucially, the present study provides further evidence of the performance and metabolic benefits of training with low carbohydrate availability. Alongside these findings, this study also documents the daily training response throughout the intervention, highlighting that

performance is still improved despite reductions in training intensity during low carbohydrate training sessions.

In collaboration with the French Triathlon Federation, the data generated during this study has been used to inform the training and nutritional strategy. Specifically, the practical application of this work has ensured that training load and dietary intake are monitored during hot weather training camps with a specific focus on previously unacclimated athletes. As a result, French Triathletes subsequently won a mixed team relay bronze medal at the Tokyo 2020 Olympic Games and multiple ITU World Championship Podiums since 2018.

5.4.2 Conclusion

Despite the hallmarks of heat acclimation, there is no immediate benefit to periodising carbohydrate intake during a 2-week heat acclimation protocol, with improvement in exercise capacity in the heat only occurring after recovery. Moreover, further important metabolic and performance data has been reported in response to an altered “sleep-low” strategy, with increased fat oxidation and improved performance following two weeks of carbohydrate periodisation in temperate conditions despite relative reductions in training exercise intensity.

Chapter 6 – Proof-of-Concept and Optimisation of Exercise-Heat Stress Model in C2C12 Myotubes

Before in vitro experimentation, previously reported cell culture models were implemented to ensure the integrity of cell population following treatment(s). Additionally, pilot experiments were conducted to optimise the treatment during subsequent in vitro experiments.

6.1 Introduction

Human *in vivo* experiments represent highly integrated physiological models to investigate the effect of environment, exercise and diet on health, performance, and adaptation. In chapters 4 & 5, the SL-TL carbohydrate periodisation strategy shows clear benefits to endurance performance and whole-body metabolism in temperate conditions. In contrast, metabolic and performance outcomes were blunted when exercise training was completed in elevated environmental temperatures. Mechanistically, understanding the tissue-specific impact of exercise and heat stress is limited to observational insights from skeletal muscle biopsies, which are limited to a small snapshot of the intramuscular environment in response to stress. Nevertheless, the advent of the muscle biopsy technique within exercise physiology has allowed the characterisation of gene expression, protein content and molecular signal transduction pathways in response to physical activity. Given the ethical and logistical constraints of manipulating specific molecular pathways in humans, the development and optimisation of *in vitro* experimental model's representative of *in vivo* exercise are essential to exercise physiologists. Utilising *in vitro* models of skeletal muscle, including immortalised cell lines (mouse C2C12 or L6) or isolated skeletal muscle from humans or animals, has allowed the elucidation of muscle-specific responses to 'exercise-like' treatments, including electrical stimulation and pharmacological agents. As such, *in vitro* exercise models have permitted the investigation of specific aspects of exercise on skeletal muscle, such as muscular contraction, heat stress or substrate availability.

Skeletal muscle contraction during physical activity induces significant intra-muscular homeostatic disturbance, which activates multiple contraction-mediated signalling pathways, leading to exercise adaptation. *In vitro,* electrical pulse stimulation (EPS) of C2C12 myotubes

has shown robust, physiologically comparable responses to skeletal muscle contraction *in vivo* and has been extensively reviewed elsewhere (Nikolic et al., 2017; Carter and Solomon, 2019). Briefly, EPS significantly increases Ca^{2+} transients (Fujita, Nedachi and Kanzaki, 2007; Valdes et al., 2008), induces exercise-mediated signal transduction pathways (Fujita, Nedachi and Kanzaki, 2007; Nedachi, Fujita and Kanzaki, 2008; Lambernd et al., 2012; Pourteymour et al., 2017), enhances glucose uptake (Lambernd et al., 2012) and induce mitochondrial biogenesis (Nikolic and Aas, 2019) as well as induce morphological changes such as sarcomeric structure formation (Fujita, Nedachi and Kanzaki, 2007), all considered critical hallmarks of endurance training and subsequent adaptation.

Additionally, during exercise *in vivo,* intrinsic heat production results in local hyperthermia because of metabolic heat production associated with ATP resynthesis. Mild heat stress alone has been shown to increase ATP flux and thus AMP levels, leading to AMPK activation (Corton, Gillespie and Hardie, 1994), with more recent *in vitro* investigations showing increased muscular transcriptional response post-exposure in C2C12 myotubes (Liu and Brooks, 2012). Chronically applying heat stress over 6-7 days (1 hr daily) increases the mitochondrial protein content and elevates respirational capacity (Liu and Brooks, 2012; Patton et al., 2018). Whilst it is well established that exercise *in vivo* involves skeletal muscle contraction and increases in muscle temperature, the effect of combined EPS and heat stress on markers of skeletal muscle adaptation is yet to be investigated.

The present study's primary aim is to combine EPS and heat stress to assess the efficacy of such a model as a more representative model of *in vivo* exercise. Secondly, using the evidence generated within this study, optimal treatment duration will be identified for metabolomic C2C12 experiments within this thesis (Chapter 7). Finally, based on the positive

"endurance-like" adaptations observed in response to heat stress and EPS independently *in vitro*, the present study hypothesised that combined heat stress and EPS would provide greater exercise-memetic stimulus leading to increased metabolic rate and an augmented post-treatment transcriptional response.

6.2 Methods

6.2.1 Cell Culture

A detailed description of cell culture approaches implemented throughout this experiment has been previously described in Chapter 3.6. Following differentiation, cells underwent 3 hrs of treatment, including a control (cells maintained at 37°C, CON), heat only (cells heated to 40°C, Heat Only), Electrical pulse stimulation (EPS, cells stimulated at 10Hz at 37°C, Stim Only) and cells simulated in 40°C (Heat & Stim). At the termination of the experiment, cells were either immediately processed or washed with cold PBS (~3°C) and dried before being transferred to -80°C until processing.

6.2.2 Assessment of Myotube Structure

Detailed methods for staining and immunofluorescence imaging have been described previously (Chapter 3.6.9), with images generated to visually assess myotube quality post-differentiation and post-treatment.

6.2.3 Assessment of C2C12 Transcriptional Response to Heat Stress

C2C12 myotubes have been shown to have significant endurance-type transcriptional response following bouts of acute heat stress (Liu and Brooks, 2012). To validate the impact

of EPS and Heat Stress on traditional transcriptional markers of endurance training adaptation (PCG-1α, SIRT1, Tfam, COX4 and NRF2), PCR analysis was completed following 1, 3 and 24 hrs of heat stress (40°C) or stimulation compared to cells maintained at 37°C. Methods of mRNA isolation, quantification and PCR are described in detail in Chapter 3.6.10. Unfortunately, PCR machines were decommissioned following initial analysis due to technical failure within the laboratory. Resultant PCR data is therefore incomplete with Stimulation and Heat stress conditions lacking transcriptional analysis. Therefore, only the data for completed conditions is presented (Control, Stimulation Only and Heat Only).

6.2.4 Assessment of Cell Viability

To elucidate the impact of stimulation, heat stress or both on cell viability, cells were subject to MTT plate-based assay after experimentation. Briefly, wells were treated with the desired experimental condition for 1, 3 or 24 hrs before the MTT solution was added. Following a 3-hr incubation, the colour change of MTT was assessed via plate reader to determine cell viability (detailed description of methodology in Chapter 3.6.11). Due to the potential for increased metabolic activity during apoptosis, validation of cell health was completed using Propidium Iodide to quantify the live cell population (Chapter 3.6.12). Propidium iodide was added to cells immediately post-treatment before assessment via flow cytometry.

6.2.5 Assessment of Metabolic Activity

Once cell health was confirmed, assessment of metabolic activity in response to treatment was investigated. To provide insight into the metabolic activity of cells during each experimental condition, MTT was added immediately before 3 hr treatment (in line with the

usual incubation period), and colour change was assessed immediately post. Any colour change observed between conditions would indicate increased metabolic activity due to the mitochondria's greater production of formazan crystals.

6.2.1 Statistical Analysis

Firstly, data were assessed for normality via the Shapiro-Wilk test (Shapiro and Wilk, 1965). For data generated by MTT assay, PI assay and PCR one-way ANOVA were used to assess differences between treatment conditions (control vs. Stim Only vs Heat Only vs Heat & Stim). Data analysis was completed using Graphpad Prism v.9 (Graphpad Software, CA, USA). All data reported as mean ± standard deviation unless otherwise stated.

6.3 Results

6.3.1 Myotube Structure

Immunofluorescent images of C2C12 myotubes pre-and post-exposure to Stim Only, Heat Only, and Heat & Stim can be seen in Figure 6.1. Upon visual inspection, no differences between pre-treatment myotube quality were observed, which was maintained post-treatment. Overall, one exposure to Stim Only, Heat Only, and Heat & Stim did not alter myotube structure. In addition, there were no visible instances of myotubes undergoing significant atrophy or hypertrophy, and all cells remained adhered to the plate.

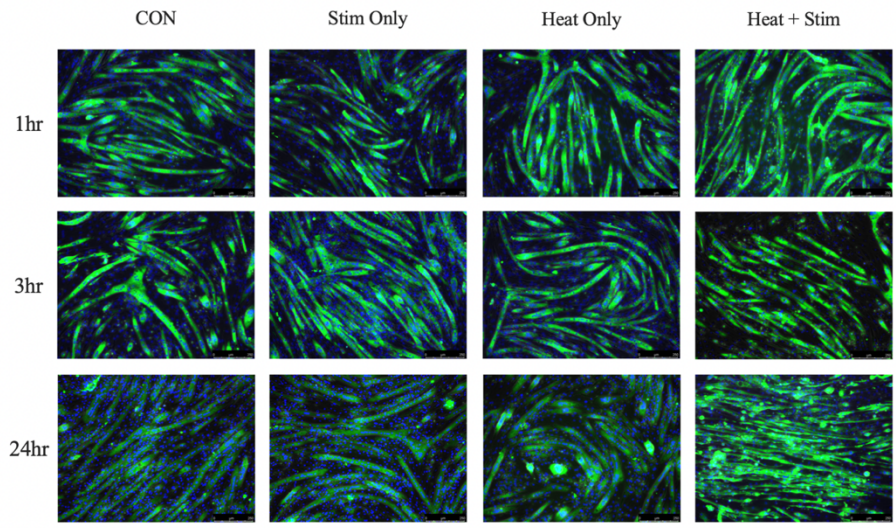

Figure 6.1 – Immunofluorescence microscopy images of C2C12 myotubes stained for myosin heavy chain (MyHC) (Green) and nuclei (Blue) following 1, 3 and 24 hrs of Stim Only, Heat Only and Heat & Stim.

6.3.2 Transcriptional Response

C2C12 myotubes displayed a significant transcriptional response following Heat Only and Stim Only. One hr of heat stress was not sufficient to induce a transcriptional response with no target genes increasing mRNA content. However, PGC-1α transcription was significantly increased ($F(3, 13) = 16.22$, $P = 0.0001$) with no significant increase following 1 hr of heat stress albeit significantly increased following 3 (2.1-fold) and 24 (2.9-fold) hrs of heat stress ($P = 0.03$ and $P = 0.0001$ respectively). In response to stimulation, PGC-1α transcription was similarly increased ($F(3, 13) = 4.530$, $P = 0.022$) with a transcription increase following 3 hours of stimulation only (2.3-fold) ($P = 0.009$).

In the case of SIRT1, transcriptional activity was significantly increased in response to Heat stress ($F(3,14) = 4.6$, $P = 0.02$) and stimulation ($F(3, 14) = 6.899$, $P = 0.004$). Three hours of heat stress induced a 2.9-fold increase ($P = 0.008$) in transcriptional activity alongside a comparable 2.8-fold increase in response to 3 hrs stimulation ($P = 0.01$) which was maintained

following 24 hours ($P = 0.02$). Tfam transcription was significantly increased in response to heat stress ($F(3, 10) = 16.38$, $P = 0.0003$) with a 2.4-fold increase following 3 ($P = 0.0034$) and 24 hours ($P = 0.0024$). Stimulation similarly increased Tfam transcription ($F(3, 16) = 233.5$, $P < 0.0001$) with a 2.1-fold increase following 1 ($P < 0.0001$) and 3 ($P < 0.0001$) hours of stimulation. NRF2 transcription was increased following heat stress ($F(3, 13) = 7.764$, $P = 0.0032$) and stimulation ($F(3, 16) = 49.99$, $P < 0.0001$). Following 3 hrs and 24hrs heat stress, an 8.2 ($P = 0.0158$) and 10.4 ($P = 0.0073$) fold increase in gene transcription was observed, respectively. One hour of stimulation increased transcription by 2.2-fold ($P < 0.0001$), with a 2.3-fold increase following 3 hours ($P < 0.0001$). COX4 transcription was increased in response to heat stress ($F(3, 14) = 4.654$, $P = 0.02$); however, Bonferroni post hoc analysis failed to report significant increases from control, specifically following 3 ($P = 0.07$) and 24 ($P = 0.06$) hours of heat stress. COX4 was unresponsive to stimulation ($F(3, 16) = 11.01$, $P = 0.0004$) with a significant decrease in transcription (0.5-fold) following 24 hours of treatment ($P = 0.0032$)

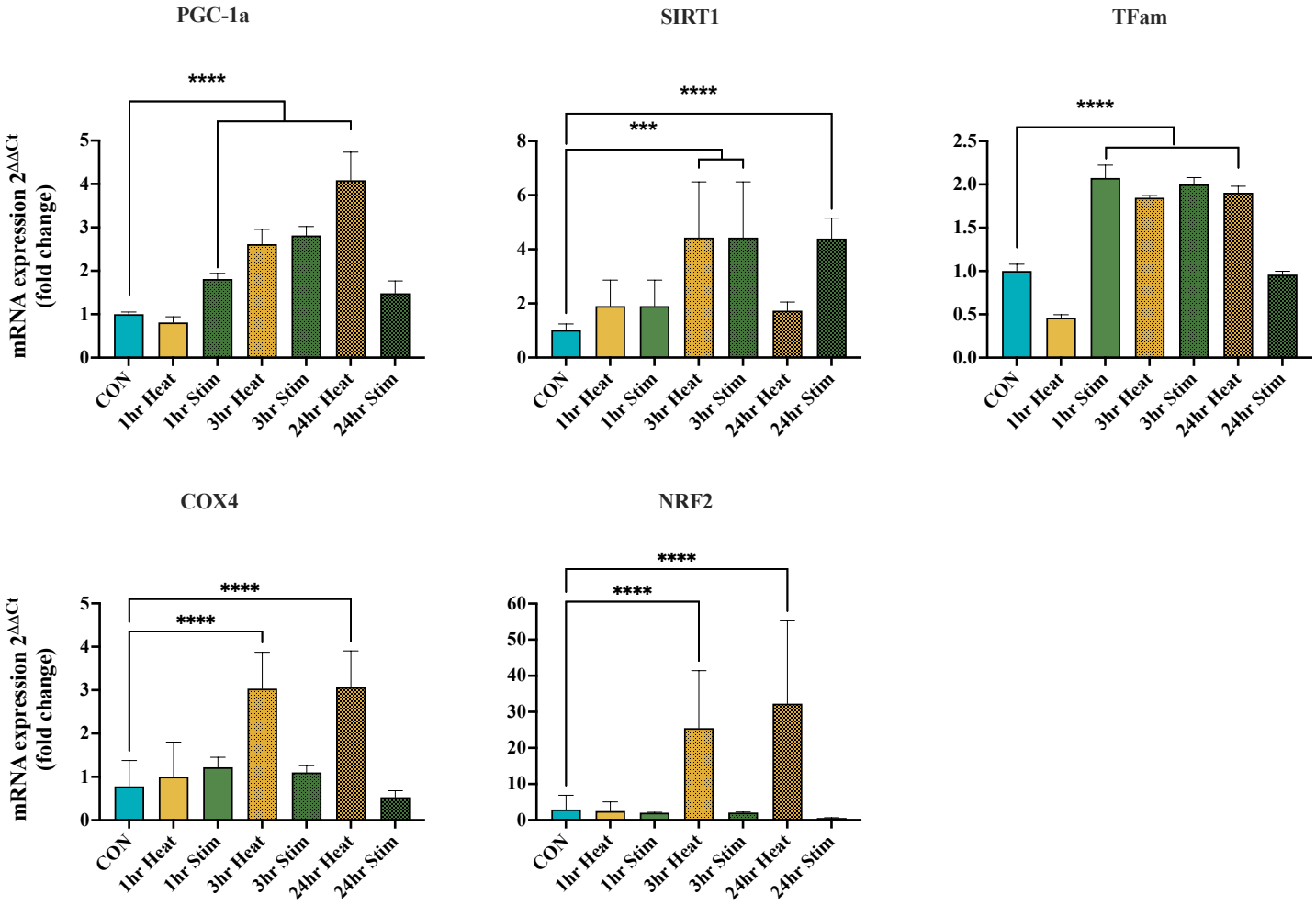

Figure 6.2 – Transcriptional response immediately following 1, 3 or 24 hours of acute Heat Only or Stim Only. Blue Bars represent transcriptional response of time match CON cells maintained at 37°C, yellow bars represent Heat Only, and green bars represent Stim Only. Shading increases as the duration of treatment increases. * Denotes significant difference from CON with a significance threshold of $P < 0.05$.

6.3.3 Metabolic Activity

Divergent metabolic responses between experimental conditions ($F(3, 8) = 18.48$, $P = 0.0006$) were observed. Metabolic activity during treatment increased in Stim only (15 ± 4 %) ($P = 0.016$) with a decrease in Heat Only (- 9 ± 5 %) ($P = 0.046$) and no change in Heat & Stim (11 ± 3%) ($P = 0.09$) conditions compared to control (Figure 6.3).

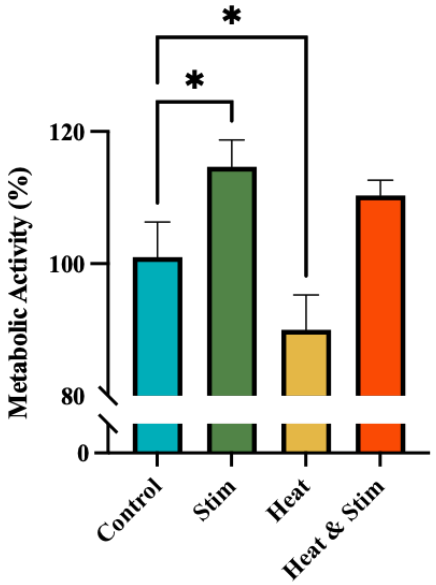

Figure 6.3 – Metabolic Activity immediately following 3 hours of Stimulation (Green), Heat Stress (yellow) or combined heat & stimulation (orange) measured via MTT assay. Data reported as mean ± SD. * Denotes significant difference from CON with a significance threshold of $P < 0.05$.

6.3.4 Cell Viability

A main effect of group ($F(3, 20) = 24.09$, $P < 0.0001$) was identified for cell viability quantified by MTT assay. No time ($F(1.94, 38.82) = 0.17$, $P = 0.84$) or interaction conditions ($F(6, 40) = 1.25$, $P = 0.3$) effects were reported. There was no difference between groups following 1 and 24 hrs of treatment. Three hours of treatment induced significant alterations to

metabolism for Stim only (+12 ± 5 %) ($P < 0.001$), Heat Only (+11 ± 4 %) ($P < 0.001$) and Heat & Stim (- 4 ± 1 %) ($P < 0.001$) compared to control (Figure 6.4A).

Propidium Iodide assay revealed significant interaction ($F(6, 16) = 8.82$, $P = 0.0003$), group ($F(3, 8) = 39.31$, $P < 0.0001$) and time effect ($F(1.59, 12.72) = 106.1$, $P < 0.0001$). Following 1 hr of treatment, Heat & Stim significantly increased the proportion of dead cells (5.46 ± 0.27 %) compared to the control (2.01 ± 0.42 %) ($P < 0.005$). There was no difference in dead cell population following 3 hrs of treatment; however, 24 hrs of treatment significantly increased dead cell population following Heat Only (5.55 ± 0.3 %) ($P < 0.01$) with Heat & Stim (5.81 ± 0.49%) trending toward a significant difference ($P = 0.05$) compared to Control cells (3.58 ± 0.14%) (Figure 6.4B). Flow Cytometry outputs are included in appendices (Figure 11.1).

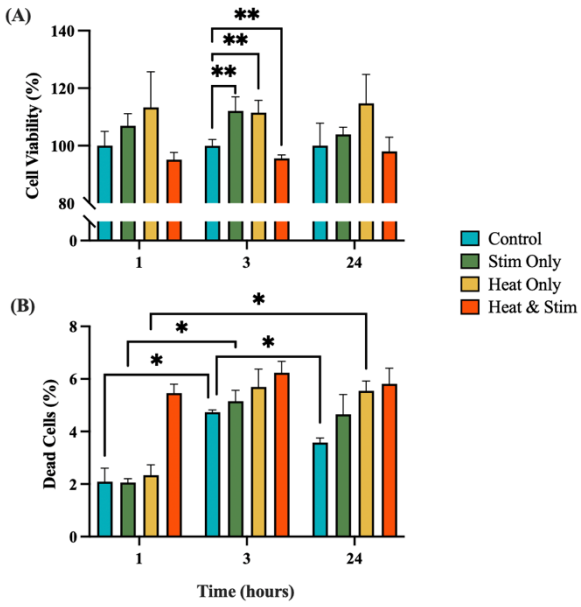

Figure 6.4 – Cell viability assessed by (A) MTT assay and (B) propidium iodide assay immediately following treatment for 1, 3 or 24 hours. Data reported as mean ± standard deviation, * represents $P < 0.05$, ** represents $P < 0.01$.

6.4 Discussion

This chapter aimed to implement a novel heat stress-EPS model and identify the optimal treatment duration for future experiments. The data presented in this chapter characterise the metabolic impact of heat stress and stimulation on C2C12 myotubes and the associated transcriptional and functional outcomes following increasing treatment periods. Visual inspection of C2C12 myotubes revealed no difference in cell structure between conditions or treatment duration, with all myotubes remaining adherent to the cell culture plates. MTT and PI assays revealed maintained cellular integrity and health across all conditions and treatment durations. Contrary to the proposed hypothesis that combined heat stress and EPS would induce greater metabolic perturbations in C2C12 myotubes, the MTT assay conducted during treatment revealed increased metabolism in response to EPS but no metabolic response following heat stress alone. When heat stress and EPS were combined, EPS increased metabolic rate in line with EPS alone.

Alterations in substrate utilisation during exercise have been attributed to multiple mechanisms, including reduced oxygen and substrate delivery and utilisation due to reduced muscle blood flow (Rowell, 1974), altered neuromuscular recruitment pattern favouring fast twitch muscle fibres (Sawka et al., 1985; Young et al., 1985), the direct (Q_{10}) effect on enzyme-mediated reactions (Kozlowski et al., 1985; Young et al., 1985) and increased circulating adrenaline levels (King et al., 1985; Yaspelkis et al., 1993). Indeed, (Febbraio, 2001) summarised these mechanisms over 20 years ago and concluded "*changes appear to be mediated by muscle temperature per se and by an augmented sympatho-adrenal response*", whilst a wealth of evidence exists to support the notion that increased circulating adrenaline in response to exercise and heat stress alters substrate metabolism (Richter et al., 1982; Jansson,

Hjemdahl and Kaijser, 1986; Chesley, Hultman and Spriet, 1995; Febbraio et al., 1998; Howlett et al., 1999), the direct impact of intramuscular temperature increases have yet to be experimentally resolved. The present chapter casts doubt on the hypothesis that temperature has a direct effect on metabolism as no increase in metabolism was observed in heat-treated compared to control and electrically stimulated C2C12 myotubes. The typical Q_{10} values for enzyme-mediated reactions are 2.0 to 3.0, meaning that with a 10°C increase, a 2- to 3-fold increase in reaction rate is observed. Intramuscular temperature can reasonably increase more than 2°C during exercise in hot conditions compared to temperate conditions. This 2°C increase could increase the enzyme-mediated reaction rate by 30 to 40%, significantly impacting substrate utilisation. The present chapter instead reports that a ~10% reduction was observed in response to heat stress rather than an increase in metabolism. The underlying metabolic impact of heat stress on C2C12 myotubes will be addressed in the following chapter and seeks to identify specific metabolic pathways that may be responsible for this change in metabolism. In humans, few studies have examined the effect of increased muscle temperature on intramuscular metabolism, with (Edwards et al., 1972) reporting increased glycogen utilisation and lactate accumulation, albeit core body temperature increased because of limb hot water immersion meaning increases in circulating adrenaline could not be excluded in the interpretation of the results. More recently, studies have bunted the rise in core body temperature and revealed increased muscle glycogenolysis and lactate accumulation, providing further for the notion that temperature directly impacts intramuscular metabolism (Febbraio et al., 1996c).

Cell models provide a highly controlled experimental model permissive to determining phenotypic adaptations and their underpinning signalling mechanisms in response to heat stress. In line with data presented within this chapter, Liu and Brooks (2012) reported that

C2C12 myotubes heated (40°C) for 1 hr did not have an augmented transcriptional response. However, in the present study, mRNA was harvested immediately post-treatment, whilst Liu and Brooks (2012) returned cells to standard cell culture conditions for 1 hour before harvest. Despite an additional hour to allow heat-induced transcriptional upregulation, none occurred, suggesting that one hour of heat stress is an insufficient stimulus to induce gene transcription. As for EPS, a robust transcriptional response was observed following 3 hrs of Stim Only in the present study, apart from COX4, which failed to increase transcriptional activity following any duration of EPS. Using EPS-treated primary skeletal myotubes (differentiated for 5 days), Pourteymour et al. (2017) implemented a transcriptomics approach to characterise the post-EPS transcriptional response, in contrast to the current findings, which showed increased PGC-1α gene expression 3 hrs of stimulation, but not 24. These authors reported that PGC-1α gene expression significantly increased after 24 hours of stimulation.

Unfortunately, investigating the combined effects of heat stress and stimulation on post-treatment transcriptional response was impossible, which would have provided further novel data and provided extra validation of the present model. Similarly, data presented here reveal that elevated heat stress and stimulation combined increase metabolic activity compared to heat stress alone; with both treatments providing additional stress to the cell population, a transcriptional response may have been induced earlier or greater. Future research should aim to complete transcriptional analysis of myotubes in response to EPS + Heat stress to compare the transcriptomes between *in vitro* experiments and human *in vivo* exercise.

6.4.1 Conclusion

The most intriguing finding of the present study is undoubtedly the lack of metabolic alteration in response to heat stress. The direct impact of heat stress on skeletal muscle metabolism has long been believed to be a crucial factor in altering substrate metabolism in response to heat stress. Future chapters aim to elucidate the underlying metabolic processes and validate the metabolic data reported here.

Considering the data presented within this chapter, 3 hours of treatment was deemed the optimal duration for future investigations. Additionally, 3 hrs represented the most time-efficient option. The selection of 3 hrs is based upon the collective data from this study, *i.e.*, metabolic activity was significantly increased during 3 hrs of stimulation. Furthermore, whilst cell viability was broadly similar between groups, there was a distinctly smaller proportion of dead cells at 3 than 24 hrs. Additionally, when considering the post-treatment transcriptional response, 3 hrs of heat stress and stimulation induced a greater or equal response than 24 hrs. Additionally, completing 3 hrs of treatment has greater practical feasibility. It allows the completion of multiple treatments per day (~3-4) (of different cell populations if stimulators are limited) compared to 24 hrs which would only permit one treatment every 2 days, severely impacting the timeliness of each experiment.

Chapter 7 – Metabolomic Profiling of C2C12 Myotubes in Response to Heat Stress and Electrical Pulse Stimulation with High and Low Glucose Availability.

Implementing an optimised in vitro model of exercise and heat stress the present chapter characterises the metabolic impact of electrical pulse stimulation, heat stress and combined stimulation and heat stress on the intramuscular metabolome. Additionally, the metabolomic impact of identical treatment in substrate depleted media is also investigated.

7.1 Introduction

Data presented in the previous chapter highlighted the impact of 3 hrs of heat stress, electrical pulse stimulation (EPS) or both heat stress and EPS on cellular metabolic activity and post-stimulation transcriptional responses. Implementing untargeted metabolomics allows biofluid phenotyping and metabolite quantification in response to treatment, *i.e*., EPS and heat stress. Similarly, ^{1}H-NMR metabolomics represents a highly repeatable and robust workflow that can be applied in cell culture and *in vivo* exercise models. Characterising a tissue or biofluid metabolome allows for a comprehensive analysis of metabolites and their respective metabolic pathways, highlighting critical biological processes in response to stimuli that would be otherwise missed when using targeted analytical approaches.

It Is well established that exercise in hot conditions increases carbohydrate oxidation with a concomitant decrease in fatty acid metabolism (Febbraio, 2001). Several mechanisms have been proposed for the alteration in substrate metabolism in response to heat stress, including reduced skeletal muscle blood flow (Fink, Costill and Van Handel, 1975; Nielsen et al., 1990; Young, 1990), the altered neuromuscular requirement (Young et al., 1985), the direct effect of temperature (Kozlowski et al., 1985; Young, 1990) and hormonal (Febbraio et al., 1994a; 1998; Hargreaves et al., 1996b) induced changes in metabolism. However, given the innate relationship between exercise and heat production, isolating the effect of heat stress on skeletal muscle metabolism *in vivo* is fraught with practical, ethical, and physiological limitations. Furthermore, data from the previous chapter provides contrary evidence to the long-held belief that heat stress directly impacts intramuscular substrate metabolism.

Recently, non-pharmacological *in vitro* exercise mimetic models have increased in popularity. Specifically, EPS shows similarities to *in vivo* exercise, including but not limited to; increased glucose uptake, lactate production, ATP and PCR turnover (Nikolic et al., 2012), exercise-mediated transcriptional response (Pourteymour et al., 2017) and increased mitochondrial biogenesis (Nikolic et al., 2012). To date, one study has investigated the effect of EPS on C2C12 skeletal muscle metabolism using 1H-NMR metabolomics (Lautaoja et al., 2021). The authors quantified 39 intra- and 37 extracellular metabolites showing EPS significantly impacted energy metabolism and was further impacted by low glucose availability, which impaired substrate turnover and presented a blunted metabolic profile.

Maintaining cells at 37°C during EPS neglects the contraction-induced heat production seen during exercise *in vivo.* Heat stress provides a potent metabolic stimulus in C2C12 myotubes (Patton et al., 2018). However, the effect of heat stress on the C2C12 myotube metabolome has not been investigated, nor has the combined effects of heat stress, EPS, and substrate availability. As such, the present chapter aimed to combine EPS with elevated ambient temperatures (40°C) to gain greater insight into the effect of exercise and heat stress on substrate metabolism at the skeletal muscle level. The hypotheses of the present chapter are:

1. EPS alone will increase energy substrate metabolism and the production of metabolites, including lactate, acetate, and branched-chain fatty acids.
2. Additional heat stress will further increase substrate utilisation in line with *In vivo* thermal-exercise physiology literature.
3. Provide metabolome level insight into the impact of heat stress on intramuscular metabolism and validate cellular metabolism data obtained in the previous chapter.

7.2 Methods

7.2.1 Cell Culture

General cell culture techniques have been previously described in Chapter 3.6. Briefly, C2C12 mouse skeletal muscle myoblasts (ATCC, Rockville, MD, USA) (passage 9-11) were resuscitated from liquid nitrogen in 2mL cryovials and seeded in T75 cell culture flasks at a cell density of 1×10^6 (As described in Chapter 3.6.4).

To achieve 80% confluency, cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for up to 72 hrs. Once at the desired confluency, GM was removed, and cells were washed twice with PBS to remove any excess media. Once washed, 1 mL trypsin was added and incubated for 5 mins at 37°C. Following confirmation of non-adherence of cells, trypsin was neutralised by adding 4 mL of GM (5 mL total). Cells were counted via the trypan blue technique previously described (Chapter 3.6.6) before being seeded on pre-gelatinised 6-well plates at 70,000 cells·ml^{-1}. Cells were incubated at 37°C for 24 hrs before GM was removed, and all plates were washed twice with PBS. To initiate differentiation, 2 mL of differentiation media was added to each well (day 0) and topped up with 200 µL daily until day 8, when terminal differentiation was reached (Described in Chapter 3.6.7).

7.2.2 Experimental Procedures

Following differentiation, DM was aspirated from C2C12 myotubes and replaced with 2 mL of fresh DM. Cells were then assigned to one of four conditions; Control (CON), whereby cells were maintained at 37°C; electrical pulse stimulation (EPS; 10 Hz at 37°C) only (Stim Only); Heat only (Heat Only), where cells were incubated at 40°C, and EPS and heat stress where cells were stimulated at 40°C (Heat & Stim). To assess the impact of reduced

carbohydrate availability (specifically media glucose concentration), an identical experiment was completed; however, treatment was completed without a prior media change, resulting in the cells being treated under conditions of reduced glucose availability. All conditions lasted 3 hrs before media were aspirated and washed with cold PBS three times to remove any differentiation media. Cells were then immediately frozen at -80°C until processing.

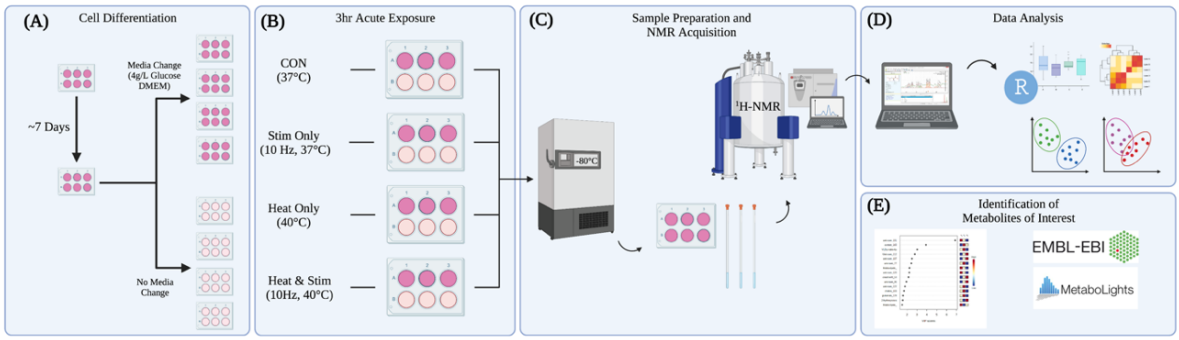

Figure 7.1 – Schematic overview of metabolomic analysis of C2C12 Myotubes. (A) C2C12 myoblasts (P9-11) were differentiated for 7 days into myotubes, differentiation media was either changed immediately before treatment, or cells maintained in "depleted media" throughout treatment. (B) Myotubes were acutely treated with Electrical pulse stimulation (EPS) (Stim Only), heated to 40°C (Heat Only), EPS and Heated (Heat & Stim), or maintained at 37°C (CON) for 3 hrs. (C) Cells were frozen and stored at -80°C until before preparation for 1H-NMR spectroscopy (D) Data analysis was completed as per the previously described workflow using in-house R-Scripts (E) Metabolites were identified before biological interpretation. Created with biorender.com.

7.2.3 Quantification of Media Glucose Availability

Throughout differentiation (Days 1-7), 200 µL of media was aliquoted into a clean Eppendorf tube and stored at -80°C until analysis. Previously stored aliquoted differentiation media (DM) was thawed, and 100 µL was diluted at 50% (v/v) with 10% NMR buffer (100 µM Trimethylsilyl propionate [TSP] [d6 deuterated, Sigma], 99.9 % $^{2}H_2O$ [Sigma], and 100 mM Na_2HPO_4:NaH_2PO_4 pH 7.4 [Thermo-Fisher]) and vortexed for 20s before centrifugation at 21,500 RCF for 5 mins at 4°C. One hundred and ninety µL of centrifuged DM was pipetted into 3 mm (outer diameter glass SampleJet NMR tubes (Bruker). Media glucose abundance was analysed per the metabolomic workflow set out in Chapter 3.8.1.

7.2.4 Intracellular Metabolite Extraction from Cell Culture – Preparation for ^{1}H-NMR

All plates were thawed from -80°C on ice before 500 µL ice-cold solvent solution (50% HPLC grade acetonitrile, 50% ddH_3O) was added to each well. Cells were immediately scraped with a pipette tip, and the resultant slurry was pipetted into Eppendorf tubes. Samples maintained on ice were sonicated at 50 kHz, 20% amplitude for 3 × 30s busts. Samples were vortexed for 20s and centrifuged at 21,500 RCF for 5 mins at 4°C. Supernatants were then aliquoted into fresh Eppendorf tubes before being snap frozen in liquid nitrogen. Samples were lyophilised overnight before the addition of 200 µL of sodium phosphate buffer (100 µM Trimethylsilyl propionate [TSP] [d6 deuterated, Sigma], 99.9 % $^{2}H_2O$ [Sigma], and 100 mM Na_2HPO_4:NaH_2PO_4 pH 7.4 [Thermo-Fisher]) and centrifugation at 12,000 RCF for 2 mins. Lastly, 190 µL of the sample was pipetted into 3mm SampleJet NMR tubes (Bruker).

7.2.5 NMR Acquisition and Sample Processing

High-resolution 1D ^{1}H NMR was acquired using 3-mm outer diameter tubes in 700MHz Avance IIIHD Bruker spectrometer equipped with a TCI cryoprobe and chilled autosampler (SampleJet). A one-dimensional ^{1}H Carr-Purcell-Meiboom-Gill (CPMG) experiment (vendor supplied cpmgpr1d) was used for all spectra acquisition and to attenuate peaks from large molecules such as protein along with standard 1D ^{1}H NOESY presat (vendor supplied noesypr1d) to check sample quality. Spectra were evaluated for quality control (QC) by ensuring consistent water suppression, baseline correction and peak line width of reference TSP signal according to best practice set out by the Metabolomics Standards Initiative (MSI) (Sumner et al., 2007; Considine and Salek, 2019).

Seven samples initially failed QC and were re-run, and all subsequently passed QC criteria. Afterwards, the spectra were divided into 'bins' using TameNMR software (github: https://github.com/PGB-LIV/tameNMR) before a 'pattern' file was generated associated metabolites with spectral peaks including unknown metabolites. Later, the 'binning' of metabolites was reviewed using TameNMR software (accessed via *galaxy.liv.ac.uk* within the University of Liverpool VPN). Peaks were binned using TameNMR by integrating each spectral region defined in the pattern file to yield a table of peak integrals corresponding to each metabolite abundance.

7.2.6 Metabolite Annotation and Identification

To convert raw NMR peaks into a biological frame of reference (e.g., relative metabolite abundances), metabolites are annotated and identified. NMR spectra underwent metabolite annotation using Chenomx NMR suite 8.2 (Chenomx, CA). The software allowed the matching of 1D-^{1}H NMR spectra of metabolite standards to an experimental spectrum using various matching algorithms. Where appropriate, identities were confirmed using an in-house library of standards.

7.2.7 Statistical Analysis

A flowchart of the statistical analysis performed within the present study is shown in Figure 3.12. Following QC, spectra were collated into a single dataset for analysis. All analytical steps were undertaken using previously published and custom-made scripts in R Studio (R Team, 2019). The scaled and normalised dataset was subject to multivariate analysis using a combination of principal component analysis (PCA) (Section 3.8.5 i) and partial least

squared–discriminant analysis (PLS-DA) (Chapter 3.8.5 ii) Variable importance of the projection (VIP) was used to select important features from the PLS-DA models. Representative bins for the selected features were identified via correlation reliability score (CRS) (Chapter 3.8.5 iv). Differences in select metabolites between groups were determined by univariate tests (T-test/ANOVA-Tukey's HSD depending on the number of groups to compare). Two-way ANOVA was performed to determine the differences between control, Stim only, Heat only, and Stim & Heat groups. Finally, pathway analysis was performed via metabolite set enrichment analysis (MSEA) (Chapter 3.8.5 vi).

7.3 Results

7.3.1 The Effect of Electrical Pulse Stimulation and Heat Stress on C2C12 Myotube Metabolome with High Glucose Availability.

7.3.1 i) Principal Component Analysis (PCA)

Initially, PCA was performed to identify major variances between all samples (Figure 7.2A) and between control samples and experimental conditions (figure 7.2B-D). PCA Scores plot of PC1 (31.89%) against PC2 (13.66%) revealed moderate condition clustering with limited separation between groups. PC1 and PC2 explain a cumulative variance of 48.55%, with 15 components required to explain 95% of the variance within the data. When groupwise myotube metabolic profile is considered, Control clustered more tightly compared to Stim Only with a distinct separation between conditions (Figure 7.2B), with Heat Only clustering more tightly compared to Control with no separation between conditions (Figure 7.2C). The overall variance was comparable between control and Heat & Stim conditions with no separation between conditions (Figure 7.2D)

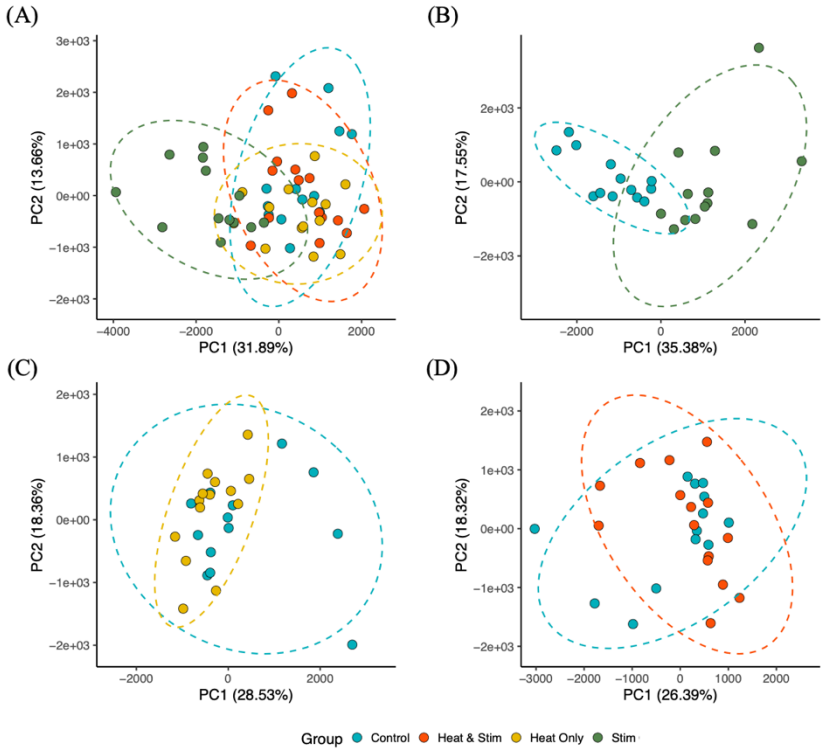

Figure 7.2 – Multivariate Principal Component Analysis of C2C12 cultured myotubes following 3 hrs acute electrical pulse stimulation (EPS) (Stim Only), heat stress (Heat Only) and combined EPS and heat stress (Heat & Stim). (A) PCA of all experimental groups, (B) Control vs. Stim Only, (C) Control vs. Heat Only, (D) Control vs. Heat & Stim. Brackets represent the variance explained by P, with 15 PC's required to explain 95% of the variance. For clarity, only PC1 and PC2 are shown in each panel. Ellipses represent 95% confidence region.

7.3.1 ii) Partial Least Squares Discriminant Analysis (PLS-DA)

To further compare metabolic profiles between conditions, a cross-validated Partial least squares – discriminant analysis (PLS-DA) model was used to enhance differences between conditions (Figure 7.3A). The optimal model complexity was found to be a 6-component model (ROC = 0.9). In contrast to the PCA scores Plot (Figure 7.2A), Control, Heat only, and Heat & Stim Conditions observed tight clustering of groups. In contrast, Stim Only displayed greater variation compared to other groups. PLS-DA models were implemented between Control and each experimental condition to highlight the difference between conditions further. As with PCA (Figure 7.2B), Stim Only had significantly more within-

condition variation than the control (Figure 7.3B). PLS-DA Scores plot showed some overlap between Control and Heat Only conditions with similar variance between conditions (Figure 7.3C) with comparable variance between Control and Heat & Stim but greater separation between conditions (Figure 7.3D). Using VIP scoring as a criterion, metabolites influential in the discrimination between conditions were extracted.

7.3.1 i) Variable of the Importance in Projection (VIP)

Metabolites expressing a VIP score greater than 1 were considered the most influential in explaining metabolic differences (Figure 7.4A) between conditions and were selected for further analysis.

Table 7.1 – Validation of PLS-DA model(s) and summary of receiver operator curve (ROC)

Model	**Model Complexity (No. of Components)**	**Comparison**	**ROC Score**
All	5	Control vs. Others	0.9
		Stim Only vs. Others	1
		Heat Only vs. Others	0.81
		Heat & Stim vs. Others	0.98
Control vs. Stim Only	2		1
Control vs. Heat Only	2		0.69
Control vs. Heat & Stim	3		1

7.3.1 i) Metabolite Set Enrichment Analysis (MSEA)

Selected metabolites were subject to MSEA to ascertain further metabolic pathway level information (Table 7.3) from KEGG curated metabolic pathways (Mus Musculus [mouse] [KEGG organism code: mmu]) using fisher's exact test with EASE correction and Holm *P*-value adjustment. Three metabolic pathways were significantly over-represented, including Aminoacyl-tRNA biosynthesis, Galactose metabolism (Glucose 1-phosphate; Uridine

diphosphate glucose; D-Glucose; D-Fructose; D-Mannose; Myo-Inositol) and Phenylalanine Metabolism (L-Phenylalanine; Phenylacetic acid; Hippuric acid; L-Tyrosine).

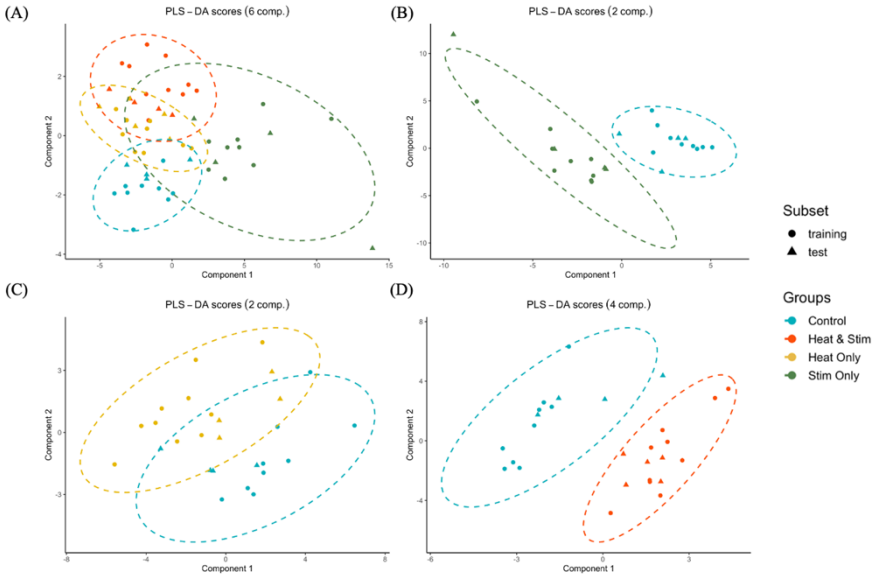

Figure 7.3 – Partial least squares discriminant analysis (PLS-DA) of Cell Extracts. (A) 4-way, 6 component PLS-DA model VIPS (n = 19) based upon differences between control and all experimental interventions (ROC = 0.9); (B) 2-way, 2 component PLS-DA model between Control and Stimulation Only (ROC = 1); (C) 2-way, 2 component PLS-DA model between Control and Heat Only (ROC = 0.69); (D) 2-way, 4 component PLS-DA model between Control and Stimulation & Heat (ROC = 1). Ellipses show the 95% confidence region.

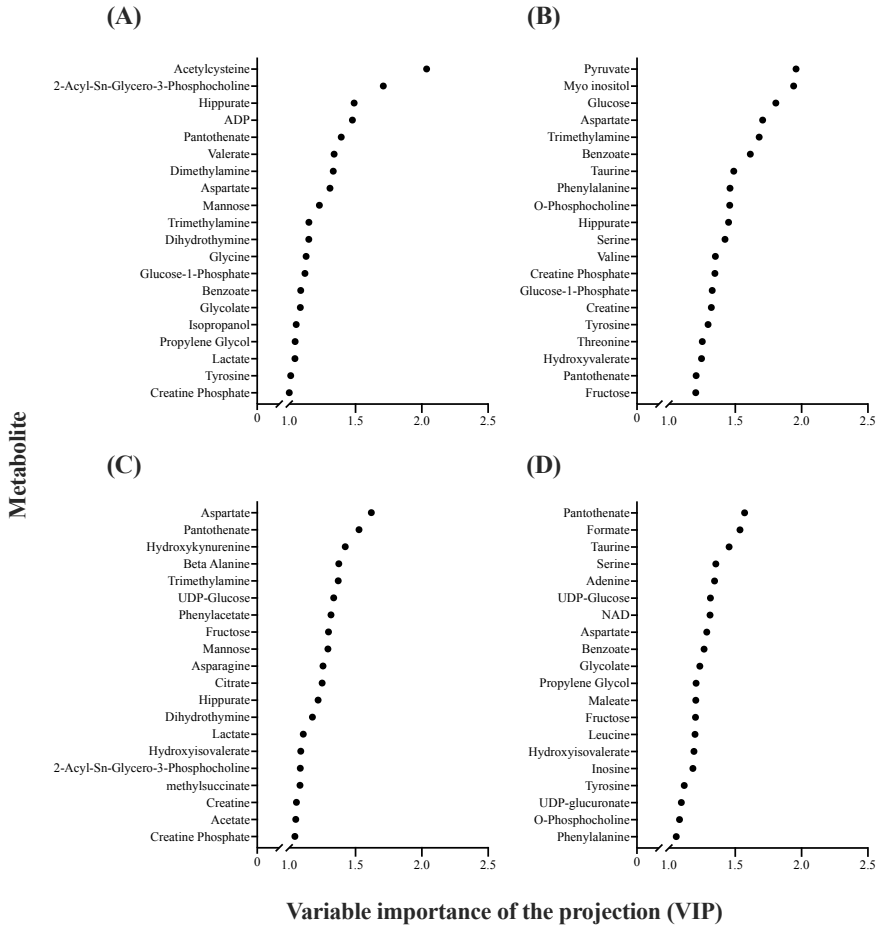

Figure 7.4 – Top 20 VIP scores of PLS-DA models built upon intervention-dependent differences in skeletal myotubes. (A) VIPS (n = 19) based upon differences between control and all experimental interventions (B); VIPS between Control and Stimulation Only (C); VIPS between Control and Heat Only (D); VIPS between Control and Heat & Stim.

7.3.1 ii) Univariate Analysis – ANOVA

To gain metabolite level information on condition-specific differences, VIP-filtered metabolites from each PLS-DA were compared via BH Adjusted ANOVA (Table 7.2).The metabolite level comparison revealed multiple significantly different metabolites in Stim Only (n = 19) and Heat & Stim (n = 3) vs. Control. However, no metabolites significantly differed between Heat Only and Control (Figure 7.5). Metabolites presented and analysed only represent VIP-filtered metabolites; complete relative abundance data, boxplots (Figure 11.1), and ANOVA outputs for the entire dataset can be found in appendices (Table 11.1).

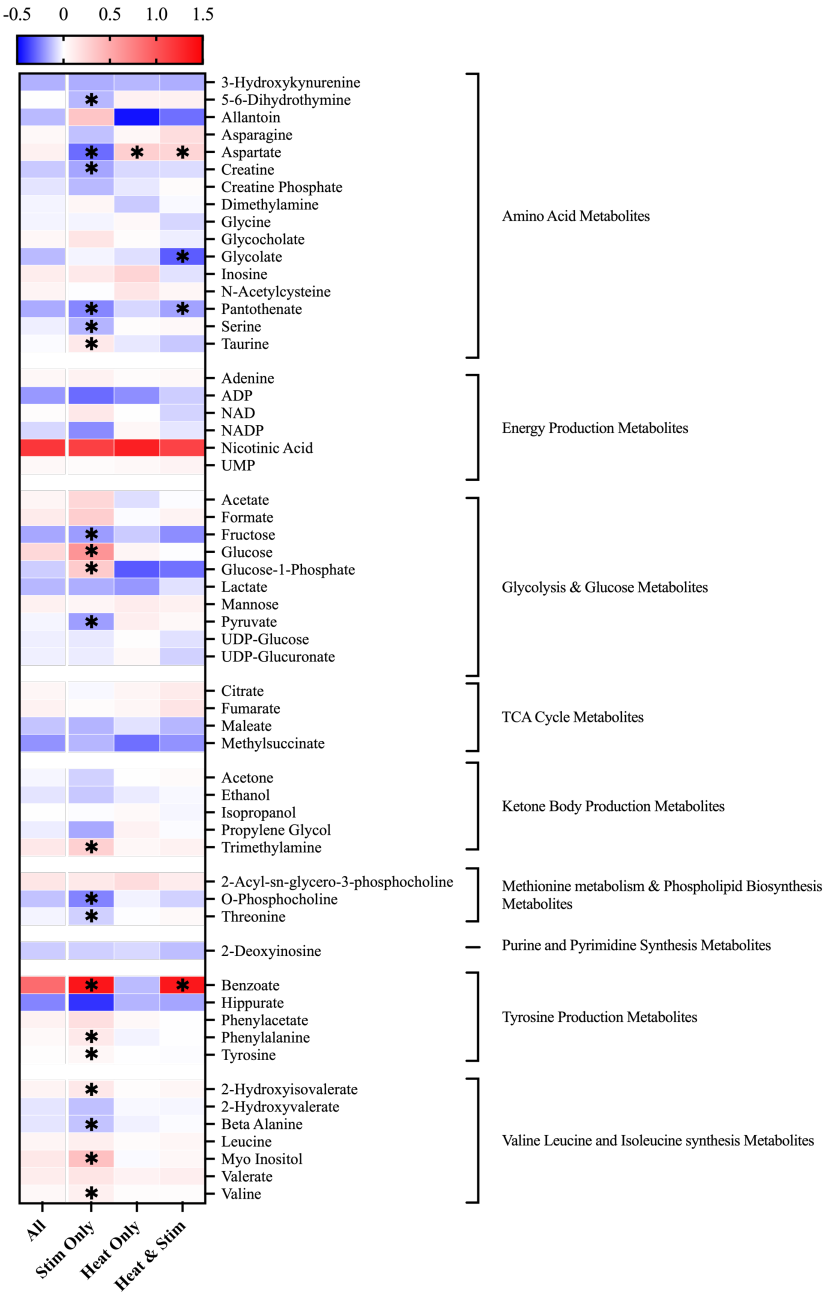

Figure 7.5 – Heatmap representing Log2 fold change in relative metabolite abundance from control C2C12 Myotubes following treatment in high glucose media. * Represents significant difference between treatment condition and Control ($P < 0.05$).

Table 7.2 – ANOVA output including Tukey-post hoc groupwise comparisons. Data presented here is filtered to include only metabolites with BH adjusted $P<0.05$. A complete metabolite abundance and ANOVA table can be found in appendices.

Metabolite	ANOVA			Pairwise Comparisons					
	F	**Raw *P*-value**	**BH Adjusted *P*-value**	**Stim Only - Control**	**Heat Only-Control**	**Heat & Stim-Control**	**Heat Only-Heat & Stim**	**Stim Only-Heat & Stim**	**Stim Only-Heat Only**
Pyruvate	26.52	$1.78x10^{-10}$	$5.08x10^{-9}$	$4.69x10^{-8}$	0.68	0.94	0.32	$1.46x10^{-7}$	$5.00x10^{-10}$
Glucose	18.12	$3.91x10^{-8}$	$7.42x10^{-7}$	$2.65x10^{-6}$	0.99	0.95	0.93	$1.94x10^{-7}$	$2.26x10^{-6}$
Myo Inositol	16.48	$1.29x10^{-7}$	$1.48x10^{-6}$	$5.25x10^{-7}$	0.98	0.27	0.49	0.0001	$1.52x10^{-6}$
Trimethylamine	16.78	$1.04x10^{-7}$	$1.48x10^{-6}$	$4.42x10^{-7}$	0.91	0.66	0.96	$1.22x10^{-5}$	$3.29x10^{-6}$
Benzoate	15.40	$2.93x10^{-7}$	$2.78x10^{-6}$	$3.32x10^{-5}$	1.00	0.0002	0.0002	0.90	$3.52x10^{-5}$
Taurine	14.85	$4.48x10^{-7}$	$3.65x10^{-6}$	0.0006	0.81	0.24	0.74	$4.38x10^{-7}$	$2.12x10^{-5}$
Serine	10.88	$1.23x10^{-5}$	$8.79x10^{-5}$	0.001	0.91	0.78	0.99	$2.87x10^{-5}$	0.0001
Pantothenate	10.25	$2.17x10^{-5}$	0.0001	0.0002	0.49	0.0003	0.02	0.98	0.01
Threonine	9.55	$4.12x10^{-5}$	0.0002	0.0005	1.00	0.46	0.59	$2.51x10^{-5}$	0.002
Phenylalanine	9.42	$4.65x10^{-5}$	0.0002	0.0003	1.00	0.97	0.97	0.0007	0.0002
Dihydrothymine	9.21	$5.70x10^{-5}$	0.0003	0.03	0.38	0.32	1.00	0.0001	0.0002
Valine	6.86	0.0006	0.003	0.003	1.00	1.00	1.00	0.003	0.002
Glycolate	6.22	0.001	0.006	0.83	0.92	0.002	0.009	0.02	1.00
Tyrosine	5.45	0.003	0.01	0.005	0.99	0.93	0.99	0.02	0.009
Glucose-1-Phosphate	5.25	0.003	0.01	0.04	0.87	0.98	0.98	0.01	0.004
Fructose	5.12	0.004	0.01	0.002	0.39	0.06	0.77	0.53	0.12
2-Hydroxyvaleric Acid	4.95	0.004	0.01	0.009	0.99	0.99	1.00	0.01	0.02
Acetone	4.38	0.008	0.02	0.06	1.00	0.87	0.84	0.006	0.06
O-Phosphocholine	4.40	0.008	0.02	0.004	0.22	0.22	1.00	0.29	0.31
Beta Alanine	4.01	0.01	0.03	0.03	0.98	1.00	0.99	0.02	0.05
Creatine	3.93	0.01	0.03	0.007	0.57	0.51	1.00	0.16	0.15

Table 7.3 – Results of the overrepresentation analysis using the hypergeometric test to evaluate whether a particular metabolite set was represented more than expected by change within the metabolites identified from PLS-DA analysis (VIPS > 1). One-tailed *p*-values are provided following multiple testing adjustment.

Biological Process	Total	Expected	Hits	Raw *p*-value	Holm adjusted *p*-value	FDR
Aminoacyl-tRNA biosynthesis	48	1.51	8	7.8×10^{-6}	0.00656	0.0045
Galactose metabolism	27	0.852	6	0.00013	0.0106	0.0045
Phenylalanine metabolism	10	0.315	4	0.00016	0.0131	0.0045
Ascorbate & aldarate metabolism	8	0.252	3	0.00148	0.12	0.0248
Valine, leucine, & isoleucine biosynthesis	8	0.252	3	0.00148	0.12	0.0248
Starch & sucrose metabolism	18	0.568	4	0.00193	0.152	0.027
Pantothenate & CoA biosynthesis	19	0.599	4	0.00238	0.186	0.0281
Glyoxylate & dicarboxylate metabolism	32	1.01	5	0.00268	0.206	0.0281
Amino & nucleotide sugar metabolism	37	1.17	5	0.00515	0.391	0.0472
Phenylalanine, tyrosine, & tryptophan biosynthesis	4	0.126	2	0.00561	0.421	0.0472
Glycolysis / Gluconeogenesis	26	0.82	4	0.00782	0.578	0.0597
Alanine, aspartate, & glutamate metabolism	28	0.883	4	0.0102	0.746	0.0663
Nicotinate & nicotinamide metabolism	15	0.473	3	0.0103	0.746	0.0663
Pentose & glucuronate interconversions	18	0.568	3	0.0172	1	0.102
Glycine, serine, & threonine metabolism	33	1.04	4	0.0182	1	0.102
Pyruvate metabolism	22	0.694	3	0.0298	1	0.156

7.3.2 The Effect of Electrical Pulse Stimulation and Heat Stress on C2C12 Myotube Metabolome with Low Glucose Availability.

7.3.2 i) Quantification of Media Glucose Availability

Before experimentation, media glucose abundance was quantified throughout differentiation to ensure glucose depletion prior to treatment. Stock differentiation media contained 4.5 g·L of glucose; throughout the 7 days of differentiation, glucose availability was depleted by ~50%, resulting in ~2.75 g·L of glucose at the onset of the low glucose condition.

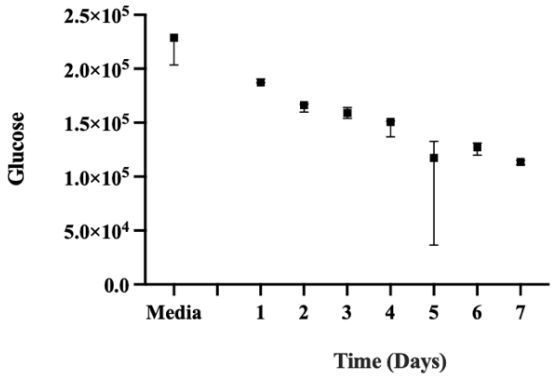

Figure 7.6 – Time course of glucose depletion throughout 7 days of differentiation. Standard deviations are not visible for some data points due to low variability between samples.

7.3.2 ii) Principal Component Analysis (PCA)

Initially, PCA was performed to identify major variances between all samples (Figure 7.7A) and between control samples and experimental conditions (figure 7.7B-D). PCA Scores plot of PC1 (23.43%) against PC2 (12.61%) revealed minimal clustering of condition in terms of separation. PC1 and PC2 explain a cumulative variance of 36.04%, with 15 components required to explain 95% of the variance within the data. There was no discernible clustering between Control and Stim Only with equal variance within each condition (Figure 7.7B), with Heat Only clustering more tightly than the Control (Figure 7.7C). The overall variance was comparable between Control and Heat & Stim conditions with minimal clustering for condition (Figure 7.7D)

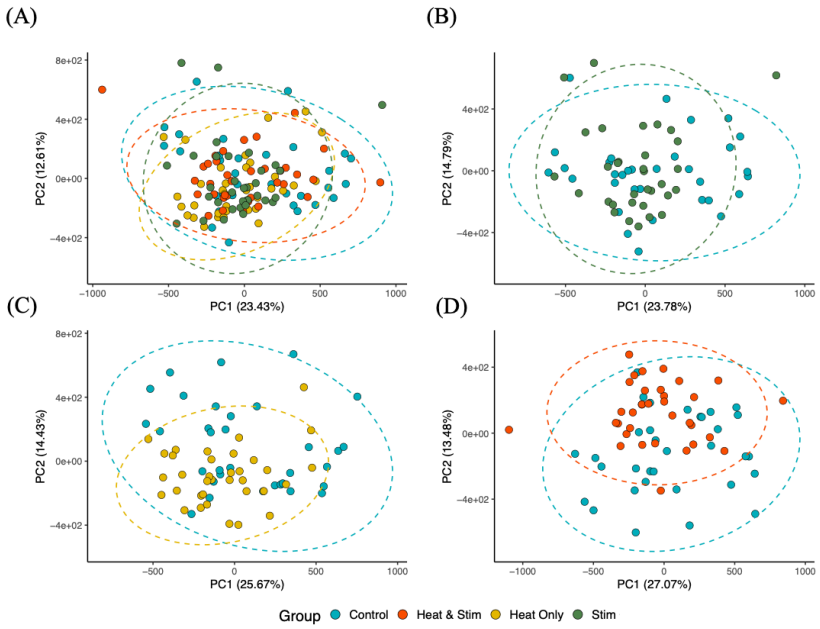

Figure 7.7 – Multivariate Principal Component Analysis of C2C12 cultured myotubes following 3 hrs acute electrical pulse stimulation (EPS) (Stim), heat stress (Heat Only) and combined EPS and heat stress (Heat & Stim) in substrate depleted media. (A) PCA of all experimental groups, (B) Control vs. Stim Only, (C) Control vs. Heat Only, (D) Control vs. Heat & Stim. Brackets represent the variance explained by P, with 15 PC's required to explain 95% of the variance. For clarity, only PC1 and PC2 are shown in each panel. Ellipses represent 95% confidence region.

7.3.2 iii) Partial Least Squares Discriminant Analysis (PLS-DA)

Per the previously described metabolomics workflow, a cross-validated PLS-DA model was used to enhance differences between conditions and compare metabolic profiles (Figure 7.8). The optimal model complexity was a 3-component model (ROC = 0.9). As with the PCA scores Plot (Figure 7.8A), no distinct clustering of groups was observed. PLS-DA models were implemented between Control and each experimental condition to highlight the difference between conditions further. Unlike the previous PCA scores plot (Figure 7.2B), more between-condition clustering was observed in Stim Only, with less variation in Stim Only compared to Control (Figure 7.8B). PLS-DA Scores plot showed some overlap between Control and Heat Only conditions with similar variance between conditions (7.8C) with comparable variance

between Control and Heat & Stim but greater separation between conditions (7.78D). Using VIP scoring as a criterion, metabolites influential in the discrimination between conditions were extracted.

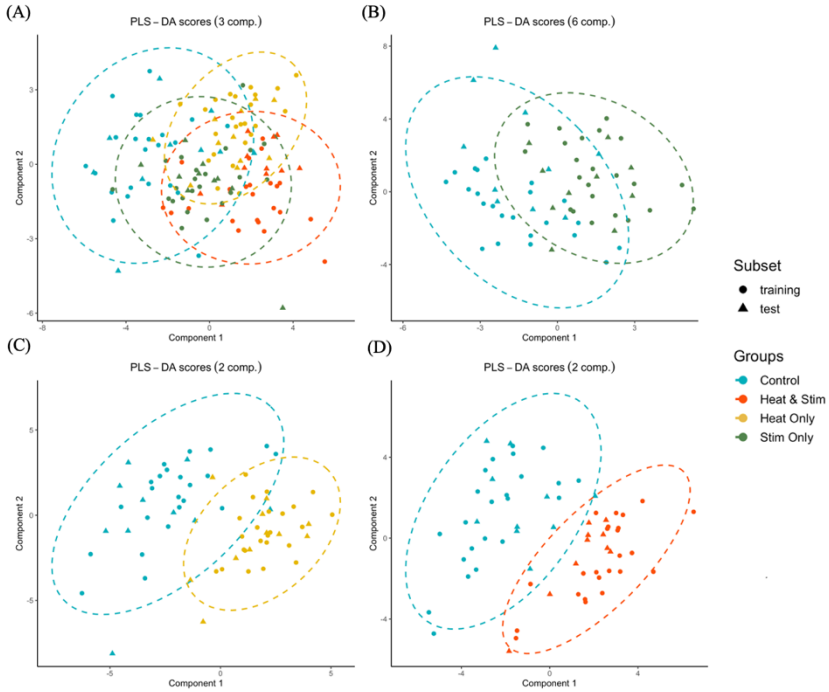

Figure 7.8 – Partial least squares discriminant analysis (PLS-DA) of Cell Extracts. (A-D) PLS-DA scores plots representing component 1 and 2 for cell extracts post intervention discriminating between groups. Ellipses show the 95% confidence region.

Table 7.4 – Validation of PLS-DA model(s) and summary of receiver operator curve (ROC)

Model	No. of Components	Comparison	ROC Score
All	3	Control *vs*. Others	0.88
		Stim Only *vs*. Others	0.83
		Heat Only *vs*. Others	0.74
		Heat & Stim *vs*. Others	0.9
Control *vs*. Stim Only	3		0.61
Control *vs*. Heat Only	2		0.95
Control *vs*. Heat & Stim	2		1

7.3.2 iv) Variable Importance for the Projection (VIP)

Metabolites expressing a VIP score greater than 1 were considered the most influential in explaining metabolic differences (Figure 7.9) between conditions and were selected for further analysis.

7.3.2 v) Univariate Analysis – ANOVA

To gain metabolite level information on condition-specific differences, VIP-filtered metabolites from each PLS-DA were compared via BH Adjusted ANOVA (Table 7.5). The metabolite level comparison revealed multiple significantly different metabolites on Stim Only (n = 7), Heat & Stim (n = 15) and Heat only (n = 15) vs. Control. Complete abundance data, ANOVA outputs (Table 11.2) and boxplots can be found in the appendices (Figure 11.2)

7.3.2 vi) Metabolite Set Enrichment Analysis (MSEA)

Selected metabolites were subject to MSEA to ascertain further metabolic pathway level information (Table 7.6) using a database curated from KEGG pathways (Mus Musculus [mouse] [KEGG organism code: mmu]) using fisher's exact test with EASE correction and Holm *P*-value adjustment. Two metabolic pathways were significantly over-represented, including Aminoacyl-tRNA biosynthesis and Alanine, aspartate, and glutamate metabolism pathway (L-Aspartate; L-Glutamate; Citrate; Fumarate; Pyruvate)

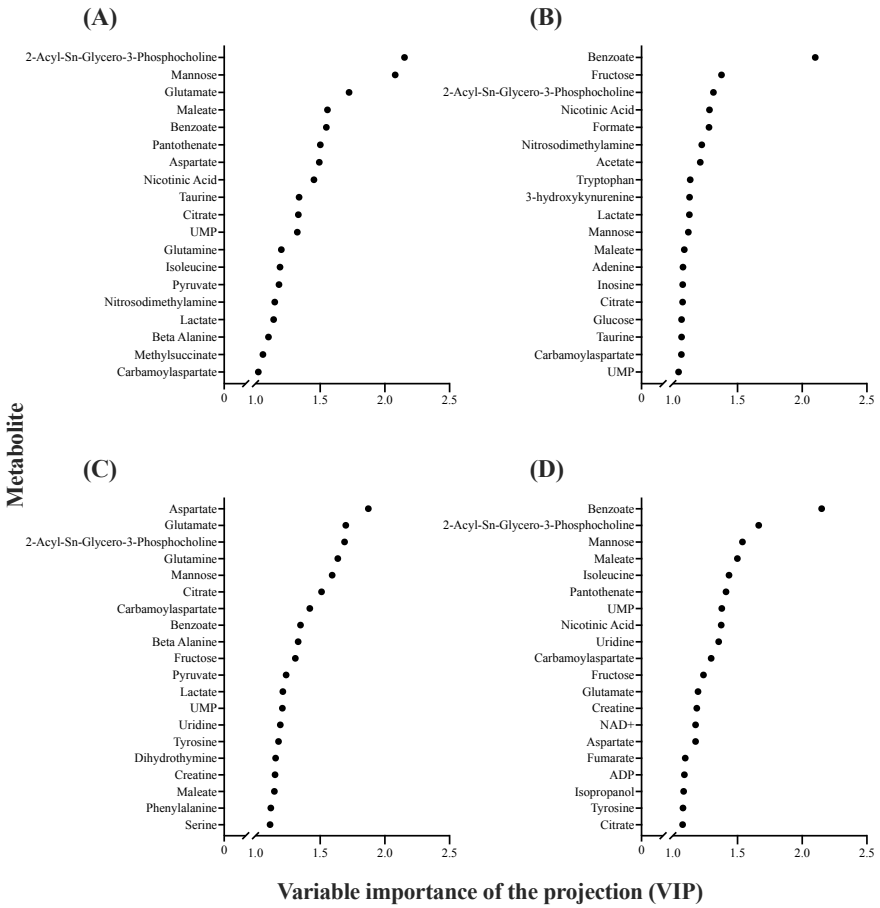

Figure 7.9 – Top 20 VIP scores of PLS-DA models built upon intervention-dependent differences in skeletal myotubes. (A) 4-way, 3 component PLS-DA model VIPS (n = 19) based upon differences between control and all experimental interventions (ROC = 0.9); (B) 2-way, 6 component PLS-DA model VIPS between Control and Stimulation Only (ROC = 0.63); (C) 2-way, 2 component PLS-DA model VIPS between Control and Heat Only (ROC = 0.95); (D) 2-way, 2 component PLS-DA model VIPS between Control and Heat & Stim (ROC = 1).

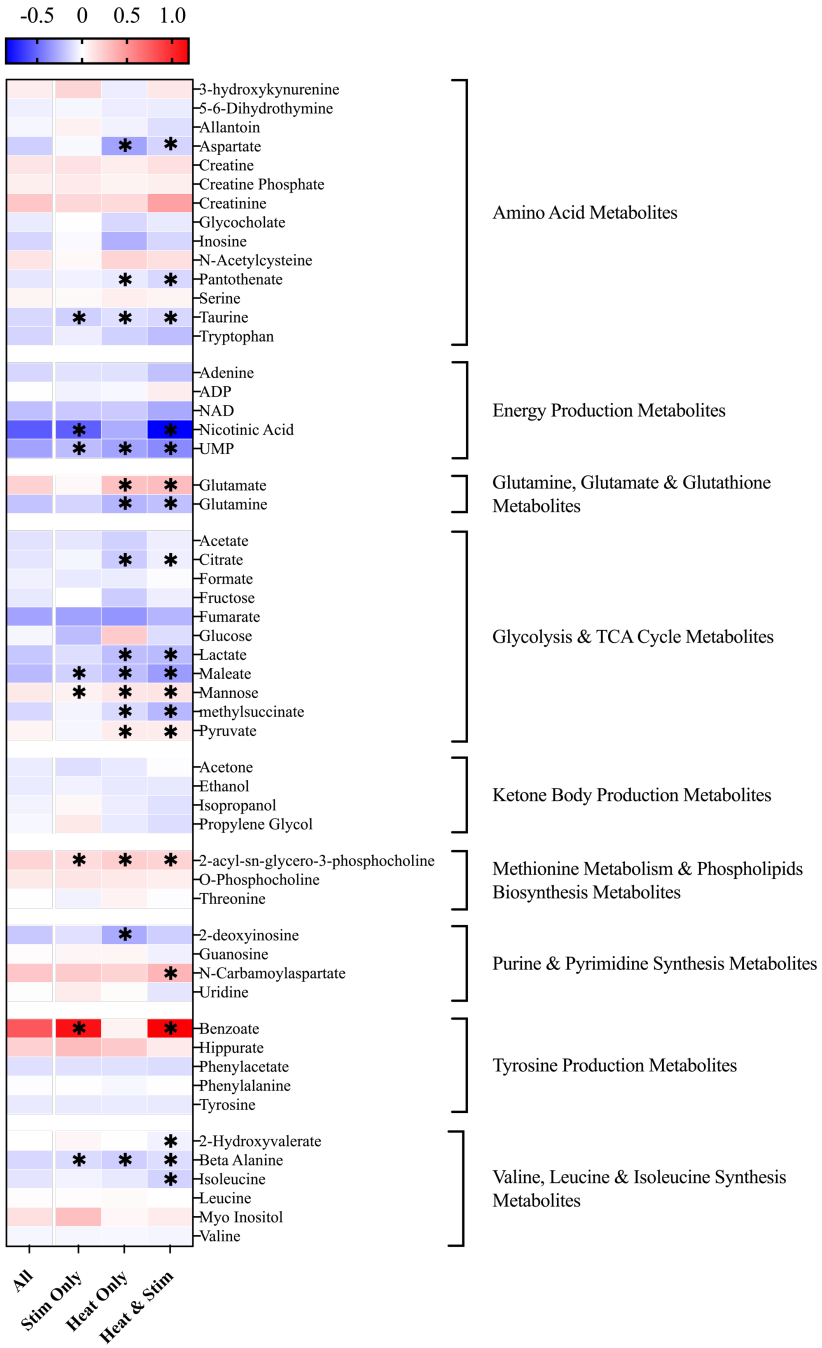

Figure 7.10 – Heatmap representing Log2 fold change in relative metabolite abundance from control C2C12 Myotubes following treatment in substrate depleted media. * Represents significant difference between treatment condition and Control ($P < 0.05$).

Table 7.5 – ANOVA output including Tukey-post hoc groupwise comparisons. Data presented here is filtered to include only metabolites with BH adjusted $P < 0.05$. A complete metabolite abundance and ANOVA table can be found in appendices.

	ANOVA			Pairwise Comparisons					
Metabolite	***F***	**Raw *P*-value**	**BH Adjusted *P*-value**	**Stim Only - Control**	**Heat Only-Control**	**Heat & Stim-Control**	**Heat Only-Heat & Stim**	**Stim Only-Heat & Stim**	**Stim Only-Heat Only**
Benzoate	24.51	$1.13x10^{-12}$	$5.02x10^{-11}$	$2.91x10^{-7}$	1.00	$6.34x10^{-8}$	$2.18x10^{-8}$	1.00	$1.07x10^{-7}$
Mannose	24.01	$1.82x10^{-12}$	$5.02x10^{-11}$	0.004	$9.26x10^{-7}$	$9.66x10^{-13}$	0.04	$3.57x10^{-5}$	0.18
2-Acyl-Sn-glycero-3-phosphocholine	21.84	$1.51x10^{-11}$	$2.76x10^{-10}$	0.002	$9.94x10^{-10}$	$7.47x10^{-10}$	1.00	0.007	0.008
Glutamate	17.12	$1.88x10^{-9}$	$2.59x10^{-8}$	0.872	$4.52x10^{-8}$	0.0002	0.24	0.003	$1.74x10^{-6}$
Pantothenate	15.27	$1.39x10^{-8}$	$1.53x10^{-7}$	0.056	0.037	$3.71x10^{-9}$	0.0006	0.0004	1.00
Nicotinic Acid	12.89	$1.95x10^{-7}$	$1.79x10^{-6}$	0.0002	0.10	$1.87x10^{-7}$	0.002	0.40	0.20
Aspartate	11.93	$5.81x10^{-7}$	$4.56x10^{-6}$	0.999	$9.21x10^{-6}$	0.021	0.15	0.02	$1.18x10^{-5}$
Maleate	10.74	$2.31x10^{-6}$	$1.41x10^{-5}$	0.004	0.02	$5.99x10^{-7}$	0.04	0.14	0.97
UMP	10.77	$2.23x10^{-6}$	$1.41x10^{-5}$	0.0004	0.004	$1.28x10^{-6}$	0.17	0.55	0.89
Citrate	10.49	$3.10x10^{-6}$	$1.70x10^{-5}$	0.319	$5.40x10^{-6}$	0.0008	0.61	0.13	0.005
Pyruvate	9.60	$8.86x10^{-6}$	$4.43x10^{-5}$	1.00	0.0004	0.008	0.82	0.005	0.0002
Methyl succinate	7.63	$9.61x10^{-5}$	0.0004	0.504	0.005	0.0002	0.78	0.02	0.20
Taurine	7.13	0.0002	0.0008	0.006	0.04	0.0001	0.29	0.73	0.89
Glutamine	6.38	0.0005	0.002	0.403	0.0003	0.64	0.01	0.98	0.05
Lactate	6.04	0.0007	0.003	0.235	0.0004	0.02	0.67	0.71	0.14
2-Hydroxyvaleric Acid	5.33	0.002	0.006	0.377	0.92	0.13	0.02	0.001	0.75
Isoleucine	5.14	0.002	0.007	0.857	0.44	0.002	0.12	0.02	0.90
Beta Alanine	4.91	0.003	0.009	0.310	0.002	0.05	0.69	0.82	0.21
Myo Inositol	4.61	0.004	0.012	0.556	0.17	0.48	0.91	0.03	0.005
Inosine	4.17	0.007	0.020	0.574	0.83	0.08	0.007	0.70	0.14
N-Carbamoylaspartate	3.86	0.011	0.027	0.137	0.19	0.006	0.53	0.65	1.00
Uridine	3.59	0.015	0.039	0.198	0.98	0.60	0.36	0.008	0.36
2-deoxyinosine	3.70	0.021	0.049	0.34	0.01	0.37	0.39	1.00	0.45

Table 7.6 – Results of the overrepresentation analysis using the hypergeometric test to evaluate whether a particular metabolite set was represented more than expected by change within the metabolites identified from PLS-DA analysis (VIPS > 1). One-tailed *p*-values are provided following multiple testing adjustment.

Biological Process	Total	Expected	Hits	Raw *p*-value	Holm adjusted *p*-value	FDR
Alanine, aspartate, & glutamate metabolism	28	0.662	5	0.000369	0.031	0.0198
Aminoacyl-tRNA biosynthesis	48	1.14	6	0.00068	0.0432	0.0198
Glyoxylate & dicarboxylate metabolism	32	0.757	5	0.000706	0.0579	0.0198
Pyruvate metabolism	22	0.52	4	0.00143	0.115	0.0299
Phenylalanine, tyrosine, & tryptophan biosynthesis	4	0.0946	2	0.00317	0.254	0.0518
Arginine biosynthesis	14	0.331	3	0.0037	0.292	0.0518
Nicotinate & nicotinamide metabolism	15	0.355	3	0.00455	0.355	0.0546
D-Glutamine & D-glutamate metabolism	6	0.142	2	0.00769	0.592	0.0807
Pantothenate & CoA biosynthesis	19	0.449	3	0.00908	0.69	0.0848
Citrate cycle (TCA cycle)	20	0.473	3	0.0105	0.788	0.0883
Phenylalanine metabolism	10	0.237	2	0.0217	1	0.152
Glycolysis / Gluconeogenesis	26	0.615	3	0.0218	1	0.152
Galactose metabolism	27	0.639	3	0.0241	1	0.156
Neomycin, kanamycin, & gentamicin biosynthesis	2	0.0473	1	0.0468	1	0.281

7.4 Discussion

The current study implemented an untargeted NMR metabolomics approach to examine the effects of exercise-like stimulation (Stim Only), heat stress (Heat Only) and combined heat stress-stimulation (Heat & Stim) on intracellular metabolism *in vitro.* Additionally, in line with this thesis's overarching theme, media substrate availability was depleted prior to stimulation and heat stress to mimic the glucose/glycogen-depleted conditions before fasted exercise *in vivo*.

The main findings of this study are three-fold. Firstly, this chapter has characterised the effect of EPS on the C2C12 skeletal muscle metabolome and confirmed previous work

from Lautaoja et al. (2021), showing increased metabolic turnover in response to exercise-like stimulation. Secondly, data from this chapter supported metabolic data from the previous chapter indicating that heat stress alone was not sufficient stimulus to increase metabolic rate or alter substrate metabolism in skeletal muscle. Finally, when substrate availability is reduced post-differentiation, metabolic perturbations are reduced following 3 hrs of heat and stimulation. Secondly, imposing additional heat stress in isolation and combination with electrical pulse stimulation to reflect exercise *in vivo* more closely does not induce significant metabolomic perturbations in C2C12 myotubes. Interestingly, heat stress alone did not induce significant metabolite level changes nor wholesale metabolome disturbance, even when substrate availability was high.

7.4.1 Intracellular Metabolomic Responses to Electrical Pulse Stimulation and Heat Stress

Initial principal component analysis (PCA) showed the greatest difference between control and Stim Only compared to control vs Heat Only and control vs Heat & Stim (Figure 7.2). Building a PLS-DA model between conditions allowed the identification of metabolites crucial for the discrimination between groups. Of the 50 metabolites identified, the univariate analysis identified 21 significantly different metabolites between conditions, with 19 significantly different from control in Stim only, 3 significantly different metabolites between control and Stim & Heat with no significant metabolites between control and heat only.

Elucidating the mechanisms responsible for altered substrate metabolism *in vivo* is difficult due to inherent heat production during skeletal muscle contraction. Here the isolation of ‘exercise’ and heat stress has revealed that heat stress alone does not significantly impact

the C2C12 intramuscular metabolome. Additionally, *in vitro* exercise stimulation and heat stress allowed the removal of systemic factors induced by exercise and heat stress. For example, it is well established that exercise and heat stress increases circulating adrenaline (Febbraio et al., 1994a; 1996c; Hargreaves et al., 1996a; 1996b). Given that glycogen phosphorylase activity is augmented via adrenal β-androgenic stimulation, any increase in circulating adrenaline likely increases muscle glycogenolysis. The lack of increase in glucose-1-phosphate abundance in both heat stress and stim & heat conditions suggests a crucial role of hormonal and metabolic regulation during exercise in high ambient temperatures.

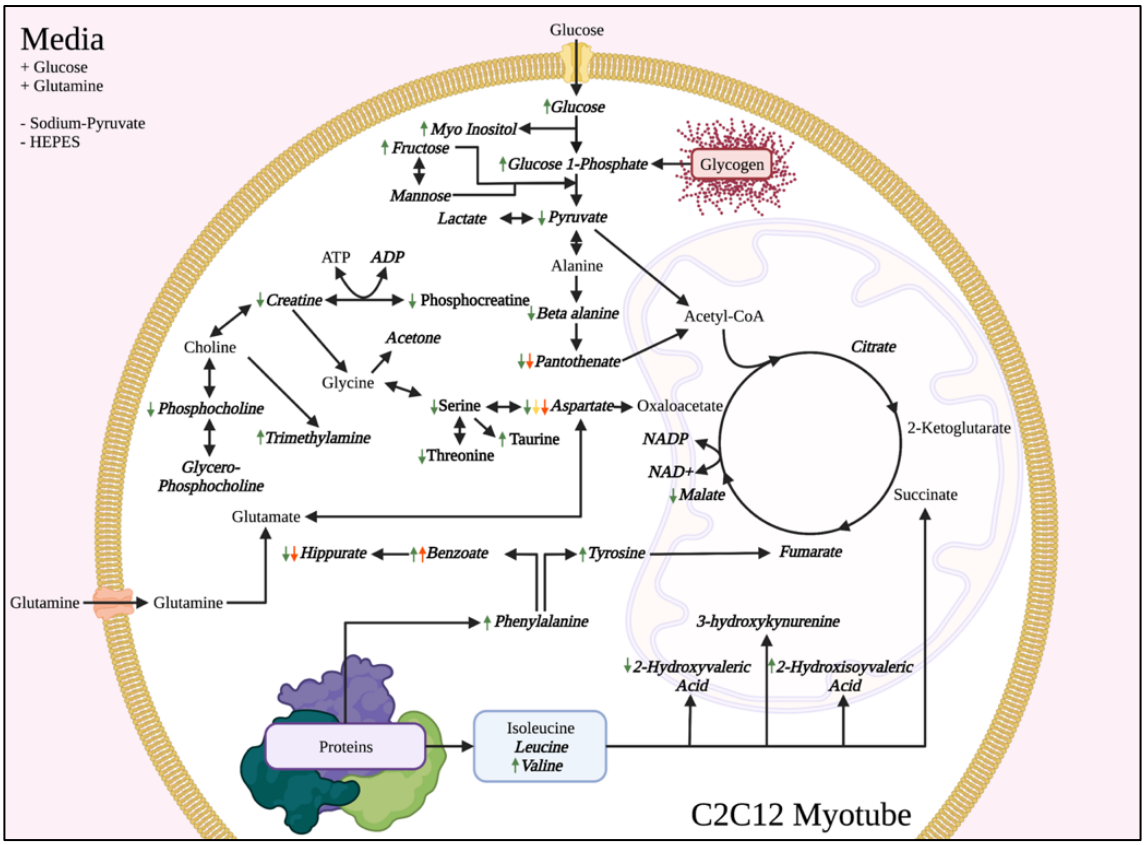

Figure 7.11 – Schematic representation of the proposed metabolic signatures of C2C12 myotubes in response to Electrical pulse stimulation (EPS) (Stim Only), Heat Stress (40°C) (Heat only) and combined EPS and heat stress (40°C) (Heat & Stim). Metabolites in italic were identified using ^{1}H-NMR Spectroscopy with change in abundance relative to control represented by coloured (Stim Only = Green, Heat Only = Yellow and Heat & Stim = Orange) arrows (Up = increased abundance, down = decreased abundance). Created with biorender.com.

Furthermore, the lack of significantly increased metabolism in response to heat stress alone casts doubt on previous suggestions that the direct temperature effect of heat stress on enzyme activity may be responsible for increased carbohydrate metabolism during exercise in the heat (Kozlowski et al., 1985; Young, 1990). To date, no *in vitro* evidence supports the notion that adrenaline is a critical regulatory factor in skeletal muscle metabolism in response to heat stress. Future research should combine EPS, heat stress and adrenaline treatment *in vitro* to understand better each factor's relative contribution to altered substrate metabolism.

In line with the initial hypothesis, increases in energy-producing metabolic pathways, specifically galactose metabolism, characterised by elevated concentrations of Glucose, Fructose, and Glucose 1-phosphate, have been reported in cells subjected to EPS only (Figure 7.13). Whilst the exact mechanism for increased glucose abundance is not identifiable within the present study, previous literature has shown increased GLUT4 translocation in C2C12 myotubes in response to EPS following 60 mins (Li et al., 2018) and 150 mins of EPS (Hu et al., 2018). Alongside the increase in intracellular glucose, elevated glucose 1-phosphate abundance indicates muscle glycogenolysis. Electrically stimulated cells have shown reductions in muscle glycogen content (Marotta, Bragos and Gomez-Foix, 2004; Heden et al., 2021), with glycogenolysis coinciding with glucose uptake (Manabe et al., 2012; Li et al., 2018). Unfortunately, the failure to characterise the post-intervention media metabolome prevents definitive conclusions on the origin of increased intracellular glucose.

Despite increases in glucose and glucose 1-phosphate, downstream glycolytic metabolites remained unchanged or decreased (in the case of pyruvate). Tricarboxylic acid (TCA) cycle metabolites, citrate, methyl succinate, fumarate and malate were unchanged, suggesting that total cellular metabolic demand may not have increased significantly in

response to stimulation or heat stress. Therefore, oxidative substrate oxidation was not required to meet energy demands within the cells. During the optimisation of EPS and heat stress models (Chapter 6), metabolic activity was quantified during treatment using an MTT assay which relies upon NAD(P)H-dependent cellular oxidoreductase enzymes, typically located within the mitochondria, to convert tetrazolium dye (MTT) into purple formazan crystals. Metabolic activity was increased by ~15% and 10% in response to stimulation only and stimulation and heat stress combined, respectively (Figure 6.3A) (within typical error of plate-based assay) which is considerably less than the 30-fold increase in TCA cycle flux observed during strenuous exercise *in vivo* (Gibala et al., 1998). Most notably, and in line with the metabolomic data, metabolic activity in heat only decreased by 10%, suggesting heat stress does not induce significant metabolic stress. Thus, changes in the metabolome are not observed. This is a particularly surprising outcome of the present work, as the metabolic stress induced by heat stress has been proposed as a mechanism for increased mitochondrial biogenesis in C2C12 cells in response to repeated heat exposures (Liu and Brooks, 2012; Patton et al., 2018).

7.4.2 Intracellular Metabolomic Response to Electrical Pulse Stimulation and Heat Stress with Low Glucose Availability

To better understand the impact of carbohydrate availability, exercise, and heat stress on skeletal muscle metabolism, the previously conducted *in vitro* metabolomics experiment was replicated but depleted media substrate availability prior to experimental treatment.

In contrast to the first experiment, reduced substrate availability, impaired intracellular metabolism and prevented significant changes to the intra-muscular metabolome (Figure 7.14), as previously reported by Lautaoja et al. (2021). The lack of change in metabolome is illustrated

by PCA scores plots indicating no apparent variance between conditions (Figure 7.7). PLS-DA used to identify important metabolites for the discrimination between groups showed greater clustering in heat and heat & stim conditions compared to stimulation and control (Figure 7.8). Of the 53 metabolites identified by ^{1}H-NMR spectroscopy, 24 were significantly different between groups, with 17 significantly different metabolites between control and Heat & Stim, 14 in heat only and 7 in Stim Only. The combination of stimulation and heat stress amplified changes in specific metabolite abundance compared to Stim Only and Heat Only conditions.

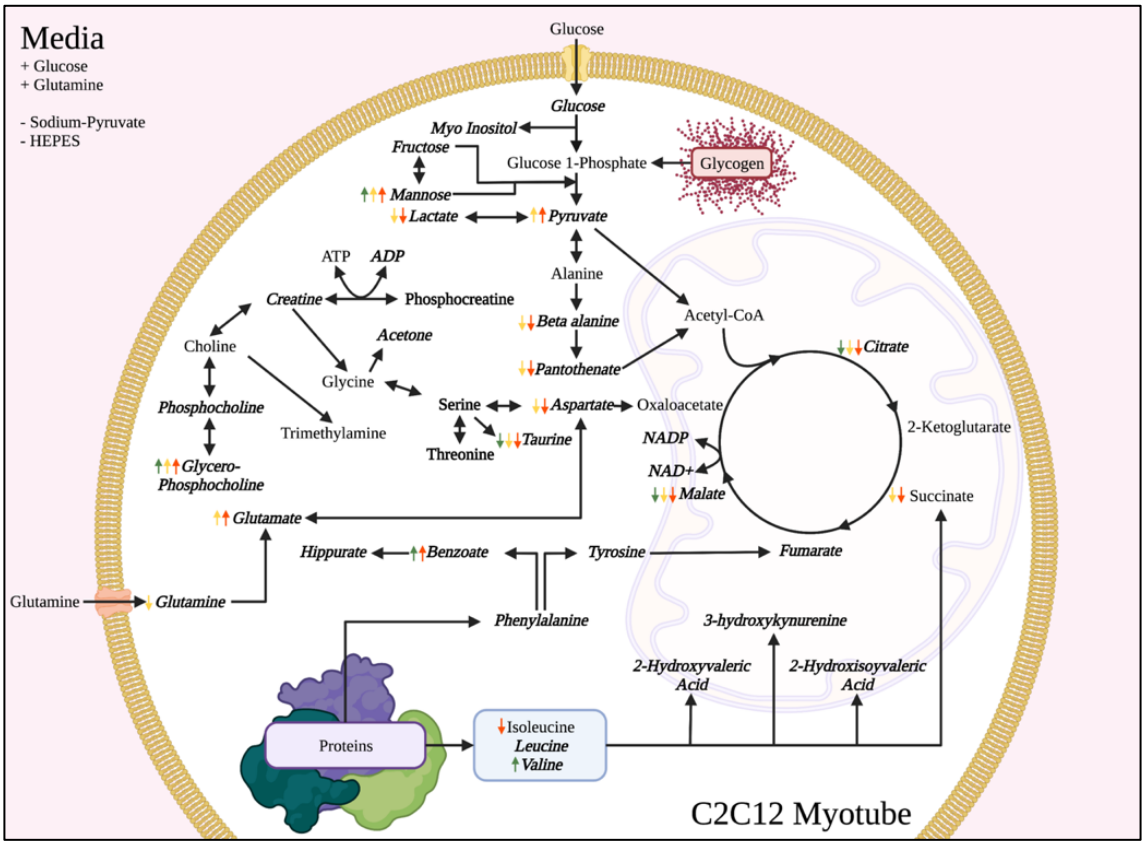

Figure 7.12 – Schematic representation of the metabolic signatures of C2C12 myotubes in response to Electrical pulse stimulation (EPS) (Stim Only), Heat Stress (40°C) (Heat only) and combined EPS and heat stress (40°C) (Heat & Stim) with low substrate availability. Metabolites in italic were identified using ^{1}H-NMR Spectroscopy with change in abundance relative to control represented by coloured (Stim Only = Green, Heat Only = Yellow and Heat & Stim = Orange) arrows (Up = increased abundance, down = decreased abundance). Created with biorender.com.

To deplete substrate availability before treatment, media was unchanged throughout differentiation; instead, 10% v/v fresh was added DM daily to prevent total depletion of

substrate and to reflect repeated bouts of substrate-depleting exercise *in vivo.* Daily media top-ups throughout differentiation result in a ~50% decrease in media glucose by day 7, resulting in ~2.75g·L^{-1} concentration, significantly higher than the 1.5g·L^{-1} typically found in low glucose DMEM. Furthermore, concomitant to a decrease in glucose, an inverse increase in media lactate accumulation was observed, a metabolite that was subsequently heavily depleted during heat stress conditions, potentially used as an alternate fuel source when glucose availability is low (Rogatzki et al., 2015).

It is well established that substrate metabolism is altered during exercise in the heat *in vivo* (Febbraio, 2001) and under conditions of altered carbohydrate availability (Achten and Jeukendrup, 2003). Additionally, low carbohydrate availability in hot conditions severely impacts performance and increases carbohydrate oxidation rate relative to exercise duration (Pitsiladis and Maughan, 1999). Data herein provide the first intra-muscular metabolome data showing the effect of heat stress and substrate availability during 'exercise' on skeletal muscle. In line with previous work by Lautaoja et al. (2021), decreased substrate availability impairs the metabolomic response to electrical stimulation in C2C12 myotubes and in contrast to previous high glucose experiments, glucose abundance is not increased in response to EPS, whilst lactate and pyruvate are decreased and increased respectively. Unexpectedly, the change in lactate availability in the present study likely provides empirical evidence to support the notion that lactate is not a by-product but an essential alternative fuel source during metabolic stress (Brooks et al., 2022).

Most notable differences in metabolite abundances occur in reduced Tricarboxylic Acid (TCA) cycle intermediates (citrate, methyl-succinate, and malate) alongside decreased TCA pre-cursor metabolites such as aspartate, beta-alanine and pantothenate. Decreases in TCA

cycle activity may be further supported by an increased abundance of pyruvate, suggestive of a potential ‘metabolic bottleneck’ between glycolysis and the mitochondrial TCA cycle.

7.4.3 Conclusion

The series of experiments presented within this chapter have characterised the metabolomic impact of electrical pulse stimulation and heat stress in C2C12 myotubes with high and reduced glucose availability. The data presented here suggest that when isolated from systemic responses to heat stress, metabolic perturbations are limited, highlighting the potentially critical role of circulating adrenaline in the modulation of intramuscular metabolism in response to heat stress.

Chapter 8 – Characterisation of the Serum Metabolome in Response to Exercise, Heat Stress and Carbohydrate Periodisation

Aspects of this work are published in Experimental Physiology

Bennett, S., Brocherie, F., Phelan, M. M., Tiollier, E., Guibert, E., Morales-Artacho, A. J., Lalire, P., Morton, J. P., Louis, J. B., & Owens, D. J. (2022).

Acute heat stress amplifies exercise-induced metabolomic perturbations and reveals variation in circulating amino acids in endurance-trained males.

Experimental Physiology, 00,1–14.

https://doi.org/10.1113/EP090911

And presented by E-Poster at the 27th Annual European Congress of Sport Science (ECSS), Seville, Spain.

8.1 Introduction

Hot conditions impair Endurance exercise in both laboratory (Tatterson et al., 2000; Tucker et al., 2004; Ely et al., 2010; Peiffer and Abbiss, 2011; Periard et al., 2011; Periard and Racinais, 2016) and 'real-world' environments (Racinais et al., 2015c) compared to thermoneutral conditions. Heat production via skeletal muscle contraction and elevated environmental temperatures induces rapid onset of hyperthermia as heat storage occurs more rapidly than head loss. In addition, increased core temperature leads to greater cardiovascular demand in an attempt to thermoregulate via increased skin blood flow via cutaneous vasodilation and increased sweat rate (Gonzalez-Alonso et al., 1999; Gonzalez-Alonso, Crandall and Johnson, 2008). Metabolically, a shift towards a greater contribution of carbohydrates to energy production is also observed at the expense of fatty acid metabolism (Febbraio et al., 1994a; 1994b).

Multiple integrated mechanisms are likely responsible for increased carbohydrate oxidation during exercise and heat stress, with elevated circulating adrenaline a significant contributor (Febbraio et al., 1994a; 1996c; Hargreaves et al., 1996a; 1996b). Via the β-adrenergic stimulation of glycogen phosphorylase, muscle glycogenolytic rate is increased with concomitant increases in hepatic glucose production, inducing a state of relative hyperglycaemia during exercise in hot conditions (Rowell et al., 1968; Hargreaves et al., 1996a; Howlett, Febbraio and Hargreaves, 1999; Howlett et al., 1999). The metabolism of liver and muscle-derived glucose appears to involve both oxidative and non-oxidative pathways resulting in increased lactate accumulation during exercise in the heat (Young et al., 1985; Young, 1990; Febbraio et al., 1994a; 1994b; Gonzalez-Alonso, Calbet and Nielsen, 1999), suggestive of increased anaerobic glycolysis during exercise in high ambient temperatures.

Despite the exponential increase in exercise metabolomics studies over the last 10 years (Figure 3.15) and investigations into the effect of environmental stressors such as altitude on the exercise metabolome (Messier et al., 2017; Margolis et al., 2021a), there are no studies investigating the effect of heat stress during exercise on the human metabolome.

Pre-exercise substrate availability also significantly impacts substrate utilisation acutely and chronically. For example, acute exercise with low carbohydrate availability increases fat oxidation (Achten and Jeukendrup, 2003; Riis et al., 2019), with long-term manipulation of carbohydrate availability translating to changes in whole-body substrate utilisation characterised by a shift towards greater fatty acid oxidation for the same relative exercise intensity (Yeo et al., 2008b; Hulston et al., 2010). As with heat stress, few studies have investigated the role of pre-exercise muscle glycogen availability on the exercise metabolome, with Margolis et al. (2021b) showing increases in branched-chain amino acids in response to acute low-glycogen exercise. Given the changes in whole-body substrate utilisation following regular exercise with low carbohydrate availability, it is logical to suggest that metabolic adaptations would be reflected in the exercise metabolome. Currently, no evidence supports the hypothesis that changes in the metabolome reflect changes in whole-body substrate metabolism.

Firstly, this chapter aimed to characterise the effect of acute heat stress and exercise on performance and the underlying impact on the human serum metabolome. Additionally, following 2 weeks of heat acclimation and nutritional intervention ("Sleep-low, train-low"), aimed to characterise the change in serum metabolome in response to exercise in hot and temperate conditions.

8.2 Methods

8.2.1 Participants and Research Design

Participant characteristics have been previously described in Chapter 3.2.1. The present study comprises two main components: an acute investigation of the impact of environmental temperature on the exercise metabolome and the metabolic characterisation of chronic exercise completed with low carbohydrate availability and/or heat stress. Initially, participants completed a previously described 30-min maximal exercise test (Chapter 3.3.3) in both hot (35°C) and temperate (21°C) conditions in a randomised order (Figure 8.1A). Venous Blood samples were collected immediately pre- and post-exercise for metabolomic analysis (blood processing protocols discussed in Chapter 3.5 and Chapter 3.8.2). Secondly, in matched, independent groups, repeated measures design participants were divided into 3 groups *(n* = 8) and completed a 2-week exercise-nutrition-environment intervention. Participants completed the same exercise training protocol; however, environmental, and nutritional conditions varied between groups. One group completed all sessions in a thermoneutral environment (21°C, 50% Relative Humidity [RH]) with "normal" carbohydrate availability for all sessions (CON). The other two groups completed the two-week training programme with periodised carbohydrate intake to reduce carbohydrate availability for specific sessions ("sleep low-train low") in either thermoneutral (SL) or hot conditions (35°C, 50% RH, SL_{Heat}) (Figure 8.2B). Standardised pre-exercise meal (CHO: 2.0g·kg·BM^{-1}, PRO: 0.3g·kg·BM^{-1}, fat: 0.3g·kg·BM^{-1}) was consumed prior to each exercise test.

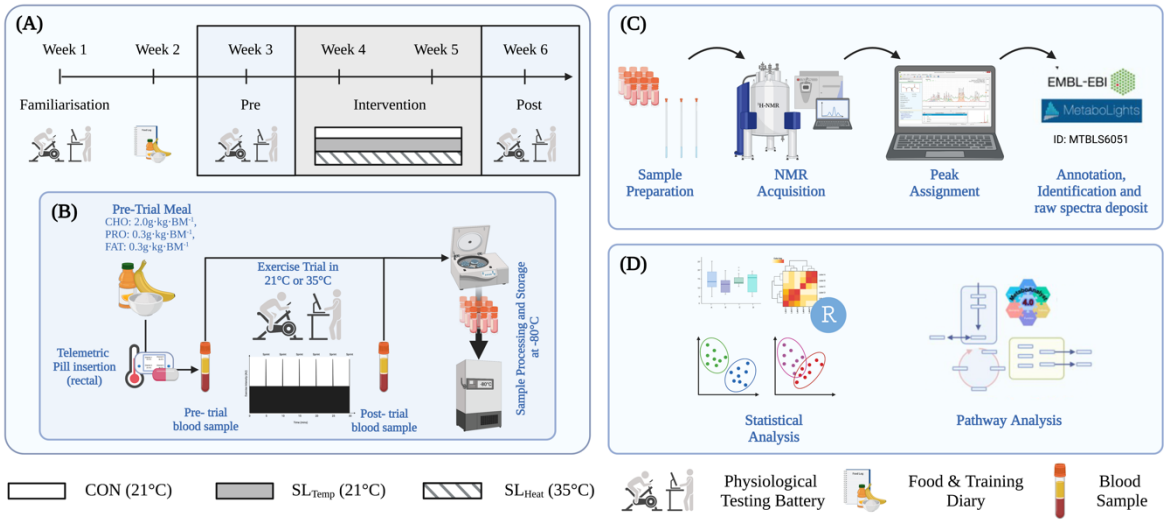

Figure 8.1 – Schematic overview of acute and chronic metabolomic studies and brief overview of sample processing and analysis. (A) Study timeline (B) Participants completed a 30-min maximal cycling test in hot and temperate conditions with blood samples collected immediately pre and post following 2 weeks of exercise, nutrition and environmental intervention, the maximal cycling test was repeated, and blood samples collected again (C) Sample were processed and analysed using ^{1}H-NMR metabolomics (D) statistical analysis was completed using in-house made R Scripts and Metaboanalyst 5.0. Figure created with biorender.com.

8.2.2 Biofluid Sample Collection

Samples were collected immediately post-exercise via an indwelling cannula inserted into the anti-cubital fossa of the forearm using standard venepuncture techniques. Samples were collected using serum tubes (Vacutainer systems, BD), free of clotting gels or additives that may interfere with metabolomics analysis. Samples were allowed to clot at room temperature for 30 mins before centrifugation at 1500 RCF for 15 mins at 4°C. The resultant serum was aliquoted into 2ml cryovials and stored at -80°C until processing.

8.2.3 Spectral Processing, Quality Control and Annotation

All spectra were automatically pre-processed at spectrometer by Fourier-transformation, phase correction and baseline correction using standard vendor routines

(apk0.noe), referenced indirectly via anomeric glucose signal at 5.24 ppm and subjected to quality control criteria as recommended by Metabolomics Standards Initiative (MSI) (Sumner et al., 2007; Salek et al., 2013). Quality control criteria consisted of appraisal of baseline, line-width, residual water signal width, phase, and signal-to-noise. Spectra were bucketed according to peaks boundaries defined with each bucket, the sum of the integral for that region divided by the region width. Metabolites were annotated via metabolite recognition software Chenomx (Chenomx v 8.2, Chenomx Ltd., Edmonton, AB, Canada), and the respective buckets were annotated prior to statistical analysis. Metabolite identities were confirmed (where possible) compared to the in-house metabolite library.

8.2.4 Data Analysis

Performance and physiological variables, including mean power output, mean HR, ΔT_{rec} and Peak RPE, were first checked for normality by the Shapiro-Wilk test (Shapiro and Wilk, 1965) before analysis via paired T-Test whilst core temperature, RPE, thermal comfort, and thermal sensation were analysed by 2-way ANOVA, using GraphPad Prism (Version 9.3.1, GraphPad Software, San Diego, CA, USA). Cohen's *d* coefficient for effect size was calculated and referenced against benchmarks suggested by Cohen (Cohen, 1988), where the thresholds for *d* to be considered small, medium, or large were < 0.2, 0.21-0.79 and > 0.8, respectively.

Metabolomics spectral processing and Data analysis have been covered in depth in Chapter 3. Briefly, spectra were probabilistic quotient normalisation (PQN) (Kohl et al., 2012) and Pareto scaling before undergoing multivariate analysis whereby samples from pre- and post-exercise samples were compared between conditions. Univariate analysis in which metabolite level differences were highlighted using t-test or ANOVA, where *p*-values were

corrected for false discovery rate by the Benjamini-Hochberg method, and an adjusted *p*-value of <0.05 was considered significant. Differences between environmental conditions were appraised with both univariate (Welch tests) and multivariate approaches, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). All statistical analyses were performed with the statistical software R (R CoreTeam). Multivariate models were applied, combining the metabolomics data (using one signal per metabolite) and the biochemistry variables measured in the participants. PLS-DA models were built using the package mixOmics (Le Cao, 2017). The number of components to retain for each model was calculated via 50 times 5-fold cross-validation using 70% of the data (training data), using the function perf within mixOmics. The 30% of data left was used to test the accuracy of the models (test data). Details of each model are presented in the results.

8.3 Results

8.3.1 Physiological and Performance Responses to Exercise in Hot and Temperate conditions

Analysed via paired two-tailed t-test, mean power was significantly greater ($t(22) = 3.934$, $P < 0.001$, $d = 0.24$) in temperate conditions (231 ± 36 W) compared to exercise in the heat (223 ± 31 W) (Figure 8.2A). Mean HR was significantly lower ($t(22) = 4.070$, $P < 0.001$, $d = 0.53$) during exercise in temperate conditions (162 ± 10 beats·min^{-1}) compared to hot conditions (167 ± 9 beats·min^{-1}) (Figure 8.2B). Peak rating of perceived exertion (RPE) was recorded at the end of exercise and was significantly higher ($t(22) = 3.102$, $P < 0.01$, $d = 0.5$) in hot conditions (19 ± 2) compared to temperate (18 ± 2). Mean core body temperature (mean T_{rec}) was not significantly different between conditions ($t(22) = 1.876$, $P = 0.07$, $d = 0.28$); however, peak core body temperature (Peak T_{rec}) was greater ($t(22) = 6.111$, $P <0.0001$, $d = 1.25$) in hot (38.8

± 0.4°C) compared to temperate (38.3 ± 0.4°C) reflected by the increased change in core temperature (ΔT_{rec}) in hot conditions (1.45 ± 0.28°C) compared to temperate (0.95 ± 0.47°C) ($t(22) = 4.426$, $P < 0.001$, $d = 1.29$) (Figure 8.2C). Analysis of Variance (ANOVA) revealed increased thermal sensation ($F(1, 44) = 19.40$, $P < 0.0001$) (Figure 8.2I) and comfort ($F(1, 44) = 31.31$, $P < 0.0001$) (Figure 8.2J) throughout the capacity test in hot conditions, with both, significantly increased throughout each exercise test (TS = $F(2.770, 121.9) = 114.0$, $P < 0.0001$; TC = $F(2.975, 130.9) = 88.53$, $P < 0.0001$). Second-by-second mean power output (Figure 8.2D) and Heart rate (Figure 8.2E) alongside 5-min-by-5-min mean core body temperature (Figure 8.2F) in hot and temperate conditions are reported throughout the exercise test. However, due to the high sampling frequency, power output and heart rate comparisons were limited to the whole trial mean data previously analysed via t-test and reported above.

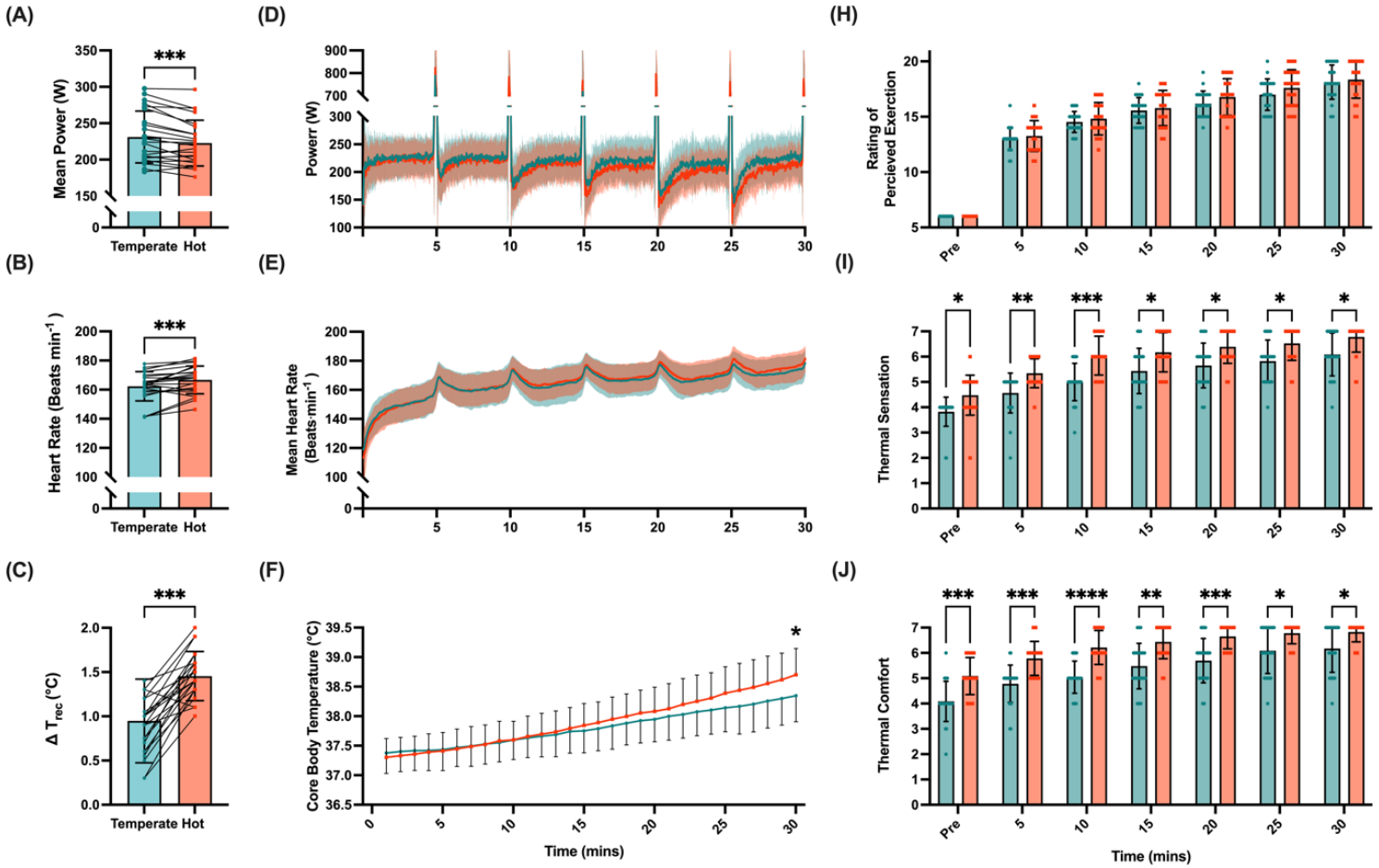

Figure 8.2 – Performance and Physiological responses during the 30-min capacity test in Hot (35°C, 50% RH) and Temperate (21°C, 50% RH) conditions. (A) Mean power output (W) (B) Mean heart rate (beats·min^{-1}) (C) Core temperature change from pre to post exercise (Δ T_{rec}) (D) Second-by-second mean power output, (E) heart rate and (F) minute-by-minute mean core temperature (°C) during 30-min capacity test. Perceptual responses to heat stress during exercise recorded every 5 minutes including (H) Rating of Perceived Exertion (RPE), (I) Thermal sensation and (J) thermal comfort.

8.3.2 Changes in Human Serum Metabolome Pre- to Post- Exercise in Hot and Temperate Conditions

8.3.2 i) Principal Component Analysis (PCA)

PCA was performed to identify any major variances between environmental conditions pre- and post-exercise (Figure 8.2). PCA Scores plot of PC1 (51.27%) against PC2 (10.39%) revealed clustering for pre- and post-exercise, with no separation between conditions. PC1 and PC2 explain a cumulative variance of 61.66%, with 9 components required to explain 95% of the variance within the data.

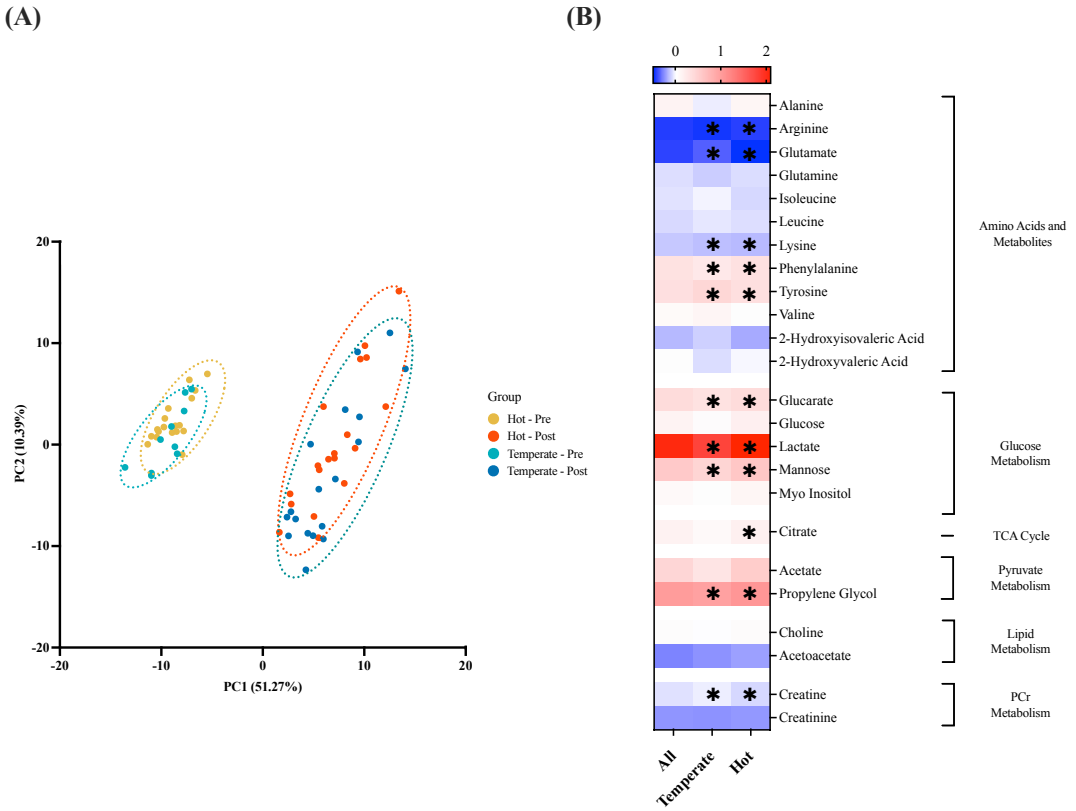

Figure 8.3 – (A) Multivariate Principal Component Analysis (PCA) of human serum pre (0 min) and post- (30 min) exercise in Hot (35°C) and Temperate (21°C) conditions. Brackets represent the variance explained by PC, 9 PCs required to explain 95% of the variance. For clarity, only PC1 and PC2 are shown on each axis. Ellipses represent a 95% confidence region. (B) Heatmap of all identified metabolites from pre- to post-exercise as the natural logarithm (Log2) to indicate whether each metabolite level had increased (greater than 0, red) or decreased (less than 0, blue). 'All' refers to the fold change of metabolites in the dataset without stratification for environmental conditions. * Denotes significant difference in metabolite abundance from pre- to post-exercise.

8.3.2 ii) Partial Least Squares – Discriminant Analysis (PLS-DA)

A cross-validated PLS-DA model was used to enhance differences between conditions and compare metabolic profiles between time points and environmental conditions (Figure 8.3A). Optimal model complexity was found to be a 2-component model with Pre-exercise in hot condition against others ROC = 0.96, Pre-exercise in temperate condition against others ROC = 0.85, post-exercise in the hot condition against others ROC = 0.87, post-exercise in the temperate condition against others ROC = 0.91. As with the PCA scores Plot (Figure 8.2), distinct clustering was observed between pre- and post-exercise, but no difference between Hot and temperate conditions. Using VIP scoring as a criterion, metabolites influential in the discrimination between conditions were extracted.

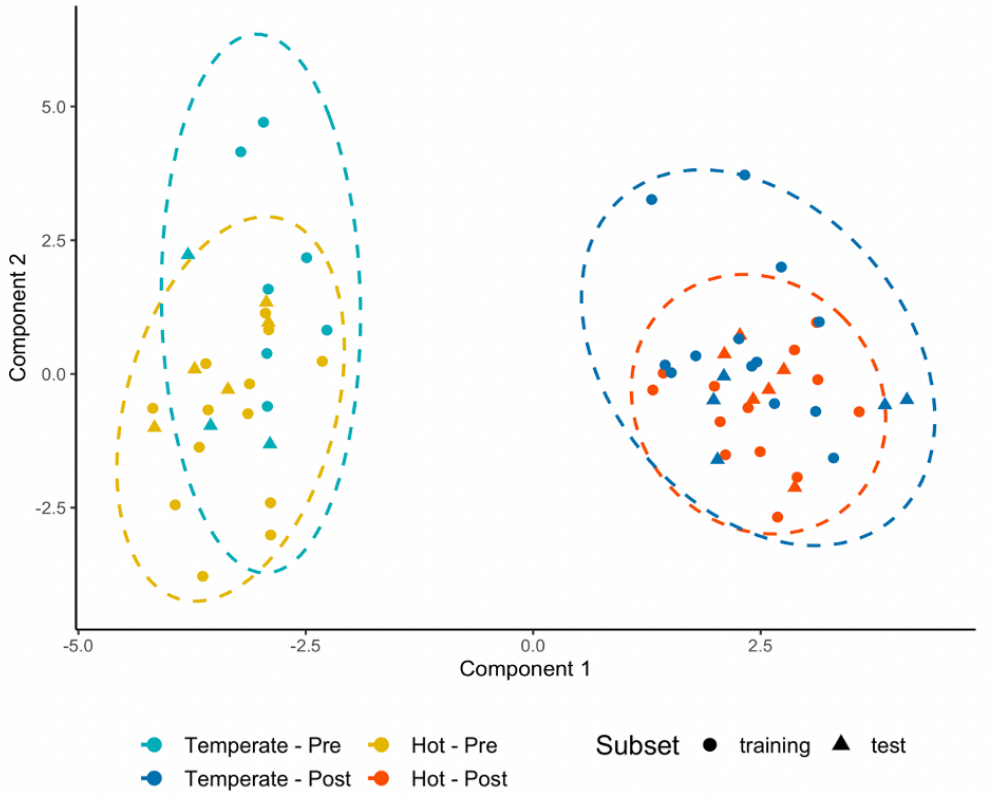

Figure 8.4 – Partial least squares discriminant analysis (PLS-DA) of human serum pre (0 mins) and post- (30 mins) exercise in Hot (35°C) and Temperate (20°C). Circular data points represent samples one which the model was built, with triangle data points representing samples used to validate the model. Ellipses show the 95% confidence region.

8.3.2 iii) Variable importance of the projection (VIP)

Metabolites expressing a VIP score greater than 1 were considered the most influential in explaining metabolic differences (Figure 8.4) between conditions and were selected for further analysis.

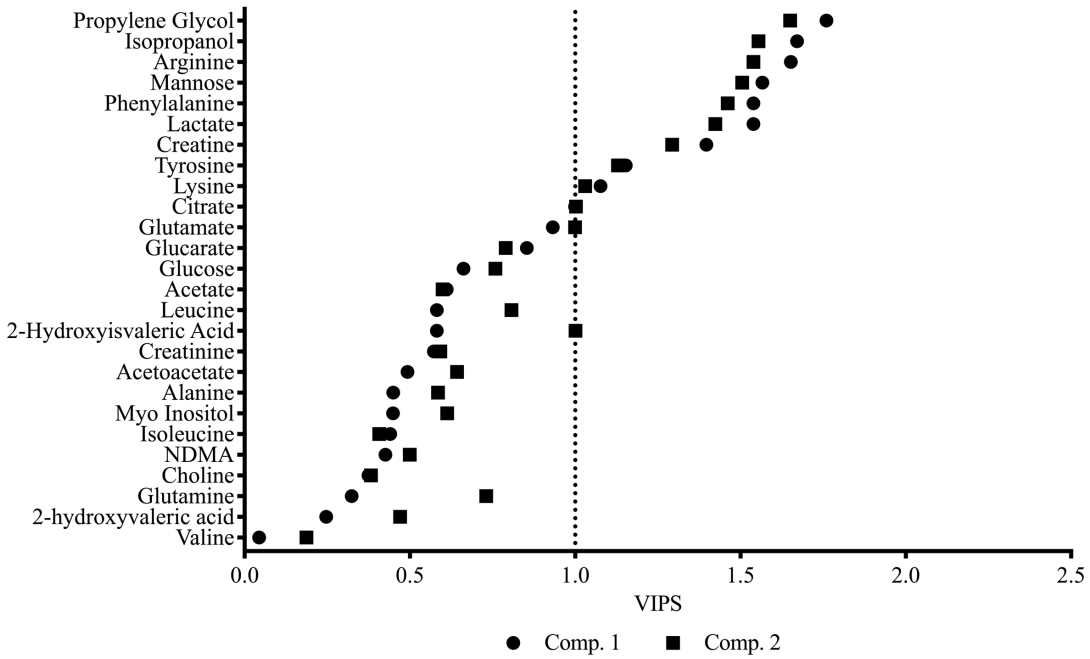

Figure 8.5 – VIP scores of PLS-DA models built upon intervention-dependent differences in human serum metabolome. Dashed line represents VIP > 1 threshold.

8.3.2 iv) Univariate Analysis – ANOVA

VIP-filtered metabolites from each PLS-DA were compared via BH Adjusted ANOVA (Table 8.1) to gain metabolite level information on environmental and time point specific differences (all metabolite data can be found in Table 11.3) 14 metabolites were significantly different pre- to post-exercise following exercise in the heat, with 11 significantly different metabolites following exercise in temperate conditions. Before exercise, the serum metabolome was not significantly different between conditions, with no significantly different metabolites

identified. Post-exercise, there were no significantly different metabolites between conditions, with only 2-Hydroxyisovalerate showing a trend toward significance ($P = 0.055$).

Table 8.1 – ANOVA output including Tukey post-hoc between conditions comparisons.

Metabolite	F-stat	*P* value	Adjusted *P* value	Pre-Temperate *vs.* Hot	Post Hot *vs.* Pre-Hot	Post Temperate *vs.* pre-Temperate	Post Temperate *vs.* Hot
Propylene Glycol	191.06	$5.81x10^{-31}$	$1.51x10^{-29}$	0.3381	$2.03x10^{-11}$	$2.03x10^{-11}$	0.6506
Isopropanol	103.26	$7.18x10^{-24}$	$9.34x10^{-23}$	0.9430	$2.03x10^{-11}$	$2.03x10^{-11}$	0.8147
Arginine	88.23	$3.70x10^{-22}$	$3.21x10^{-21}$	0.9583	$2.03x10^{-11}$	$2.04x10^{-11}$	0.9902
Lactate	59.01	$5.06x10^{-18}$	$3.29x10^{-17}$	0.9754	$2.04x10^{-11}$	$1.80x10^{-10}$	0.9994
Mannose	47.14	$6.86x10^{-16}$	$3.57x10^{-15}$	0.0498	$2.46x10^{-11}$	$2.09x10^{-7}$	0.3386
Phenylalanine	39.95	$2.08x10^{-14}$	$9.01x10^{-14}$	0.9511	$2.24x10^{-10}$	$1.49x10^{-8}$	0.7814
Creatine	27.06	$2.98x10^{-11}$	$1.11x10^{-10}$	1.0000	$5.24x10^{-8}$	$1.06x10^{-6}$	0.9798
Tyrosine	13.15	$1.00x10^{-6}$	$3.25x10^{-6}$	0.9725	0.0029	0.0001	0.3983
Lysine	12.08	$2.63x10^{-6}$	$7.59x10^{-6}$	0.9955	0.0001	0.0028	0.9154
Glutamate	11.33	$5.29x10^{-6}$	$1.37x10^{-5}$	0.1782	0.0011	0.0025	0.2084
Glucarate	9.34	$3.60x10^{-5}$	0.0001	0.9693	0.0003	0.0228	0.9943
Citrate	8.61	0.0001	0.0002	0.2010	0.0004	0.2794	0.9903
2-Hydroxyisovalerate	6.90	0.0004	0.0009	0.1013	0.0725	0.1244	0.0553
Glucose	4.30	0.0081	0.0150	1.0000	0.0124	0.3798	0.6038
Acetate	3.54	0.0197	0.0341	0.9988	0.0322	0.3645	0.9078
Acetoacetate	3.43	0.0224	0.0364	0.6442	0.1404	0.2497	0.5402
Alanine	3.13	0.0320	0.0489	0.2598	0.1187	0.9357	0.9107

8.3.2 v) Metabolite Set Enrichment Analysis (MSEA)

Selected metabolites were subject to MSEA to ascertain further metabolic pathway level information (Table 9.2) using a database curated from KEGG pathways (Homo Sapiens [Human] [KEGG organism code: hsa]) using fisher's exact test with EASE correction and Holm *P*-value adjustment. Two metabolic pathways were significantly over-represented, including aminoacyl-tRNA biosynthesis and phenylalanine, tyrosine, and tryptophan biosynthesis pathway (L-Phenylalanine, L-Tyrosine) with D-Glutamine and D-glutamate metabolism failing to reach statistical significance ($P = 0.0689$).

Table 8.2 – Results of the overrepresentation analysis using the hypergeometric test to evaluate whether a particular metabolite set was represented more than expected by change within the metabolites identified from PLS-DA analysis (VIPS > 1). One-tailed *p*-values are provided following multiple testing adjustment.

Biological Process	Total	Expected	Hits	Raw *p*-value	Holm adjusted *p*-value	FDR
Aminoacyl-tRNA biosynthesis	48	0.378	5	$1.7x10^{-5}$	0.00143	0.001
Phenylalanine, tyrosine, & tryptophan biosynthesis	4	0.0315	2	0.00034	0.0281	0.014
D-Glutamine & D-glutamate metabolism	6	0.0473	2	0.00084	0.0689	0.024
Phenylalanine metabolism	10	0.0788	2	0.00248	0.201	0.052
Arginine biosynthesis	14	0.11	2	0.00492	0.394	0.083
Arginine & proline metabolism	38	0.3	2	0.0342	1	0.479
Nitrogen metabolism	6	0.0473	1	0.0465	1	0.558

8.3.3 Changes in Serum Metabolome in Response to Maximal Exercise in Hot and Temperate Conditions.

8.3.3 i) Principal Component analysis (PCA)- Δ Relative Abundance

PCA was performed to identify any major variances between changes in metabolome in response to exercise in hot and temperate conditions (Figure 8.5). PCA Scores plot of PC1 (48.61%) against PC2 (16.05%) revealed clustering for hot and temperate conditions, with greater variability in temperate than hot. PC1 and PC2 explain a cumulative variance of 64.66%, with 8 components required to explain 95% of the variance within the data.

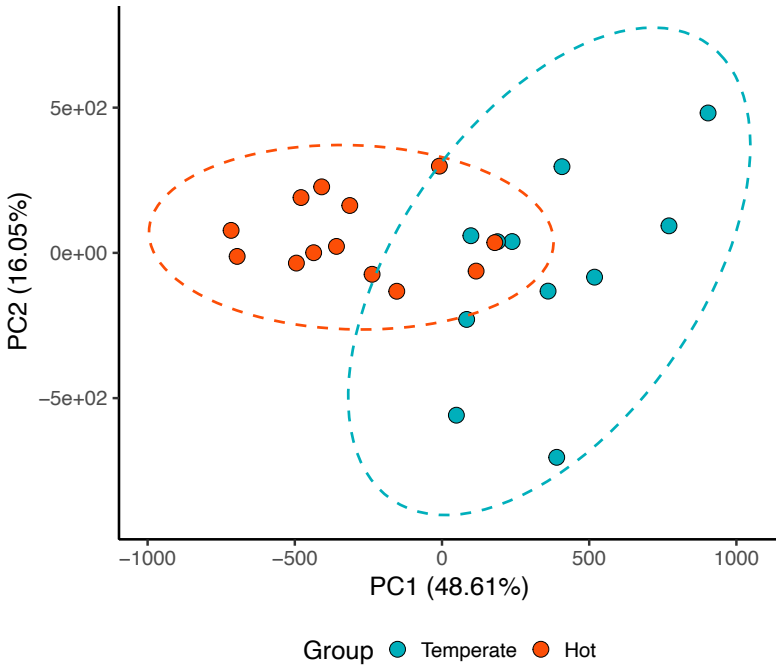

Figure 8.6 – Multivariate Principal Component Analysis of change in human serum metabolite concentration from Pre- to Post-exercise in Hot (35°C) and Temperate (21°C). Brackets represent the variance explained by P, with 8 PC's required to explain 95% of the variance. For clarity, only PC1 and PC2 are shown in each panel. Ellipses represent 95% confidence region.

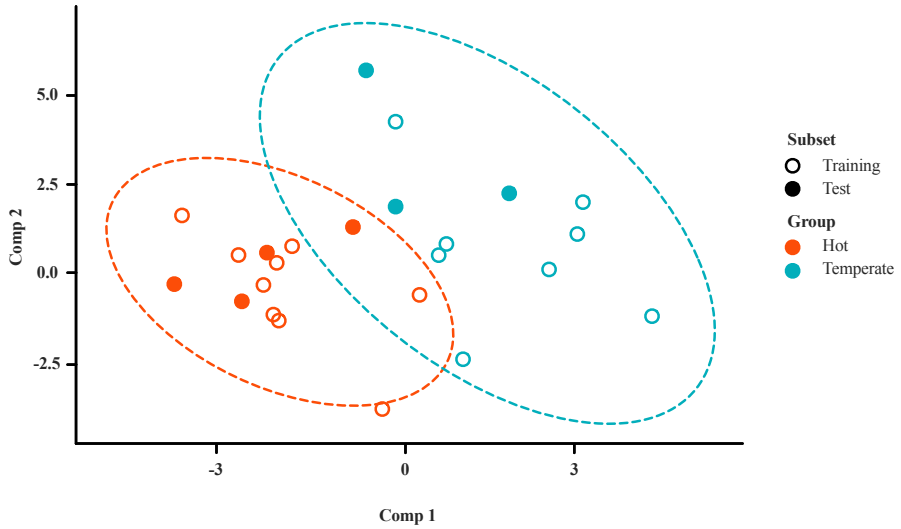

Figure 8.7 – Partial least squares discriminant analysis (PLS-DA) of change in human serum metabolite concentration from Pre- to Post-exercise in Hot (35°C) and Temperate (21°C). (A-D) PLS-DA scores plots representing component 1 and 2 for change in human serum metabolite concentration from Pre- to Post-exercise in Hot (35°C) and Temperate (20°C) discriminating between groups. Circular data points represent samples one which the model was built (training), with triangle data points representing samples used to validate the model (test). Ellipses show the 95% confidence region.

8.3.3 i) Partial Least Squares – Discriminant Analysis (PLS-DA) – Δ Relative Abundance

A cross-validated PLS-DA model enhanced differences between environmental conditions (Figure 8.6). The optimal model complexity was a 3-component model (ROC = 1). As with the PCA scores Plot (Figure 8.5), distinct clustering of environmental conditions was observed with, greater-within group variability observed in temperate conditions. In addition, using VIP scoring as a criterion, metabolites influential in the discrimination between conditions were extracted.

8.3.3 ii) Variable Importance for the Projection (VIP)

Metabolites expressing a VIP score greater than 1 were considered the most influential in explaining metabolic differences (Figure 8.8) between conditions and were selected for further analysis.

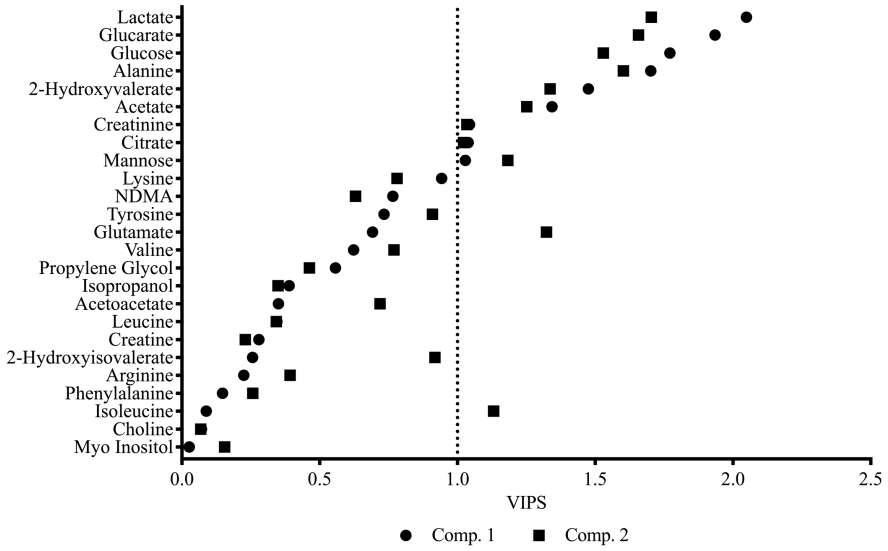

Figure 8.8 – VIP scores of PLS-DA models built upon intervention-dependent differences in human serum metabolome. Dashed line represents VIP > 1 threshold.

8.3.3 iii) Univariate Analysis – T-Test

VIP-filtered metabolites from each PLS-DA were compared via BH adjusted T-test (Table 8.3) to gain metabolite level information on condition-specific differences, 4 metabolites were significantly different, including Lactate, Glucarate, Alanine and Glucose. As previously, 2-Hydroxyvaleric Acid and Acetate exhibited a trend toward significance but did not reach the $P < 0.05$ threshold.

Table 8.3 – T-Test output including Tukey post-hoc comparisons.

Metabolite	**95% Confidence Intervals**	**Raw *P*-value**	**BH adjusted *P*-value**	**Bonferroni adjusted *P*-value**
Lactate	(-719.56996-330.99815)	1.00×10^{-9}	0.00031	0.00031
Glucarate	(-165.57186-55.26784)	4.00×10^{-4}	0.0052	0.0104
Alanine	(-194.12485-58.1585)	9.00×10^{-4}	0.00584	0.02183
Glucose	(-512.71964-151.91368)	9.00×10^{-4}	0.00584	0.02335
2-Hydroxyvaleric Acid	(-119.99505-17.28262)	0.0111	0.05017	0.27812
Acetate	(-291.33357-40.14385)	0.012	0.05017	0.30103
Creatinine	(9.19737,148.03724)	0.0282	0.10085	0.70592

8.3.3 iv) Metabolite Set Enrichment Analysis (MSEA)

Selected metabolites were subject to MSEA to ascertain further metabolic pathway level information (Table 8.4) using a database curated from KEGG pathways (*Homo Sapien* [Human] [KEGG organism code: hsa]) using fisher's exact test with EASE correction and Holm *P*-value adjustment. No metabolic pathways were identified as overrepresented during this analysis.

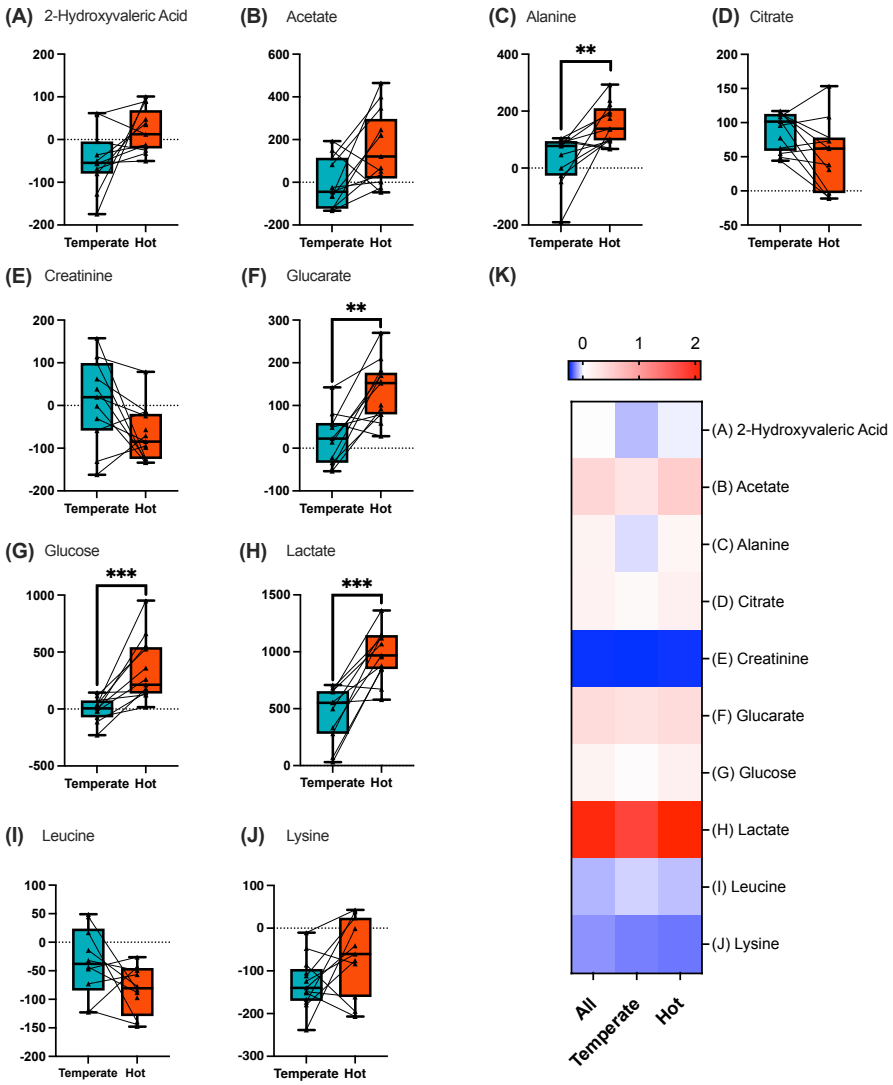

Figure 8.9 – Box-plots (A-J) representing median Δ abundance and Heat-map (K) of metabolite fold change from pre to post of VIP filtered serum metabolites following exercise in Temperate (21°C) and Hot (35°C) conditions. Significant difference between temperate and hot conditions denoted by *, where $P < 0.05$. For the heat-map, red represents positive metabolite fold change, whilst blue represents negative metabolite fold change. "All" refers to wholescale change in relative metabolite abundance from pre to post exercise irrespective of group. Box-plots of all measured metabolites can be found in Figure 11.3.

Table 8.4 – Results of the overrepresentation analysis using the hypergeometric test to evaluate whether a particular metabolite set was represented more than expected by change within the metabolites identified from PLS-DA analysis (VIPS > 1). One-tailed *p*-values are provided following multiple testing adjustment.

Biological Process	Total	Expected	Hits	Raw *p*-value	Holm adjusted *p*-value	FDR
Glucose-Alanine Cycle	13	0.127	2	0.0063	0.62	0.62
Gluconeogenesis	35	0.342	2	0.043	1	1
Biotin Metabolism	8	0.0781	1	0.0758	1	1
Pyruvate Metabolism	48	0.469	2	0.0762	1	1
Lactose Degradation	9	0.0879	1	0.0849	1	1

8.3.4 Effect of Previous Heat Acclimation and Periodised Carbohydrate Intake on Human Serum Metabolome Following Maximal Exercise in Hot and Temperate Conditions.

Following 2 weeks of exercise training and nutritional intervention whereby participants consumed the same total daily carbohydrate intake ($6g \cdot kg \cdot day^{-1}$) but the timed differently to achieve either high or low carbohydrate availability before specific training sessions in either temperate (20°C) hot conditions (35°C) human serum metabolome was characterised in response to maximal exercise in hot (35°C) and temperate (20°C) conditions following a standardised pre-exercise meal (CHO: $2.0g \cdot kg \cdot BM^{-1}$, PRO: $0.3g \cdot kg \cdot BM^{-1}$, Fat: $0.3g \cdot kg \cdot BM^{-1}$). As shown previously, comparison of metabolome pre- to post-exercise does not differentiate between conditions and as such, metabolome data is representative of change (Δ) in relative abundance here necessary. The number of samples per group and condition is shown in Table 8.5.

Table 8.5 – Number of participants included in analysis per group and per condition.

	Condition	
Group	**Hot (*n*)**	**Temperate (*n*)**
Control (CON)	6	3
Sleep-low, Train-low (SL)	4	6
SL + Heat (SL_{Heat})	5	6

8.3.4 i) Principal Component analysis (PCA) – Group-wise Δ Relative Abundance

PCA was performed to identify any major variances between changes in metabolome in response to exercise in temperate (Figure 8.10a) and conditions (Figure 8.10b) following 2 weeks of exercise, nutrition, and heat acclimation. In temperate conditions, the PCA Scores plot of PC1 (42.91%) against PC2 (24.12%) revealed clustering for hot and temperate conditions, with the greatest variability observed within the SL_{Heat} group; no clear separation was present between groups. PC1 and PC2 explain a cumulative variance of 67.03%, with 6 components required to explain 95% of the variance within the data.

During the Hot condition, the PCA Scores plot of PC1 (51.78%) against PC2 (17.32%) revealed no distinct clustering between groups, with the greatest variability observed within the Control group; no clear separation was present between groups. PC1 and PC2 explain a cumulative variance of 69.1% with 6 components required to explain 95% of the variance within the data.

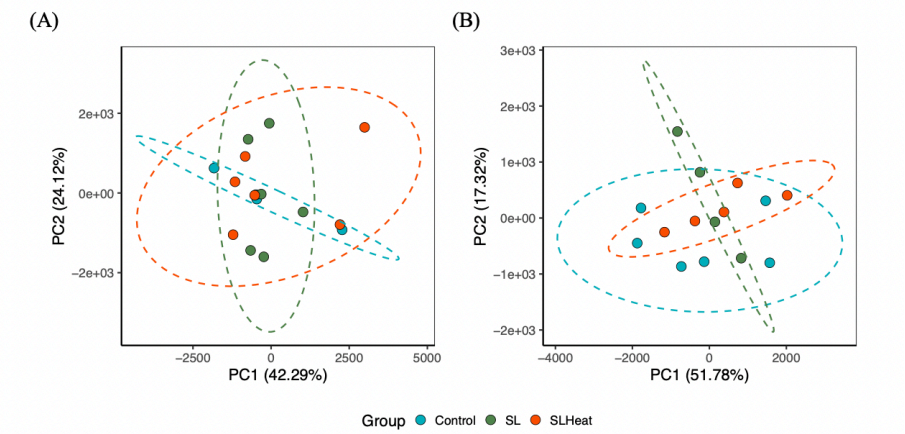

Figure 8.10 – Multivariate Principal Component Analysis (PCA) of change in human serum metabolite concentration pre (0 mins) to post (30 mins) exercise in (A) Temperate (21°C), and (B) Hot (35°C) conditions following 2 weeks of exercise-nutrition-environmental intervention. Brackets represent the variance explained by PC, with 6 PC's required to explain 95% of the variance in both temperate and hot conditions. For clarity, only PC1 and PC2 are shown in each panel. Blue represents Control group, Green represented Sleep Low (SL) group and Orange represented Sleep low – Heat group (SL_{Heat}). Ellipses represent 95% confidence region.

8.3.4 ii) Partial Least Squares – Discriminant Analysis (PLS-DA) – Groupwise Δ Relative Abundance

To calculate Δ metabolite relative abundance, only participants with both pre- and post-exercise samples were included in the initial analysis reducing the number of participants upon which the model can be built (Table 8.5). Furthermore, due to the limited sample size, the whole group were randomly removed from the model and preventing the separation of groups; as such, PLS-DA data is representative of all participants' serum metabolome pre- to post-exercise in hot and temperate conditions.

A cross-validated PLS-DA model was used to enhance differences between environmental conditions and groups (Figure 8.11). Optimal model complexity was a 2-component model with ROC scores reported in Table 8.6. As with the PCA scores Plot (Figure 8.10A/B), distinct clustering of environmental condition was observed, with greater within-

group variability observed in temperate conditions. In addition, using VIP scoring as a criterion, metabolites influential in the discrimination between conditions were extracted.

Table 8.6 – Summary of PLS-DA cross-validation ROC scores. ROC = 1 represents a perfectly accurate model compared to ROC = 0 showing no predictive capacity of the model.

Condition	Comparison (vs. Others)	ROC Score
Hot	CON	0.93
	SL	0.63
	SL_{Heat}	0.88
Temperate	CON	0.38
	SL	0.57
	SL_{Heat}	0.86

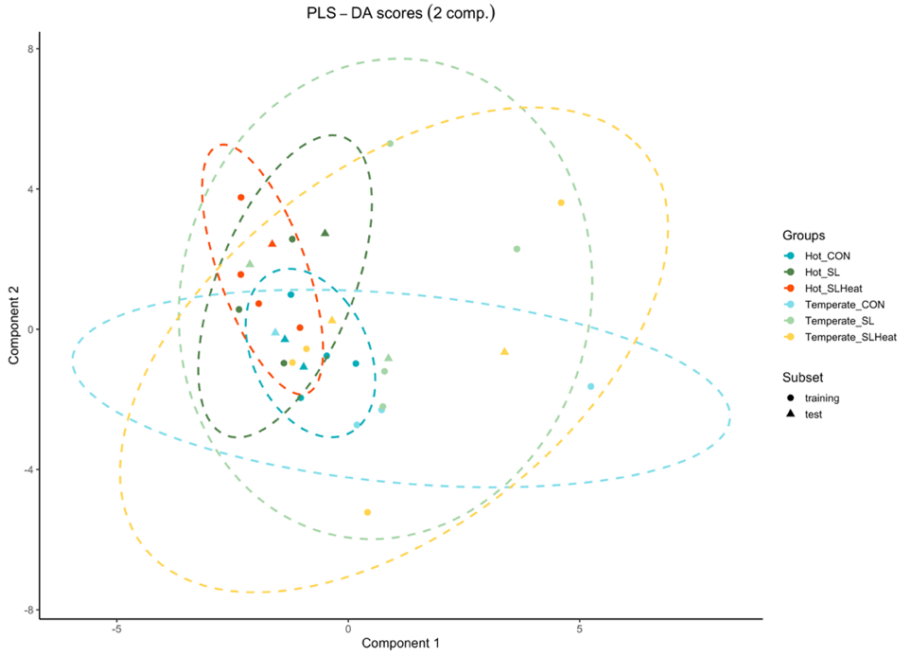

Figure 8.11 – Partial least squares discriminant analysis (PLS-DA) of change in human serum metabolite concentration from pre (0 mins) to post (30 mins) exercise in Hot (35°C) and Temperate (20°C) conditions separated by experimental group. Ellipses show the 95% confidence region.

8.3.4 iii) Variable Importance for the Projection (VIP)

Due to the low PLS-DA model validation scores (Table 8.6), the accuracy of associated VIP metabolites is limited. Therefore, to generate a subset of influential metabolites, 10 2-component PLS-DA models were generated, and a mean VIP score across all models was

calculated per metabolite. Metabolites with a mean VIP score above one were used in further analysis.

8.3.4 iv) Univariate Analysis – ANOVA

VIP-filtered metabolites from each PLS-DA were compared via BH Adjusted ANOVA in temperate and hot to gain metabolite level information on group-specific differences in metabolite abundance. No metabolites were significantly different between groups or environmental conditions. Therefore, metabolome-wide data can be found for all measured metabolites in temperate conditions (Table 11.4), hot conditions (Table 11.5) and boxplots for all metabolites across both conditions in Figure 11.4.

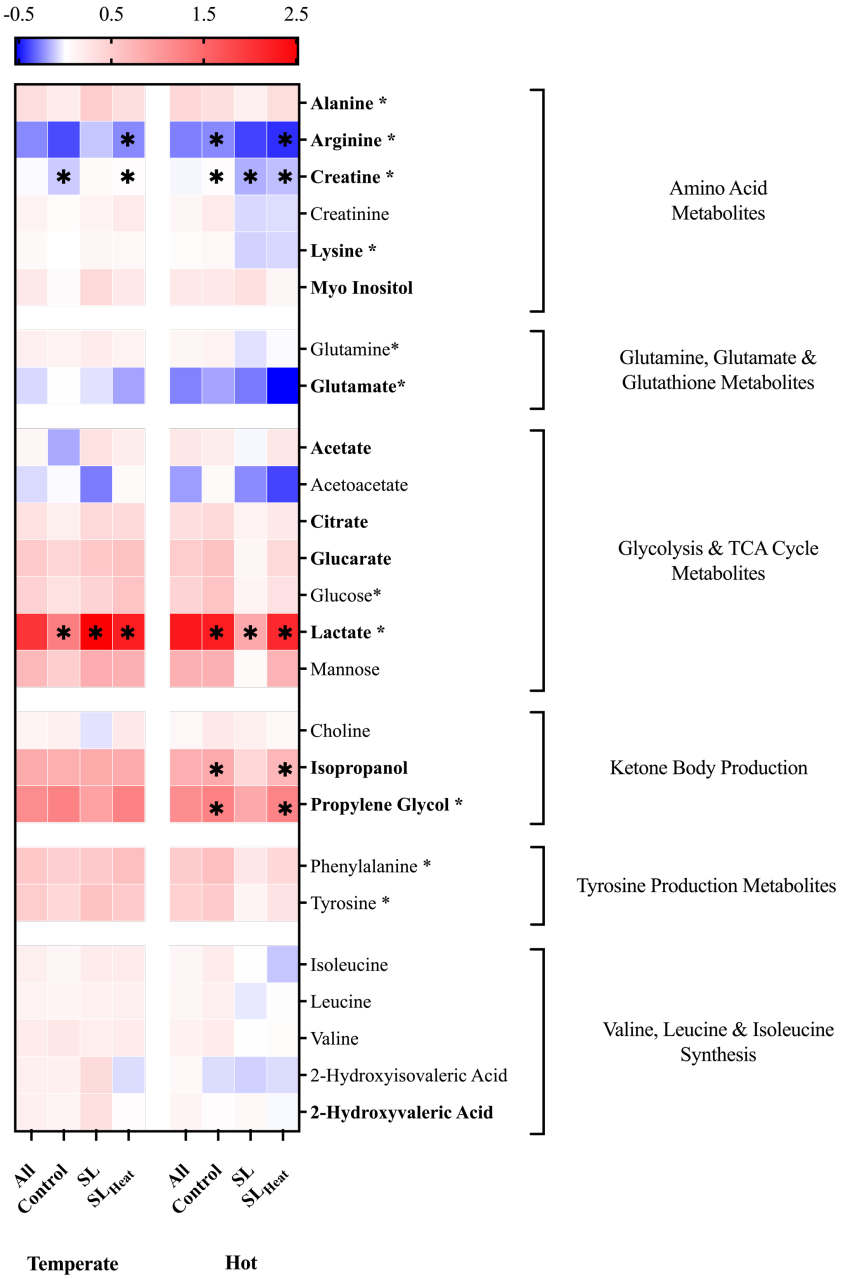

Figure 8.12 – Heatmap representing Log2 fold change in relative metabolite abundance from pre to post exercise in temperate (20°C) and hot (35°C) conditions for each group prior to intervention. Red represents positive whilst blue represents a negative metabolite fold change. "All" refers to wholescale change in relative metabolite abundance from pre to post exercise irrespective of group. Influential metabolites identified by 10 x PLS-DA VIP scoring (> 1) are highlighted in bold.

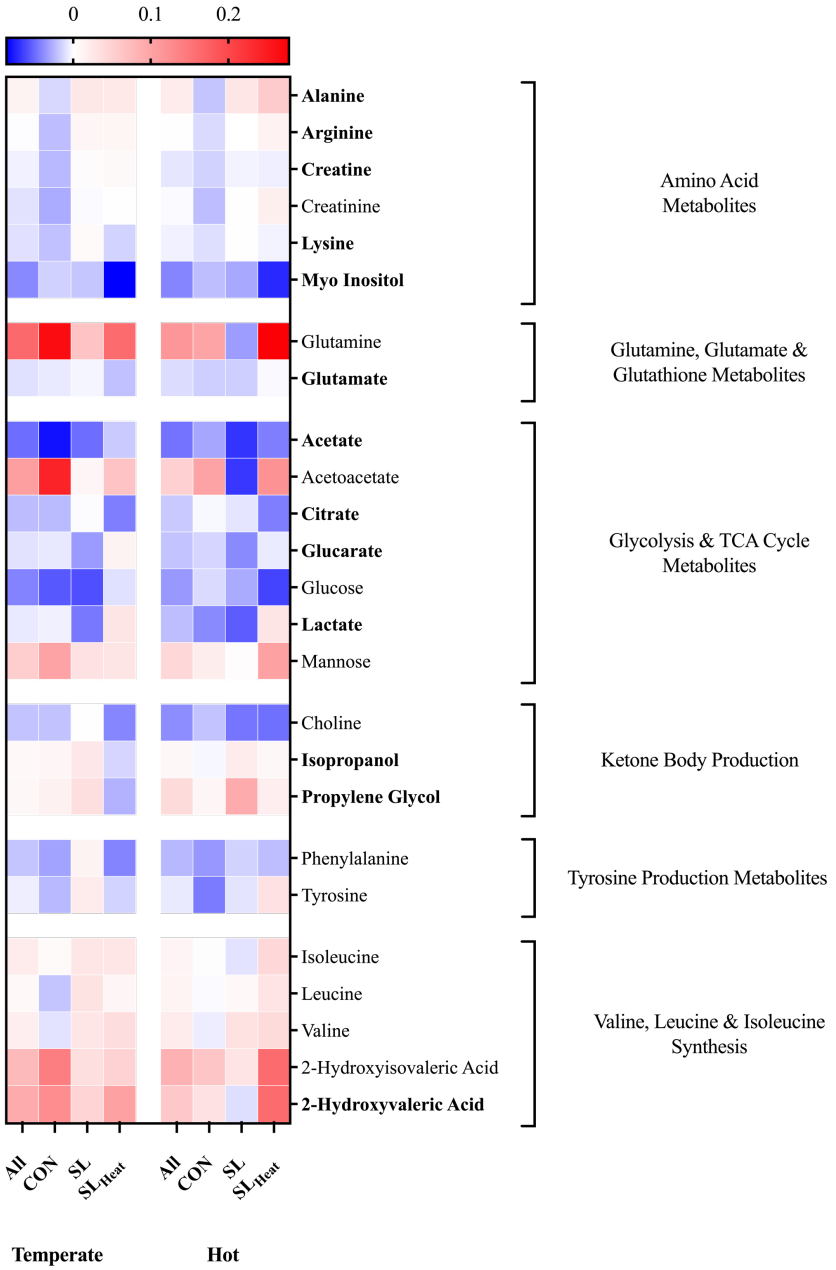

Figure 8.13 – Heatmap representing Log2 fold change in relative metabolite abundance for each experimental group (CON, SL and SL_{Heat}) from pre- to post intervention in both temperate (20°C) and hot (35°C) conditions for each group. Red represents positive whilst blue represents a negative metabolite fold change. "All" refers to wholescale change in relative metabolite abundance from pre to post intervention irrespective of group. Influential metabolites identified by 10 x PLS-DA VIP scoring (> 1) are highlighted in bold.

8.3.4 v) Metabolite Set Enrichment Analysis (MSEA)

Selected metabolites were subject to MSEA to ascertain further metabolic pathway level information (Table 9.2) using a database curated from KEGG pathways (*Homo Sapien* [Human] [KEGG organism code: hsa]) using fisher's exact test with EASE correction and Holm *P*-value adjustment. No metabolic pathways were returned as overrepresented following Holm *P*-value adjustment.

Table 8.7 – Results of the overrepresentation analysis using the hypergeometric test to evaluate whether a particular metabolite set was represented more than expected by change within the metabolites identified from PLS-DA analysis (VIPS > 1). One-tailed *p*-values are provided following multiple testing adjustment within the metabolites identified from PLS-DA analysis (VIPS > 1).

Biological Process	Total	Expected	Hits	Raw *P*-value	Holm adjusted *P*-value	FDR	Impact
Aminoacyl-tRNA biosynthesis	48	0.46452	4	0.0009	0.07	0.07	0
Glycolysis / Gluconeogenesis	26	0.25161	3	0.002	0.14	0.07	0.03
Pyruvate metabolism	22	0.2129	2	0.02	1	0.51	0.06
Alanine, aspartate, and glutamate metabolism	28	0.27097	2	0.03	1	0.51	0
Glyoxylate and dicarboxylate metabolism	32	0.30968	2	0.04	1	0.51	0.03
Phenylalanine, tyrosine, and tryptophan biosynthesis	4	0.03871	1	0.04	1	0.51	0.5
Synthesis and degradation of ketone bodies	5	0.048387	1	0.05	1	0.51	0.6

8.4 Discussion

Investigating the metabolic impact of heat stress during exercise has largely been overlooked for the last 20 years, with seminal works published in the latter years of the previous century. Studies using whole-body indirect calorimetry, stable isotope and biopsy techniques have ubiquitously shown increased carbohydrate utilisation and decreased lipid oxidation during exercise in high ambient temperatures (Febbraio et al., 1994a; 1996c; Hargreaves et al., 1996a; 1996b; Parkin et al., 1999; Starkie et al., 1999; Febbraio, 2001). Untargeted metabolomics analysis provides insight into the metabolic mechanisms underlying the alterations to substrate metabolism during exercise in elevated environmental temperatures (Nicholson, Lindon and Holmes, 1999). Furthermore, metabolomics analysis has proven to be an effective, sensitive and comprehensive approach for detecting changes in whole-body substrate metabolism during acute or chronic exercise and in response to nutritional availability(Sakaguchi et al., 2019; Schranner et al., 2020). For the first time, the impact of maximal acute exercise in temperate (21°C) and hot (35°C) conditions on the human serum metabolome has been investigated using untargeted ^{1}H-NMR spectroscopy. Additionally, the impact of 2 weeks of endurance training with periodised carbohydrate availability in hot or temperate conditions has also been investigated. The endurance training programme was completed in temperature conditions with high carbohydrate intake (CON), in temperate conditions with periodised carbohydrate availability (SL) or in hot conditions with periodised carbohydrate intake (SL_{Heat}).

8.4.1 Characterising the Serum Metabolome in Response to Exercise in Hot and Temperate Conditions

Although the metabolic impact of acute heat stress during exercise has been relatively well defined (Febbraio, 2001), to our knowledge, the present study is the first to characterise its impact on the exercise metabolome. Furthermore, the application of metabolomics in exercise physiology, specifically in trained populations, is in its relative infancy, with all of the current literature being conducted in temperate conditions (Schranner et al., 2020). Here, we provide novel exercise metabolomics data related to environmental heat stress, highlighting that high-intensity endurance exercise elicits comparable disturbances to the serum metabolome in hot and temperate conditions. Despite this, several metabolites were altered in response to exercise and heat stress when considered at the metabolite level, highlighting potential novel metabolic implications for exercise in hot conditions.

Principle component analysis revealed differences in the serum metabolome from pre- to post-exercise with little difference between hot and temperate conditions (Figure 2A), confirmed by univariate analysis, 11 and 14 metabolites were significantly different following exercise in hot and temperate conditions, respectively (Figure 2B). When considering within-subject metabolite changes from pre- to post-exercise, building a PLS-DA model identified 10 metabolites critically important for differentiation between conditions (Figure 4A), with 4 significantly different between conditions following univariate analysis (Figure 5). The present analysis highlights the relatively small contribution of heat stress to alterations in serum metabolome during exercise in hot conditions. Nevertheless, the data reveal increased glycolytic metabolite abundances following exercise in the heat, supporting previously reported increases in glycolysis during heat stress and exercise. Additionally, the data presented

here provide novel insight into the impact of heat stress during exercise on circulating amino acid concentration, highlighting potential practical implications and future research considerations.

Increased relative hyperglycaemia is consistently observed during exercise under heat stress (Febbraio et al., 1994a; Febbraio et al., 1994b; Febbraio et al., 1996c; Fernandez-Elias et al., 2015). Whilst the exact source of circulating glucose is experimentally challenging to identify, increased blood glucose during exercise in hot conditions is potentially due to increased liver glucose output with minimal alterations in the whole-body glucose metabolism (Hargreaves et al., 1996a). Mechanistically, altered glucose kinetics during exercise in the heat is partly due to elevated circulating plasma adrenaline, which is increased during exercise in hot conditions, inducing hepatic glucose production and increased rates of muscle glycogenolysis (Febbraio et al., 1998). Although we did not quantify circulating adrenaline here nor characterise muscle glycogen utilisation, the rationale that increased circulating adrenaline alters glucose metabolism during exercise and is amplified with heat stress is well established (Chesley, Hultman and Spriet, 1995; Febbraio et al., 1998; Howlett, Febbraio and Hargreaves, 1999). Furthermore, during exercise in hot conditions, increases in blood glucose are accompanied by increases in circulating lactate, suggesting greater flux via anaerobic glycolysis (Febbraio et al., 1994a; Febbraio et al., 1994b). The metabolomic dataset provided here highlights several glycolytic metabolites, including acetate, glucarate, glucose and lactate, all are increased in hot relative to temperate conditions, suggesting increased glycolytic flux in response to environmental heat stress. The aetiology of altered substrate metabolism during heat stress is multifactorial and encompasses multiple organ systems. Mechanisms include reduced oxygen provision to skeletal muscle as competition for blood flow occurs following cutaneous vasodilation for thermoregulation (Rowell et al., 1968), greater recruitment and

utilisation of fast-twitch muscle fibres (Young et al., 1985), the direct effect of muscle temperature increasing the rate of enzyme-mediated reactions (Q10 effect) (Young et al., 1985) and increased circulating adrenaline concentration (King et al., 1985; Yaspelkis et al., 1993). Implementing targeted -omics such as ^{13}C-fluxomics to determine metabolite concentrations and absolute flux through large networks would permit metabolic phenotyping of cells or tissues, allowing greater elucidation of specific metabolic pathway's contribution to energy provision during exercise and the origin and fate of specific metabolites of interest.

Lactate, historically considered a metabolic by-product and terminal metabolite during exercise, contributing to fatigue and limiting exercise capacity (Brooks et al., 2022), has undergone a significant paradigm shift. Lactate is increasingly considered a crucial metabolic intermediate functioning as an energy source, major gluconeogenic precursor, and signalling molecule (Brooks, 2020). Whilst lactate in skeletal muscle is oxidised by the mitochondria (Hashimoto, Hussien and Brooks, 2006) or converted into glycogen (Hargreaves and Spriet, 2020), of the lactate that is released into circulation, the metabolic fate includes utilisation as an energy substrate in the heart (Gertz et al., 1988) and brain (Glenn et al., 2015), whilst the remainder of circulating lactate will be used as gluconeogenic substrate in the liver (Bergman et al., 2000). Crucially, circulating lactate is a critical metabolic precursor for liver gluconeogenesis and increased production during exercise in hot conditions may directly or indirectly increase circulating glucose concentration in the blood; the exact mechanisms by which this occurs remain to be resolved.

Alongside increases in glycolytic metabolite abundance, alanine similarly increased during exercise and was further augmented in response to heat stress. Alanine concentrations are elevated following a bout of high-intensity interval training (Peake et al., 2014) and

exhaustive sub-maximal exercise (Zafeiridis et al., 2016), consistent with the nature of our exercise protocol (30-min maximal capacity test with 6 high-intensity sprint intervals). Given the glucogenic role of alanine, we hypothesise that increases in circulating alanine during exercise in hot conditions are representative of the multi-tissue alanine cycle whereby alanine is liberated at the peripheral tissue level (skeletal muscle) and transported via the bloodstream to the liver for gluconeogenesis (Felig and Wahren, 1971; Felig, 1973; Williams, Chinkes and Wolfe, 1998). It has also been proposed that an increase in circulating alanine with a concomitant decrease in glutamate, as observed here, is indicative of a rightward shift in the alanine aminotransferase catalysed reaction, a predominant anaplerotic mechanism during exercise (Sahlin, Katz and Broberg, 1990; Gibala et al., 1997). Therefore, reductions in circulating glutamate during exercise are expected (Felig and Wahren, 1971; Ahlborg et al., 1974), with the established consensus that a significant source of carbon for expansion of Tricarboxylic acid (TCA) cycle intermediate production is glutamate derived; as such, intramuscular glutamate concentration decreases sharply at the onset of exercise (Katz et al., 1986), leading to greater intramuscular glutamate uptake. Additionally, decreases in serum glutamate concentrations may result from increased liver uptake due to the critical role of glutamate in the alanine cycle as it is converted to alanine by alanine aminotransferase, serendipitously corroborating our reported increase in circulating alanine (Wagenmakers, 1998).

Alterations to circulating amino acid concentrations, specifically the branched-chain amino acids (BCAA) isoleucine and leucine, have been previously reported during exercise (Felig and Wahren, 1971; Ahlborg et al., 1974). Leucine and isoleucine are ketogenic amino acids oxidised by skeletal muscle during exercise (McKenzie et al., 2000). Initially, BCAA catabolism requires the TCA cycle intermediate 2-oxoglutarate; as such, TCA cycle

intermediates are reduced during exercise when BCAA metabolism is required (Gibala, Gonzalez-Alonso and Saltin, 2002). It is well established that protein intake following exercise increases skeletal muscle protein synthetic rates (Churchward-Venne et al., 2020), with leucine increasingly considered a vital amino acid for stimulating protein synthesis following endurance exercise. Increased amino acid turnover during exercise and heat stress may harm post-exercise recovery and adaptation; as such, future research should aim to elucidate the impact of heat stress on the circulating amino acids and the potential impact on recovery. As with carbohydrate recommendations for exercise and heat stress, protein requirements may also require revision.

A relatively unknown metabolite in exercise is 2-hydroxyvaleric acid; nevertheless, the increases in abundance of this metabolite may interest exercise physiologists. Elevations in urinary 2-hydroxyvaleric acid have been associated with lactate academia induced by succinate acidaemia (Asano et al., 1988) and Propionyl-CoA carboxylase deficiency (Bergstrom et al., 1981). Most interestingly, increases in lactic acid, 2-hydroxyvaleric acid and succinic acid were associated with impaired protein activities involved in succinate metabolism (succinate dehydrogenase) and mitochondrial respiratory complexes I and III. Increased 2-Hydroxyvalerate may result from increased isoleucine metabolism, converted to propionyl-CoA, a metabolic precursor of succinyl-CoA, a TCA intermediate. Due to increased glycolytic rate and a concomitant decrease in citrate abundance in the heat, excess acetyl-CoA and propionyl-CoA may lead to increased 2-Hydroxyvalerate and attenuation of isoleucine catabolism (Stokke et al., 1973; Goodman et al., 1978; Sweetman et al., 1978). Whilst requiring further investigation in the context of exercise in hot conditions, 2-Hydroxyvalerate may represent an important biomarker for branch chain amino acid metabolism and impaired mitochondrial oxidation during exercise.

Practically, endurance exercise increases skeletal muscle protein synthesis rates (Harber et al., 2010; Mascher et al., 2011), utilising amino acids as 'building blocks' for *de novo* protein synthesis. As such, amino acids must be available for this process to allow optimal recovery and protein synthesis rates. The reduction in circulating amino acids reported in the present study following exercise may lead to sub-optimal recovery as insufficient amino acids are available to support post-exercise skeletal muscle protein synthetic rates. Protein intake immediately following endurance exercise has been shown to increase circulating amino acid concentrations and muscle protein synthesis in a dose-dependent manner (Churchward-Venne et al., 2020).

Notably, the samples collected pre-exercise were not in a fasted state; instead, approximately 2-3 h post-standardised meal, which coincides with peak post-prandial gluconeogenic amino acid appearance (Bos et al., 2003), meaning circulating alanine abundance may be influenced by prior dietary intake and not solely exercise. Nevertheless, the standardisation of pre-exercise food intake means that there is likely an equal impact of diet on each environmental condition. Therefore, the attestation that alanine is increased following exercise in hot conditions remains true. However, identifying the precise origin of circulating alanine remains challenging due to the inherent difficulty in quantifying tissue-specific protein breakdown.

It is widely accepted that a period of heat acclimation reduces the physiological impact of heat stress and thus reduces the metabolic demands of exercise (Febbraio et al., 1994a). Despite this, the time course of metabolic adaptation in response to heat stress is not characterised. The potential for readily accessible capillary blood collection for metabolomics analysis (Catala et al., 2018) in conjunction with indirect measures of whole-body metabolism

represents a promising strategy for the elucidation of heat-induced changes in metabolism during exercise and the impact of heat acclimation on the exercise metabolome. Although a consistent and robust analytical approach [54, 55], NMR metabolomics constitutes an untargeted technique that only reports primary metabolites (Psychogios et al., 2011). Being limited to primary metabolites poses specific limitations for biological interpretation; much of the discussion within this manuscript has focused on carbohydrate and amino acid metabolism, with no discussion of the impact of exercise and heat stress on lipid metabolism. Few studies have investigated the impact of heat stress on lipid metabolism, with plasma free fatty acid (FFA) concentrations consistently reported unchanged during exercise in hot conditions (Fink, Costill and Van Handel, 1975; Nielsen et al., 1990; Yaspelkis et al., 1993). Critically, plasma FFA concentrations reflect the net difference between whole-body lipolysis and tissue uptake during exercise. Likely due to the high variability in analytical techniques, the sole literature reporting intramuscular lipid metabolism by Fink, Costill and Van Handel (1975) reported intramuscular triglyceride utilisation was reduced in hot conditions. The application of NMR spectroscopy for metabolomics utilises a CPMG pulse train, which attenuates signals from large molecules, such as proteins. Given the mechanism of FFA transportation in the blood via albumin or within chylomicrons, much of the serum lipidome data is suppressed and thus preventing investigations of the systemic lipid response following exercise.

A further limitation of this study is the exclusively male population; as such, the generalisability of the results is limited to endurance-trained males and should not be extrapolated to females. Generally, the impact of heat stress on metabolism is widely under-investigated in females, with no literature investigating the impact of heat stress during exercise on female metabolism. Whilst it is beyond the scope of this research, the application of serum metabolomics in the investigation of female exercise metabolism in response to heat stress

could provide further insight into the endocrinological impact of the menstrual phase on metabolic regulation during exercise and thermal stress.

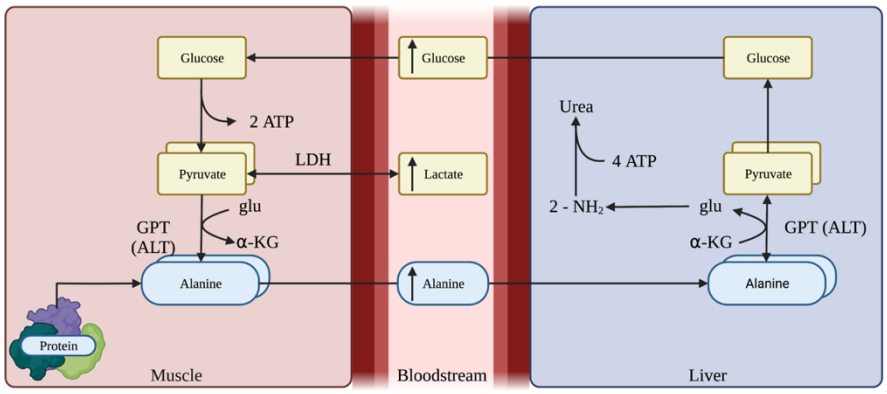

Figure 8.14 – Schematic overview of the Alanine cycle, including key metabolites, changed during exercise in hot environmental conditions. On the left, reactions take place in skeletal muscle. Alanine is liberated from skeletal muscle breakdown or synthesised from other amino acids. It is released into the bloodstream and carried to the liver, which converts it to pyruvate by glutamate-pyruvate aminotransferase (ALT). Pyruvate is then converted into glucose via the gluconeogenic pathway. The alanine-derived amino group is converted to urea via the urea cycle and excreted. Liver-derived glucose can then return to the bloodstream for use within skeletal muscle. ALT, Alanine Transaminase; Glu, Glutamate; GPT, glutamate-pyruvate aminotransferase (or ALT); LDH, Lactate dehydrogenase, α-KG, alpha-ketoglutarate. Created with biorender.com.

8.4.2 Effect of Exercise Training, Heat Acclimation and Carbohydrate on Exercise Serum Metabolome in Hot and Temperate Conditions

Following aerobic exercise, metabolomics analysis has revealed a robust increase in fatty acid metabolism (Sakaguchi et al., 2019; Schranner et al., 2020) and increased reliance on BCAA metabolism for oxidative purposes (Stander et al., 2018; Stander et al., 2020). It has been previously reported that increased rates of muscle glycogen induced by hypoxia similarly alter the amino acid profile, with decreases in leucine, isoleucine and valine with increased downstream BCAA metabolism (Margolis et al., 2021a). Additionally, concomitant increases in tricarboxylic acid (TCA) metabolites further supported the hypothesis that BCAA

metabolism was required to contribute to energy metabolism during periods of low muscle glycogen availability.

Participants repeated maximal exercise tests in hot and temperate conditions to gain insight into the impact of chronic exercise with low carbohydrate availability and the impact of environmental heat stress. No distinct clustering in Δ relative abundance during PCA between groups in either condition was observed. PLS-DA failed to accurately discriminate between groups and environmental conditions, with univariate analysis failing to identify metabolite-level differences between groups or conditions. The null findings presented here may be attributed, in part, to the reduced sample size and significant intra-group metabolite. Initially, the cohort of 23 participants was divided into 3 groups of 8, further reduced by the analytical approach implemented. In addition, the changes in metabolite abundance (Δ Relative Abundance) were calculated as within-subjects difference pre- to post-exercise, meaning only participants with matched pre- and post-exercise samples were included in this analysis, significantly reducing participant number (Table 8.5). Nevertheless, the metabolomic workflow employed throughout this thesis was completed, and despite the null findings from univariate analysis, interesting metabolite level trends can still be observed.

Firstly, during exercise in hot conditions, circulating blood glucose was reduced in the SL_{Heat} group only, suggesting a positive heat acclimatory response following 2 weeks of exercise in the heat (Figure 8.13). To date, the metabolic impact of heat acclimation has yet to be well understood, with Febbraio et al. (1994a) providing evidence of decreased blood glucose following acclimation, whilst King et al. (1985) reported no effect of heat acclimation. Interestingly, it has been previously reported that during exercise with low glycogen availability (Margolis et al., 2021b) or high rates of muscle glycogenolysis, such as those

induced during hypoxia (Margolis et al., 2021a), BCAA metabolism is increased to support energy production. In line with acclimation-induced changes in glycolytic and TCA metabolites, it would be appropriate to speculate that reductions in muscle glycogenolysis and relative physiological strain would lead to reductions in BCAA metabolism, as observed in this study with increased isoleucine, leucine and valine concentrations following exercise in hot conditions in the SL_{Heat} group.

As with blood glucose, conflicting data also exists regarding alterations to circulating lactate after heat acclimation. Febbraio et al. (1994a) previously reported a positive effect of heat acclimation, reducing circulating blood lactate, whilst no difference was observed by King et al. (1985). Notably, in the present study, blood lactate was increased in hot conditions in the SL_{Heat} group following acclimation. However, the self-selecting intensity of the exercise test implemented means lactate is likely increased due to improved exercise capacity and the enhanced thermoregulatory response of heat-acclimated subjects during exercise in the heat. Had the serum metabolome been investigated during fixed-intensity sub-maximal exercise, greater differences may have been observed between groups and in response to acclimation.

It is widely accepted that acute exercise in the heat results in increased glycolytic flux, which is reduced following a period of heat acclimation (Febbraio et al., 1994a). Previous work in this chapter highlighted increased glycolytic metabolite abundance following exercise in hot environmental conditions, which was somewhat attenuated following 2 weeks of carbohydrate periodisation and heat acclimation. However, as with lactate, it is difficult to directly compare the impact of the chronic training and nutrition intervention on the serum metabolome due to the non-standardised exercise workloads observed between groups from pre- to post-intervention.

It should be noted that carbohydrate flux via PDH activity closely corresponds to exercise intensity, with higher exercise intensities seeing greater PDH activation (Howlett et al., 1998b). Pyruvate Dehydrogenase has been the subject of intense research concerning metabolic regulation and adaptation in response to hypoxia (Parolin et al., 2000a; 2000b) and low carbohydrate high-fat diets (Stellingwerff et al., 2003; 2006); however, little is known about the impact of heat stress during exercise on the function of this critically important enzyme. The decreased citrate abundance following 2 weeks of heat acclimation and carbohydrate periodisation heightens the speculation that carbohydrate flux capacity is improved in response to chronic heat stress; however, the direct impact on PDH warrants further investigation.

Unfortunately, metabolomics does not allow characterising specific metabolites' origin or fate. It only provides a temporal change in total abundance throughout exercise, i.e., a metabolomic 'snapshot'. Fluxomics, an innovative, new '-omics' approach that aims to characterise the rate of intracellular metabolite flux using ^{13}C labelled molecules, may represent a novel approach to understanding the movement of specific metabolites in and out of tissues and their ultimate metabolic fates (Emwas et al., 2022). Since the reaction rates of metabolic pathways cannot be directly measured due to the intrinsic properties of metabolism, the indirect measure via the quantification of metabolite level changes in response to stimuli between tissues provides a unique opportunity to understand better the turnover of specific metabolites (Winter and Kromer, 2013). It must be noted that metabolite quantification by NMR, as used in this thesis, may not be appropriate for Fluxomics due to the inherent low sensitivity of the approach. Nevertheless, the contrasting response of circulating alanine following exercise in hot conditions following heat acclimation and carbohydrate periodisation is undoubtedly of interest and requires further investigation.

Choline is an essential micronutrient with limited endogenous synthesis from methionine and must be supplemented with diet (Food and Nutrition Board of the Institute of Medicine, 1998) and is found in various foods (Cho et al., 2006). Playing a critical role in phospholipid synthesis, triglyceride metabolism and cell membrane function (Cai, 2019), choline has been associated with poor performance in deficient individuals (Zhao, Frohman and Blusztajn, 2001; Penry and Manore, 2008). Similarly, choline appears to be an exercise-responsive metabolite, with multiple studies showing decreased circulating "free" choline concentration following strenuous exercise, such as following a marathon (Conlay et al., 1986; Buchman, Jenden and Roch, 1999). It has previously been proposed that reductions in free choline may be due to increased demand for methyl-group donation from choline during physiological stress (Kanter and Williams, 1995), a response thought to inhibit muscle performance due to reduced choline available for acetylcholine synthesis, inhibiting neuromuscular junction function (Conlay, Sabounjian and Wurtman, 1992). Within the present study, serum choline remained unchanged during 30 mins of maximal cycling exercise in both hot and temperate conditions, in line with current literature across multiple endurance exercise modalities reporting significant changes in free choline concentration (von Allworden et al., 1993; Spector et al., 1995; Deuster et al., 2002). This may suggest that exercise duration was insufficient to induce sufficient metabolic stress to require choline for methyl-group donation.

Following 2 weeks of periodised carbohydrate intake and heat acclimation, exercise-induced changes in serum choline concentration were observed pre- to post-exercise during exercise in thermoneutral and hot conditions, with all groups reducing circulating choline abundance. The mechanisms responsible for changes in choline following exercise are currently unknown, with no chronic investigations seeking to understand the effect of regular exercise on long-term changes in circulating choline abundance. Free choline may be decreased

for many reasons, including decreased ingestion of choline-rich food, which is not to be overlooked during the present study as the postprandial time-course of choline appearance in circulation is not well characterised and may be influential. Despite a controlled pre-exercise meal, residual effects of the previous day's dietary intake may be present. Practically, athletes consuming high carbohydrate diets may compensate by increasing choline-rich food intake; consequently, the investigation of combined carbohydrate periodisation and lecithin ingestion, which contains ~35% phosphatidylcholine (choline precursor), may pose a novel strategy to increase choline availability during high carbohydrate diets.

8.4.3 Conclusion

The present chapter has presented novel metabolomic data following acute exercise in hot and temperate conditions and following 2 weeks of carbohydrate periodisation with or without heat acclimation. Data presented here show apparent differences in metabolome pre- to post-exercise, with slight differences in metabolomic signature between environmental conditions. Glycolytic metabolites were elevated in hot conditions, suggestive of increased glycolytic flux, with novel metabolites of interest identified for future research. Despite being hampered by limited sample size, clear trends within metabolites and between groups were observed following 2 weeks of intervention. Together, exercise metabolomics data from both acute and chronic interventions have provided critical early insight into the potential differences in metabolism between environmental conditions and following exercise, nutrition and heat acclimation interventions and have highlighted the need for further research in exercise-heat stress metabolism.

Chapter 9 – Synthesis of Findings

The overarching aim of this chapter is to provide an overview of the experimental findings presented throughout this thesis and how they relate to the achievement of the aims set out in Chapter 1. Following this, a general discussion will pay specific attention to the scientific and practical impact of this work concerning carbohydrate periodisation and heat stress and the subsequent metabolic adaptation and performance outcomes. Finally, limitations associated with the interpretation of the data will be discussed before recommendations for future research are suggested and hypothesis generation.

9.1 Achievement of Aims and Objectives

This thesis aimed to characterise the performance and metabolic effects of training with low carbohydrate availability via the application of the sleep-low, train-low (SL-TL) model and whether the implementation of a simultaneous heat acclimation protocol provided additional benefit to adaptation and performance. Chapters 4 and 5 provide data showing that 3 and 2 weeks of SL-TL improve cycling performance in thermoneutral conditions, respectively, with the latter highlighting changes in sub-maximal substrate metabolism, characterised by increased fat oxidation at matched exercise intensities, following 2 weeks of SL-TL. However, when SL-TL was completed in hot conditions (~35°C), performance and metabolic adaptation were initially impaired, with performance only improving following 1 week of recovery, drawing into question the efficacy of SL-TL for metabolic adaptation in hot environments.

Secondary to characterising the phenotypic adaptations associated with SL-TL, this thesis aimed to elucidate the intra-muscular and serum metabolomic responses to exercise, heat stress and reduced carbohydrate availability, using novel ^{1}H-NMR metabolomics both *in vitro*

and *in vivo.* Following the optimisation of an *in vitro* model of exercise and heat stress in C2C12 myotubes (Chapter 6), the intramuscular metabolomic response to exercise, heat stress and reduced carbohydrate availability was characterised. This data showed significant intra-muscular metabolomic perturbations in response to electrical stimulation; however, these alterations were attenuated under low glucose availability. Furthermore, *In vivo* investigations revealed distinct pre- and post-exercise metabolomic signatures with several metabolites differing between Temperate and Hot conditions. Lastly, following 2 weeks of intervention, no marked difference in serum metabolome was observed between groups, with no metabolite level differences following univariate analysis. This thesis's lack of metabolite-level differences is unsurprising due to the significantly reduced sample size and high interindividual variability. Nevertheless, several metabolites were identified as showing interesting trends and discussed with potential for future research interest.

Objective 1: The implementation of a large-scale, home-based exercise-nutrition intervention to investigate the effect of 3 weeks of SL-TL on performance outcomes in trained individuals compared to exercise completed in a consistently high carbohydrate state (Study 1, Chapter 4).

This study supported the "fuel for the work required" approach (Impey et al., 2018), whereby periodising carbohydrate intake around specific exercise bouts can enhance endurance performance outcomes compared to individuals consuming consistently high carbohydrate intake. For example, Chapter 4 revealed a 5.5% improvement in FTP following 2 weeks of SL-TL compared to only a 1.3% improvement in consistently high carbohydrate intake participants. A caveat of these findings is the apparent intensity-specific performance outcomes observed dependent on the mode of carbohydrate intake. For instance, the SL group improved

peak power output (PPO) for 20- and 5-mins, whilst the control group (consistently high carbohydrate intake) improved in 5- and 1-min power tests. This provides essential empirical data to inform the application of SL-TL in practice with the proviso that SL-TL may impair short-duration PPO and should be avoided during pre-competition preparation phases in specific athletic populations. For example, road and track sprinters reliant on high peak power output in cycling may be acutely impaired by SL-TL. In contrast, cycling climbers, rouleurs and general classification contenders may benefit from higher 20-min PPO outputs and FTP.

Objective 2: To examine the effects of periodising carbohydrate intake (SL-TL) during 2 weeks of heat acclimation on exercise adaptation and performance in hot and temperate conditions compared to a high carbohydrate control group and a SL-TL group in temperate conditions (Study 2, Chapter 5).

Having previously reported performance improvements following 3 weeks of SL-TL carbohydrate periodisation, chapter 5 aimed to investigate whether training with periodised carbohydrates in conjunction with a heat acclimation protocol (daily exposures to heat stress [~35°C, 50% RH] during exercise) conferred a greater metabolic and performance benefit compared to SL-TL in a temperate environment and a control group (exercise with consistent high carbohydrate intake) in unacclimated athletes. To do this, an altered SL-TL strategy was implemented to allow the amalgamation of an active heat acclimation protocol and SL-TL. The altered regimen consisted of daily low-intensity training (LIT) sessions interspersed with high-intensity training sessions on alternate evenings intended to decrease muscle glycogen availability. All sessions were either completed in thermoneutral (~20°C, 50% RH) or hot (~35°C, 50% RH) conditions, dependent on the experimental group. In support of data presented in chapter 4, SL-TL improved performance capacity in temperate conditions and

altered whole-body substrate metabolism during sub-maximal endurance exercise, increasing the relative contribution of fat to exercise energy expenditure compared to high carbohydrate control participants.

Conversely, when SL-TL was conducted in a hot environment (~35°C, 50% RH), performance and metabolic benefits associated with SL-TL were initially impaired, with performance benefits only recorded following 1 week of recovery. Therefore, to assess the efficacy of SL-TL and heat acclimation in different environmental conditions, all participants completed pre-, post- and follow-up performance tests (after 1 week) to assess whether the beneficial effects of each strategy transferred between environmental conditions. Interestingly, in contrast to previous heat acclimation literature, no performance improvement in hot conditions was observed despite 2 weeks of exercise training in high ambient temperatures. Additionally, following 1 week of recovery, performance benefits were present in the heat-acclimated participants, suggesting acute fatigue may have played a critical role in the impairment of exercise capacity immediately following training intervention.

Objective 3: To understand the metabolomic impact of exercise completed under conditions of heat stress, *in vivo* and *in vitro,* using a combination of electrically stimulated C2C12 myotubes and human serum (Chapters 7 and 8).

To understand the metabolic impact of exercise training under high ambient temperatures and the effect of periodised carbohydrate intake, a translational *in vitro – in vivo* study was conducted. Firstly, to replicate exercise-heat stress stimuli *in vitro*, electrical pulse stimulation (EPS) of C2C12 myotubes was completed in standard cell culture conditions (37°C) (Stim Only), heated to 40°C with EPS (Heat & Stim) or heated to 40°C (Heat Only).

Cells exposed to Heat Only exhibited no increase in metabolic activity compared to metabolic perturbations in response to Stim Only and Heat & Stim. Data from this experiment highlights the impact of "exercise-like" stimulation and the limited impact of additional heat stress. In addition, metabolome-wide data identified metabolites and metabolic pathways of interest during heat stress and exercise, highlighting the potential importance of circulating adrenaline in regulating intra-muscular metabolic responses.

Having identified metabolites and metabolic pathways of interest *in vitro*, the effect of acute exercise and heat stress on the human serum metabolome was investigated. Chapter 8 demonstrated increased glycolytic carbohydrate metabolism during exercise in hot conditions, in line with previous studies investigating exercise metabolism in heat. Lastly, the effect of exercise in hot and temperate conditions on the serum metabolome was investigated following 2 weeks of exercise with high carbohydrate intake, periodised carbohydrate intake and periodised carbohydrate intake in hot conditions. Several metabolites of interest were identified as necessary in explaining the differences between exercise in each environmental condition following the nutritional or environmental intervention. Human metabolomics data presented in this chapter highlights the importance of the broader metabolome in exercise metabolism and raises the need for a greater understanding of heat stress on exercise metabolism.

9.2 General Discussion and Practical Implications

Based on current molecular evidence, athletes, coaches, and physiologists may speculate that combining physiological stressors during exercise would maximise post-exercise cell signalling responses and increase exercise training adaption and, thus, performance. The central dogma of endurance training adaptation is founded on the principle of the General

Adaptation Syndrome (GAS), that periodic exposures to an environmental stressor (exercise) induce a positive adaptive response (Selye, 1951). However, when completed in excessively high volume and with inadequate recovery, the same environmental stressor induces a state of fatigue proving detrimental to training quality and, ultimately, adaptation. The induction of positive training adaptation improves intramuscular metabolic capacity, requiring less of the muscles' maximal respiratory capacity to sustain a given absolute workload. Thus, lesser cellular homeostatic disturbance and a blunted post-exercise molecular adaptive response occur (Wojtaszewski et al., 2000; Nielsen et al., 2003). Consequently, multiple strategies (reduced carbohydrate availability, hot/cold stress, and hypoxia) have been proposed to maximise cellular homeostatic disturbance, adaptive response, and performance capacity (Hawley et al., 2018). However, the efficacy of these proposed strategies is seldom researched; whether strategies in combination further augment adaptation and performance outcomes has not been investigated.

Does SL-TL provide a performance benefit when compared to high carbohydrate intake?

The SL-TL carbohydrate periodisation strategy has previously been shown to benefit exercise performance in endurance-trained individuals (Marquet et al., 2016a; 2016b). In contrast, several studies have not shown any additional benefit to periodising carbohydrate intake during specific training bouts in highly trained endurance athletes (Burke et al., 2017; 2020; Gejl et al., 2017; Riis et al., 2019). While these studies represent highly controlled laboratory investigations, more ecological validity is needed to generalise results to real-world applications. Similarly, the greatest performance improvements have been observed during exercise trials that more closely reflect field-based assessments. For example, data from chapter

4 shows greater performance responses than typically recorded in the literature due to participants' high familiarity with exercise tests, supported by the reliability data presented in the same chapter. Additionally, combined with data from chapter 5, showing 2 weeks of SL-TL improved performance, albeit following a slightly altered training and nutrition programme, provide a compelling case supporting SL-TL as a strategy to enhance endurance performance.

These data reinforce using the SL-TL exercise-nutrition strategy to improve endurance training adaptation and performance. However, whilst participants in the present study were trained/highly trained endurance athletes, glycogen depletion for elite athletes will take considerably longer due to increased exercise capacity, glycogen storage, and reduced utilisation rate. SL-TL, therefore, requires consideration of the impact on surrounding training sessions as simply completing the SL-TL approach used in this thesis would a) represent a suboptimal training load and b) fail to deplete muscle glycogen during HIT sessions in elite athletes sufficiently. Practically, the integration of low-carbohydrate has been reported to be easiest and tolerated by athletes best when sessions are completed in the morning (Stellingwerf, 2012). This evidence supports the implementation of SL-TL over other carbohydrate periodisation approaches.

The more the merrier? Does combining SL-TL and heat acclimation improve endurance training outcomes?

Endurance training aims to induce significant physiological and metabolic adaptation and is permitted through activating specific cellular signalling pathways in response to homeostatic disturbance. It is well established that manipulating muscle glycogen availability impairs exercise capacity, and the impact of intensity and duration should be ignored (Egan et

al., 2010). Within chapters 4 and 5, exercise commenced with reduced carbohydrate availability, resulting in initial impairments to exercise training intensity. Despite reductions in exercise intensity during LIT sessions, consuming a high carbohydrate diet ($6g \cdot kg^{-1} \cdot day^{-1}$) restored exercise capacity during HIT sessions in both studies, ultimately leading to improved performance. However, the completion of SL-TL in hot conditions significantly impaired exercise intensity during both LIT and HIT sessions compared to high carbohydrate control participants and SL-TL in thermoneutral conditions.

Consequently, there was no immediate improvement in performance or metabolic adaptation following two weeks of carbohydrate periodisation in hot environmental conditions, albeit performance was improved following 1 week of recovery. Collectively, the data here support the notion that whilst one strategy may improve performance, the addition of others may impair exercise training quality and subsequent adaption, leading to suboptimal exercise outcomes (Hawley et al., 2018). Additionally, whilst increasing the number of stimuli may be detrimental, special attention is needed regarding athlete recovery and training load, as this is likely a crucial factor in optimising performance outcomes when implementing multiple strategies.

When considering the impact of combined heat acclimation and periodised carbohydrate, this thesis highlights a counterproductive strategy with the benefits associated with SL-TL impaired and no immediate improvement in performance in hot or temperate conditions. This does not say that periodising carbohydrates during heat stress would not work for elite athletes with typically higher training loads and greater exercise tolerance. However, caution is advised before implementing such an approach, and serious consideration should be given when in an athlete's training cycle; this approach would be most effective.

Coming in Hot! Exercise metabolomic investigations reveal increased metabolic rate in response to exercise and heat stress.

In a series of translational studies using *in vitro* and *in vivo* approaches, Chapters 7 and 8 primarily aimed to characterise the metabolomic perturbations induced by exercise in hot environments. Secondly, chapter 8 aimed to characterise the serum exercise metabolome in response to exercise in hot and temperate conditions following 2 weeks of heat acclimation and carbohydrate periodisation. To date, combining electrical pulse stimulation (EPS) and heat stress *in vitro* represents a novel investigative strategy to assess the impact of 'exercise' on the intracellular metabolome. A noteworthy study by Lautaoja et al. (2021) characterised the impact of 24 hours of EPS on C2C12 myotubes with high and low carbohydrate availability using a 1H-NMR metabolomics approach. The authors provided evidence that the metabolic perturbations observed during EPS in high-glucose media were blunted when EPS was conducted in low-glucose media. Specifically, glycolytic and TCA cycle metabolite abundance in low glucose media increased, indicative of increased energy production. Conversely, data presented in chapter 7 failed to demonstrate marked increases in glycolytic metabolites.

Additionally, TCA cycle metabolites (Citrate, succinate, fumarate, malate) decreased after 3 hours of stimulation, indicating that substrate deletion may impair cellular metabolism. Conversely, under conditions of high glucose availability, glucose and glucose 1-phosphate were increased, likely due to increased intra-muscular glucose uptake (Hu et al., 2018; Li et al., 2018) and glycogenolysis. Unlike Lautaoja et al. (2021), increased glycolytic metabolite abundance did not translate to increased TCA cycle metabolites. When considering the addition of heat stress to EPS, no additional metabolomic perturbations were observed compared to control and heat stress only.

The impact of exercise and heat stress on the human serum metabolome has not been experimentally investigated; acute exercise significantly altered the serum metabolome profile, with greater increases in glycolytic metabolites (acetate, glucose, glucarate, lactate) in hot conditions. These findings contradict data from *in vitro* experiments presented in this thesis, showing no greater metabolic response when combined with EPS and heat stress. Notably, the physiological simplicity of C2C12 cells means that systemic factors, including increased plasma adrenaline observed during exercise in the heat, cannot act upon the cultured muscle. The data presented here between *in vitro* and *in vivo* studies may highlight the role of hormonal regulation of energy metabolism during exercise and heat stress. Furthermore, the present *in vitro* model, combined with adrenaline treatment, would shed light on the role of circulating adrenaline in exercising muscles.

Following 2 weeks of intervention (exercise-only controls, SL-TL group, and SL-TL in hot environmental conditions [SL_{Heat}]), no marked differences were observed in the serum metabolome following exercise in hot and temperate conditions with no metabolite level differences during univariate analysis. Due to the study design implemented here, a limited sample size combined with sizeable interindividual variability hampered the statistical power of the ANOVA implemented for metabolite-level comparisons. Nevertheless, hyperglycaemia and reductions in other glycolytic metabolites were observed in both SL and SL_{Heat} groups during exercise in hot conditions. Interestingly, in the temperate condition, choline was increased in SL and SL_{Heat} following exercise, potentially indicating increased triglyceride metabolism following 2 weeks of carbohydrate periodisation, in line with whole-body changes in substrate metabolism observed in Chapter 5.

9.3 Limitations

Remote Exercise Prescription & Research Design – The entirety of Study 1 was completed remotely, with participants required to complete all dietary and exercise interventions at home without direct supervision from the research team. Whilst this approach facilitated larger sample sizes than traditional laboratory-based studies, completing home-based research is not without significant drawbacks. Firstly, participants are responsible for conducting their test batteries and collecting data. In addition, whilst participants were given detailed written and verbal instructions, including calibration protocols, pre-exercise nutrition and hydration guidelines and controls for the time of day and prior exercise, the true extent of between trial standardisation was impossible to assess. Nevertheless, all participants were given identical instructions, and there is no reason to conclude that one group was more affected by any of the above variables so that the results can be interpreted the same, nonetheless.

Research requiring participants to self-report data to researchers is inherently fraught with bias as participants attempt to provide desirable responses (Sallis and Saelens, 2000). It is noteworthy that all exercise training data, whilst self-reported *per se*, was uploaded directly to an online training platform from the subject's power meters and heart rate monitors. Data could not be tampered with or subject to participant interference. Truly self-reported data included subjective data (session RPE), body mass and dietary intake and were at higher risk of participant bias when reporting data. Fortunately, the primary outcome measures from this chapter which included power output and heart rate, were not dependent on participant feedback. Notwithstanding, it remains essential that detailed instructions and information are

provided to participants to ensure accurate data collection and reporting whilst maintaining avenues for contact should any participants need guidance during any home-based testing.

Standardisation of Exercise Intensity – Within Chapters 4 and 5, the standardisation of exercise intensity within participants and between groups was essential. Due to the home-based nature of chapter 4, functional threshold power (FTP) was used to standardise exercise intensity due to the ease of calculation based upon a single 20-min maximal exercise bout. FTP is the theoretical maximal power an individual can sustain for 60 mins of exercise (Allen, 2019) and is theoretically a field-based equivalent of the maximal lactate steady state (Allen, 2019). Whilst highly popular amongst coaches and athletes, with highly repeatable results (Mackey and Horner, 2021), the relationship between FTP and other physiological performance outcomes is contentious. It has been shown that FTP consistently over/under predict maximal 60- min power output (Mackey and Horner, 2021). Using critical power (CP), the hyperbolic power-asymptote of time-to-exhaustion may provide better standardisation of exercise intensity (Vanhatalo, Jones and Burnley, 2011) and provide greater similarity between field and laboratory measures.

In chapter 5, the varied environmental conditions increased the complexity of standardising physiological and absolute exercise intensity. It is well established that heart rate increases at a given exercise intensity in the heat (Febbraio et al., 1994a). It reduces the workload for a given physiological threshold (Lorenzo et al., 2010). Logistically, the use of a constant work rate regimen (daily exposures at 60% MAP) may have been sub-optimal compared to progressive overload approaches such as controlled hyperthermia (to a core temperate of 38.5ºC) (Taylor, 2000; 2014). Utilising the latter would allow continual adaptation due to the forcing function (i.e., metabolic heat production) being continually increased

through the manipulation of core temperature (as work capacity increases over time) (Taylor, 2000). The use of a controlled heart rate approach to prescribing exercise intensity may have been preferred, as an initial decrease in power output would be diminished as heat acclimation occurs (Febbraio et al., 1994a) whilst maintaining comparable physiological loads between hot and temperate conditions (Maunder et al., 2021b). Furthermore, the constant work rate approach (fixed power output) used in Chapter 5 likely induced a state of physiological habituation as tolerance to heat stimulus increased over time (Taylor, 2014), the influence of which becomes progressively reduced as adaptations develop (Eichna et al., 1950; Fox et al., 1963; Rowell et al., 1967).

Validation of Heat Acclimation Model – Chapter 5 highlighted no immediate performance benefit following 2 weeks of heat acclimation with carbohydrate periodisation. Heat acclimation literature ubiquitously reports improved exercise capacity in hot conditions following repeated heat exposures, drawing into question the validity of the present heat acclimation protocol. Given the consensus that heat stress induced positive performance outcomes in hot conditions, a null finding following this study is cause for concern. Unfortunately, the omission of a heat acclimation-only group, whereby participants completed the same exercise and environmental intervention with consistently high carbohydrate availability, prevents the categorical conclusion that training with low carbohydrate availability impaired improvements following heat acclimation.

^{1}H-NMR Metabolomics - To gain a comprehensive insight into the metabolic effect of exercise, heat stress and carbohydrate availability, chapters 7 and 8 implemented an untargeted ^{1}H-NMR metabolomics approach. Unlike other -omics approaches, metabolomics is in its relative infancy; as such, difficulties identifying metabolites in complex biofluids such

as serum still exist (Wishart, 2011). Additionally, NMR metabolomics, although a consistent and robust analytical approach (Dona et al., 2014; Jimenez et al., 2018), constitutes an untargeted technique that only reports primary metabolites (Psychogios et al., 2011). Being limited to primary metabolites poses specific limitations for biological interpretation, with KEGG pathways being non-exhaustive metabolite maps with significant overrepresentation of disease state metabolism, potentially skewing pathway analysis in the context of exercise metabolomics (Viant et al., 2017). Collectively, these limitations highlight the necessity of completing further exercise-metabolomics studies to improve the biological context of exercise and nutrition on tissue metabolomes.

9.4 Recommendations for Future Research

Recommendation 1 – Based on data presented in Chapters 4 and 5, traditional SL-TL presents a practical strategy to increase performance in trained individuals. However, much of the literature showing improved performance following chronic application of SL-TL lacks intra-muscular data to provide skeletal muscle level information regarding changes in post-exercise transcriptional responses, protein expression and maximal activity. A wealth of data highlights increased transcriptional responses following acute low-carbohydrate exercise and evidence to suggest improved performance following chronic carbohydrate periodisation. However, the impact of such a strategy on protein-level remodelling and evidence of mitochondrial adaptation is lacking (Figure 9.1). Changes in skeletal muscle protein turnover and subsequent abundance underpin tissue-level phenotypic changes in response to exercise and nutrition. The application of exercise-proteomics to characterise the intra-muscular response to chronic training with low carbohydrate availability would permit a greater understanding of the molecular events preceding altered metabolism and performance.

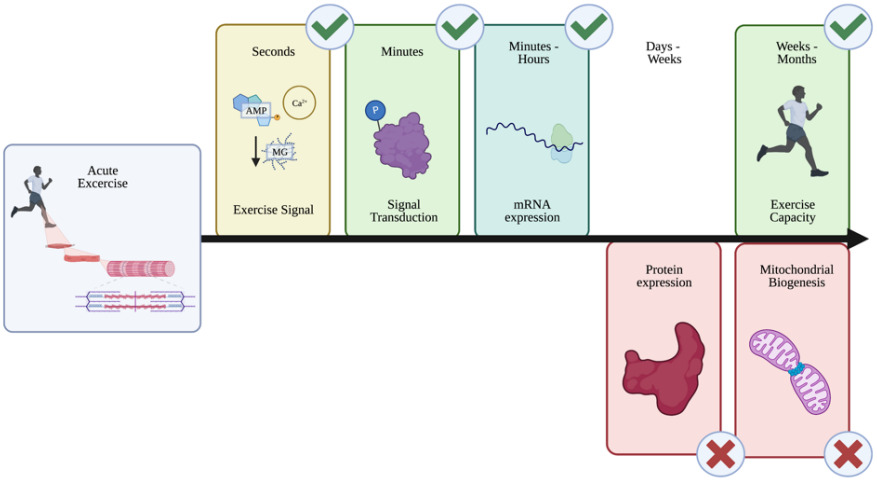

Figure 9.1 – Gaps in the molecular time-course of endurance training adaptation in response to training with low carbohydrate availability. Created with biorender.com

Recommendation 2 – The translational applicability of C2C12 exercise-like stimulation *in vivo* is inherently tricky; however, comparisons between human primary, C2C12 and rat L6 *in vitro* skeletal muscle models have shown broadly similar myosin content and glycogen storage characteristics between C2C12 and human muscle (Abdelmoez et al., 2020). Therefore, using C2C12 as an *in vivo* exercise metabolism model within the present thesis is appropriate. The present work investigates the role of acute heat stress during exercise on the human serum metabolome and C2C12 intramuscular metabolome. Whilst some parallels exist between both experiments (increased phenylalanine metabolism and hyperglycaemia), each experiment represents a different tissue, and the use of either skeletal muscle biopsies or primary human myoblasts would provide greater physiological comparisons. Similarly, cell culture models represent glucose-dependent populations with reduced dependence on fatty acid oxidation during energy metabolism. Therefore, enhancing the translational impact of *in vitro* exercise-heat stress models would require using the same species tissue in culture (*i.e.,* isolated human myoblasts), combined with *in vivo* exercise-heat stress human investigations, including skeletal muscle biopsies pre- and post-exercise allowing more appropriate comparison between models.

Recommendation 3 – Exercise metabolomics is undoubtedly a field in its infancy, and as such, every effort to integrate this new '-omics' approach into the current exercise physiology landscape should be made. As such, a reductionist approach may be necessary. Exercise metabolomics studies represent novel experiments, and datasets should aim to revisit underlying physiological principles and investigate whether changes in the exercise metabolome match expected changes in exercise whole-body or tissue-specific metabolism. For example, Margolis et al. (2021b) recently investigated the role of muscle glycogen availability on serum metabolites, indicating increased branched-chain amino acid metabolites, validating the previous work recommending leucine-enriched whey protein intake to mitigate detrimental effects of low muscle glycogen on skeletal muscle during exercise (Impey et al., 2016). Furthermore, the work presented in chapter 8 progresses previously dormant exercise-heat stress metabolism literature. Supporting previous findings, this chapter has identified several metabolites of interest during exercise in hot environments. The benefits of an untargeted metabolomics approach are two-fold: validating metabolomic datasets against previous literature improves data credibility and places the exercise-induced changes in metabolome with fundamental exercise physiology literature, serving as a foundation for future, more complex metabolomic investigations. This thesis evidences this recommendation with several effective metabolomics investigations, which were well-designed, executed, and appropriate for a metabolomics approach. A less successful metabolomics experiment whereby sample size and study design likely impaired the outcome of the data. Characterising the serum metabolome in response to exercise (Chapter 8, Hot vs Temperate exercise) was a success and aligned with the current evidence base.

Conversely, an example of the limitations of human metabolomics is presented in the same chapter. The comparison of serum metabolome across 3 groups following regular

exercise and dietary intervention was not appropriately designed (or statistically powered) to characterise each strategy's effect on serum metabolome. Future studies should characterise the serum metabolome in response to acute exercise prior to the chronic changes in metabolome in response to the exercise intervention.

Sample size and study design are crucial. Intra-individual variability is often greater and inter-individual variability is often apparent in repeated-measures experimental designs. As such, ensuring a sufficient sample size and robust statistical analysis will help elucidate the experimental story. In addition to sample size, ensuring appropriate standardisation and pre-sampling control measures are essential due to the susceptibility of the metabolome to exercise and diet. When considering metabolomics as an analytical technique, the impact of study design must be considered.

Recommendation 4 – Throughout Chapter 2, special attention was given to the impact of heat stress on metabolism, with the scholarly consensus suggesting increased carbohydrate utilisation at the expense of fat oxidation (Febbraio, 2001). Similarly, it is accepted that a period of heat acclimation reduces the physiological impact of heat stress and thus reduces carbohydrate utilisation (Febbraio et al., 1994a). However, whilst the time course of induction of many physiological adaptations to heat acclimation are well characterised (displayed in figure 2.6) (Periard, Racinais and Sawka, 2015), the time course of metabolic adaptation in response to heat stress remains unknown. As such, no evidence exists to inform nutritional recommendations in response to heat acclimation-induced alterations in substrate metabolism during exercise in hot conditions. The question remains, should high carbohydrate intake be maintained indefinitely during exercise in the heat, or is there a time at which carbohydrate

requirements are reduced, as such, the manipulation of substrate availability could be introduced as per the "fuel for the work required" paradigm?

Recommendation 5 – Nutritional recommendations for endurance exercise in the heat have not been revisited for over 20 years (Burke, 2001), with guidelines focused primarily on carbohydrate fuelling and hydration strategies. More recently, Periard, Eijsvogels and Daanen (2021) provided updated recommendations for mitigating the effects of environmental conditions, but nutritional recommendations, specifically carbohydrate provision and supplementation, should have been considered. Additionally, metabolomics data presented throughout Chapters 7 & 8 have highlighted potentially increased protein metabolism under exercise in high ambient temperatures; as such, future research should better understand the protein requirements of exercise in hot environmental conditions and form the basis for updated nutritional guidelines for exercise under heat stress. Not least, the realisation of recommendations 4 and 5 have the potential to reshape nutritional recommendations for exercise and heat stress.

9.5 Closing Thoughts

To summarise, the data presented in this thesis demonstrate how the strategic manipulation of carbohydrate availability around specific training sessions can improve exercise capacity and induce metabolic adaptation. However, such adaptations are negated when completed in elevated ambient temperatures. These data suggest that training with reduced carbohydrates during exercise in temperate conditions improves performance across 2 and 3 weeks of exercise-nutrition intervention; however, these benefits are blunted and delayed by simultaneous exercise in high ambient temperatures. As such, it remains advisable to follow traditional nutritional recommendations and maintain high carbohydrate intake on arrival in hot environments or at the onset of heat acclimation protocols (Burke, 2001). Unlike several other adaptations induced by heat acclimation (Periard, Racinais and Sawka, 2015), the time course of changes in substrate utilisation is not characterised (Febbraio et al., 1994a). It remains to be investigated whether or when carbohydrate periodisation strategies could or should be introduced following an initial period of adaptation to heat stress. The increase in major sporting events in hot conditions should make environmental exercise metabolism a hot topic.

Chapter 10 – References

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Chapter 11 – Appendices

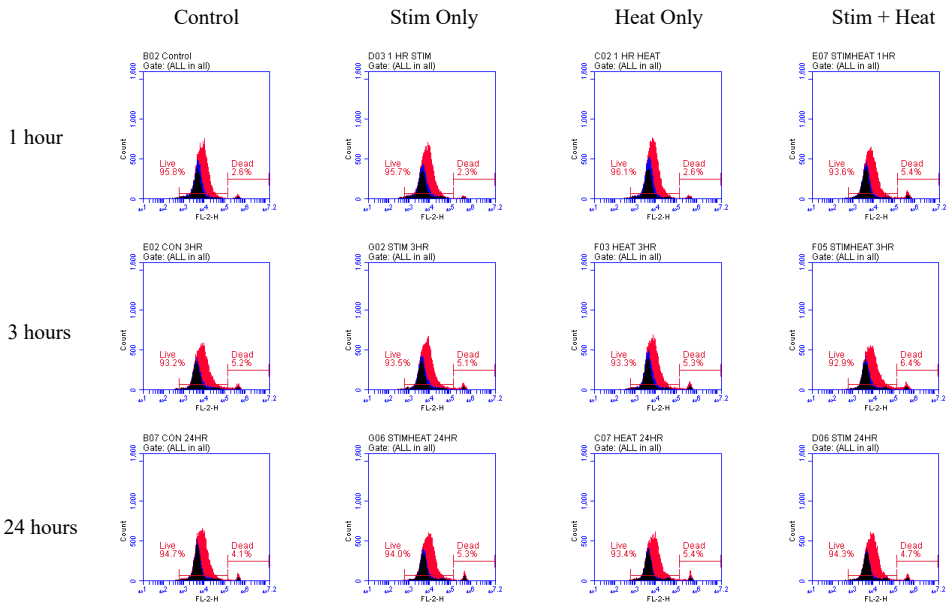

Figure 11.1 – Cell viability quantified by propidium iodide assay via flow cytometry. Flow cytometry outputs following 1, 3 and 24 hours of treatment of C2C12 with either electrical pulse stimulation (EPS) (Stim Only), Heat stress (40°C) or combined EPS and heat stress (Stim + Heat). Fluorescence was gated to quantify “Live” and “Dead” cell populations.

Table 11.1– Overview of mean relative metabolite abundance in C2C12 myotubes in high glucose media with ANOVA outputs (Chapter 7).

	Mean Relative Metabolite Abundance				ANOVA			Pairwise Comparisons					
Metabolite	**Control**	**Stim Only**	**Heat Only**	**Heat & Stim**	**F-stat**	***P*-Value**	**BH Adjusted *P*-Value**	**Stim Only-Control**	**Heat Only-Control**	**Heat & Stim-Control**	**Stim Only-Heat Only**	**Stim Only-Heat & Stim**	**Heat Only-Heat & Stim**
Aspartate	19806 ± 1351	14539 ± 6630	24559 ± 1988	23547 ± 3514	33.74	$3.7x10^{-12}$	$2.11x10^{-10}$	0.00346	$7.58x10^{-5}$	0.0001	$1.25x10^{-10}$	$1.57x10^{-10}$	0.9968
Pyruvate	46270 ± 2729	34407 ± 14072	48074 ± 2716	45647 ± 2829	26.52	$1.78x10^{-10}$	$5.08x10^{-9}$	$4.69x10^{-8}$	0.6752	0.9448	$5.00x10^{-10}$	$1.46x10^{-7}$	0.3175
Glucose	94073 ± 25286	125428 ± 58408	94682 ± 27864	91500 ± 29948	18.12	$3.91x10^{-8}$	$7.42x10^{-7}$	$2.65x10^{-6}$	0.9999	0.9532	$2.26x10^{-6}$	$1.94x10^{-7}$	0.9324
Myo Inositol	253315 ± 19435	289708 ± 118165	255870 ± 24729	282749 ± 56881	16.48	$1.29x10^{-7}$	$1.48x10^{-6}$	$5.25x10^{-7}$	0.9764	0.2775	$1.52x10^{-6}$	0.0001	0.4886
Trimethylamine	40892 ± 2022	43069 ± 17561	41860 ± 2856	42677 ± 3141	16.77	$1.04x10^{-7}$	$1.48x10^{-6}$	$4.42x10^{-7}$	0.9068	0.6623	$3.29x10^{-6}$	$1.22x10^{-5}$	0.9648
Benzoate	102716 ± 3686	81393 ± 34274	102329 ± 6203	103107 ± 4586	15.40	$2.93x10^{-7}$	$2.78x10^{-6}$	$3.32x10^{-5}$	0.9995	0.0002	$3.52x10^{-5}$	0.8986	0.0002
Taurine	287462 ± 20275	310057 ± 145711	270829 ± 24033	261166 ± 22160	14.85	$4.48x10^{-7}$	$3.65x10^{-6}$	0.0006	0.8076	0.2356	$2.12x10^{-5}$	$4.38x10^{-7}$	0.7367
Serine	100806 ± 3373	78427 ± 32606	102616 ± 5031	102345 ± 7816	10.88	$1.23x10^{-5}$	$8.79x10^{-5}$	0.0011	0.9132	0.7757	0.0001	$2.87x10^{-5}$	0.9911
Pantothenate	128874 ± 3818	7540 ± 1975	124041 ± 8597	114185 ± 5138	10.25	$2.17x10^{-5}$	0.0001	0.0002	0.4855	0.0003	0.0103	0.9783	0.0225
Threonine	523542 ± 29377	413471 ± 169274	527200 ± 27611	536209 ± 41109	9.55	$4.12x10^{-5}$	0.0002	0.0046	0.9951	0.4596	0.0018	$2.51x10^{-5}$	0.5942
Phenylalanine	17117 ± 1309	16940 ± 7001	16965 ± 1339	17278 ± 1144	9.42	$4.65x10^{-5}$	0.0002	0.0003	1.0000	0.9738	0.0002	0.0007	0.9732
Dihydrothymine	33115 ± 1312	27315 ± 11512	35330 ± 1663	34511 ± 3155	9.21	$5.70x10^{-5}$	0.0003	0.0323	0.3761	0.3210	0.0002	0.0001	0.9998

Valine	67531 ± 4020	63799 .± 25561	67424 ± 4524	67832 ± 3996	6.86	0.0006	0.0025	0.0029	1.0000	0.9994	0.0021	0.0028	0.9987
Glycolate	549240 ± 65549	455796 ± 187910	531815 ± 50321	439589 ± 88773	6.22	0.0011	0.0045	0.8393	0.9228	0.0016	0.9962	0.0197	0.0090
Tyrosine	26537 ± 1466	24944 ± 9999	26616 ± 1481	26785 ± 1181	5.45	0.0025	0.0095	0.0046	0.9908	0.9253	0.0090	0.0185	0.9881
Glucose-1-Phosphate	28069 ± 4847	30612 ± 15253	25177 ± 6462	27476 ± 11218	5.25	0.0031	0.0111	0.0398	0.8666	0.9736	0.0042	0.0100	0.9832
Fructose	59792 ± 5397	38553 ± 24362	54662 ± 4863	50731 ± 3901	5.12	0.0036	0.0120	0.0022	0.3873	0.0608	0.1210	0.5348	0.7701
2-Hydroxyvalerate	22442 ± 1807	18138 ± 7192	22458 ± 1962	22479 ± 1795	4.95	0.0043	0.0136	0.0089	0.9851	0.9929	0.0198	0.0138	0.9998
Acetone	12966 ± 893	10512 ± 4253	13100 ± 1135	13373 ± 1370	4.38	0.0081	0.0229	0.0597	1.0000	0.8658	0.0585	0.0061	0.8402
O-Phosphocholine	88755 ± 7910	67775 ± 28070	82887 ± 8373	82467 ± 8577	4.40	0.0079	0.0229	0.0036	0.2242	0.2161	0.3061	0.2869	1.0000
Beta Alanine	2101 ± 493	6277 ± 5229	2050 ± 384	5769 ± 1848	4.01	0.0122	0.0331	0.0253	0.9829	0.9999	0.0549	0.0226	0.9885
Creatine	174716 ± 12079	133588 ± 57047	167588 ± 12629	166329 ± 15222	3.93	0.0134	0.0346	0.0072	0.5653	0.5078	0.1516	0.1627	0.9999
2-Hydroxyisovalerate	110357 ± 7565	102681 ± 41462	110069 ± 6855	112333 ± 5782	3.56	0.02	0.05						
Leucine	167738 ± 9680	154709 ± 62166	167495 ± 10101	170427 ± 7513	3.38	0.03	0.06						
Mannose	198005 ± 8500	171456 ± 70379	212485 ± 5746	202508 ± 18331	3.28	0.03	0.06						
Phenylacetate	15108 ± 1120	15159 ± 6899	15058 ± 1238	15165 ± 1671	3.26	0.03	0.06						
Allantoin	2794 ± 749	3461 ± 2934	2045 ± 789	2676 ± 795	3.10	0.03	0.07						
Creatine-phosphate	719567 ± 82710	580756 ± 241499	702611 ± 65244	745422 ± 87248	3.06	0.04	0.07						

Hippurate	11080 ± 1755	7498 ± 3323	9612 ± 2184	9825 ± 2716	3.00	0.04	0.08
Acetylcysteine	15463 ± 1293	13601 ± 5659	16845 ± 1261	16215 ± 1794	2.56	0.06	0.12
Propylene Glycol	7220 ± 1503	5630 ± 2379	7509 ± 1212	6976 ± 1189	2.52	0.07	0.13
NADP	11056 ± 3515	3540 ± 11289	9542 ± 3268	9825 ± 4161	2.44	0.08	0.13
Maleate	17225 ± 1082	14082 ± 6019	16631 ± 1249	15754 ± 913	2.31	0.09	0.15
Glycine	892758 ± 40743	762519 ± 309875	910144 ± 50688	857634 ± 55732	2.25	0.09	0.16
2-Acyl-sn-glycero-3-phosphocholine	424278 ± 37238	378298 ± 154930	467769 ± 25093	432194 ± 64864	2.02	0.12	0.20
Asparagine	9329 ± 1749	7408 ± 3257	9770 ± 2054	9854 ± 2356	1.82	0.15	0.24
NAD+	6652 ± 871	7540 ± 1975	6764 ± 820	6360 ± 1005	1.72	0.18	0.27
Adenine	6653 ± 754	6544 ± 2936	6871 ± 611	6636 ± 868	1.59	0.20	0.31
Acetate	295191 ± 24786	379209 ± 347793	284723 ± 27565	309081 ± 52026	1.49	0.23	0.32
Citrate	36934 ± 3912	30705 ± 12255	38680 ± 5859	38527 ± 5853	1.51	0.22	0.32
Lactate	513270 ± 94527	410896 ± 177319	464223 ± 62642	504898 ± 61087	1.33	0.28	0.38
Dimethylamine	9314 ± 6898	5593 ± 3032	5700 ± 1261	8181 ± 6381	1.16	0.33	0.45
Glycocholate	26294 ± 2334	31446 ± 27383	26674 ± 2074	25981 ± 2016	0.79	0.51	0.67
Formate	92172 ± 16934	112264 ± 103547	89628 ± 12321	93163 ± 18125	0.72	0.55	0.71

UDP-glucuronate	15074 ± 3250	12868 ± 5955	15691 ± 3722	13937 ± 3421	0.64	0.59	0.75
2-deoxyinosine	4251 ± 707	3948 ± 2574	4033 ± 527	3742 ± 625	0.59	0.63	0.77
Nicotinic Acid	705 ± 712	1645 ± 3704	1128 ± 839	1100 ± 778	0.57	0.64	0.77
UDP-Glucose	7886 ± 758	8174 ± 6108	7770 ± 1026	7411 ± 1221	0.50	0.68	0.81
ADP	3889 ± 511	3256 ± 1687	3447 ± 874	3760 ± 1360	0.46	0.71	0.83
Ethanol	21318 ± 2658	19774 ± 10087	20980 ± 1413	21793 ± 2068	0.40	0.76	0.84
Valerate	15910 ± 5344	15916 ± 8710	17378 ± 6813	17020 ± 5699	0.41	0.74	0.84
methyl succinate	7872 ± 1907	6938 ± 3243	7397 ± 1514	7909 ± 1767	0.31	0.81	0.89
Isopropanol	11717 ± 1153	11873 ± 7441	11997 ± 1481	11693 ± 1615	0.22	0.88	0.95
Inosine	4680 ± 2053	5158 ± 4525	4954 ± 1493	4714 ± 2745	0.18	0.91	0.96
3-hydroxykynurenine	3202 ± 544	3135 ± 2900	2962 ± 431	3021 ± 699	0.15	0.93	0.96
Fumarate	5042 ± 722	4540 ± 2634	5073 ± 1140	4977 ± 950	0.02	1.00	1.00
UMP	2984 ± 591	2691 ± 1259	2980 ± 572	3095 ± 558	0.04	0.99	1.00

Table 11.2 – Overview of mean relative metabolite abundance and ANOVA outputs in C2C12 myotubes following electrical pulse stimulation (EPS) (Stim Only), Heat Stress (40°C) (Heat Only) or combined EPS and Heat Stress (Heat and Stim) (Chapter 7).

	Mean Relative Metabolite Abundance				ANOVA			Pairwise Comparisons					
Metabolite	**Control**	**Stim Only**	**Heat Only**	**Heat & Stim**	**F-stat**	***P*-value**	**BH Adjusted *P*-value**	**Stim Only-Control**	**Heat Only-Control**	**Heat & Stim-Control**	**Stim Only - Heat Only**	**Stim Only-Heat & Stim**	**Heat Only-Heat & Stim**
Nicotinic Acid	28645 ± 12887	18980 ± 9102	23147 ± 7563	15152 ± 8202	12.89	$1.95x10^{-7}$	$1.79x10^{-6}$	0.0002	0.0956	$1.87x10^{-7}$	0.2016	0.3987	0.0025
Benzoate	2606 ± 1418	5817 ± 3254	2502 ± 1296	5782 ± 2165	24.51	$1.13x10^{-12}$	$5.02x10^{-11}$	$2.91x10^{-7}$	0.9993	$6.34x10^{-8}$	1.07x10-7	0.9956	$2.18x10^{-8}$
Mannose	5852 ± 1323	5327 ± 1242	4933 ± 1156	4641 ± 1264	24.01	$1.82x10^{-12}$	$5.02x10^{-11}$	0.004	$9.26x10^{-7}$	$9.66x10^{-13}$	0.1825	$3.57x10^{-5}$	0.0362
2-Acyl-sn-glycero-3-phosphocholine	145323 ± 15252	160636 ± 14490	171300 ± 17090	171999 ± 14986	21.84	$1.51x10^{-11}$	$2.76x10^{-10}$	0.0021	$9.94x10^{-10}$	$7.47x10^{-10}$	0.0083	0.007	0.9999
Glutamate	37030 ± 5358	34745 ± 5658	31541 ± 3596	35266 ± 6480	17.12	$1.88x10^{-9}$	$2.59x10^{-8}$	0.8724	$4.52x10^{-8}$	0.0002	$1.74x10^{-6}$	0.0031	0.2359
Pantothenate	78563 ± 8210	75823 ± 8188	73833 ± 5739	68511 ± 8814	15.27	$1.39x10^{-8}$	$1.53x10^{-7}$	0.056	0.0368	$3.71x10^{-9}$	0.9993	0.0004	0.0006
Aspartate	69006 ± 6768	66946 ± 6659	63322 ± 5497	65362 ± 6479	11.93	$5.81x10^{-7}$	$4.56x10^{-6}$	0.9999	$9.21x10^{-6}$	0.0209	$1.18x10^{-5}$	0.0245	0.1464
UMP	5225 ± 1094	4391 ± 1214	4374 ± 839	3998 ± 1105	10.77	$2.23x10^{-6}$	$1.41x10^{-5}$	0.0004	0.0044	$1.28x10^{-6}$	0.8917	0.545	0.1668
Maleate	79076 ± 6984	83818 ± 5778	87035 ± 4033	90378 ± 5916	10.74	$2.31x10^{-6}$	$1.41x10^{-5}$	0.0044	0.0156	$5.99x10^{-7}$	0.9678	0.142	0.0441
Citrate	69937 ± 13815	77065 ± 15634	76444 ± 9771	77419 ± 14956	10.49	$3.10x10^{-6}$	$1.70x10^{-5}$	0.319	$5.40x10^{-6}$	0.0008	0.0047	0.1301	0.6058

Pyruvate	26866 ± 2771	26688 ± 2938	30128 ± 3239	29383 ± 3723	9.6	$8.86x10^{-6}$	$4.43x10^{-5}$	0.9988	0.0004	0.0082	0.0002	0.005	0.8205
Methyl succinate	272217 ± 58430	287389 ± 49520	245697 ± 47872	255695 ± 44564	7.63	$9.61x10^{-5}$	0.0004	0.504	0.0048	0.0002	0.1963	0.021	0.7799
Taurine	86162 ± 6627	80069 ± 8420	81233 ± 5869	77650 ± 9282	7.13	0.0002	0.0008	0.0056	0.0397	0.0001	0.8897	0.7291	0.2876
Glutamine	9745 ± 2363	9805 ± 2455	8662 ± 1470	9418 ± 2025	6.38	0.0005	0.0018	0.4025	0.0003	0.6367	0.0473	0.9781	0.0137
Lactate	19832 ± 1823	19926 ± 1697	19949 ± 1223	19534 ± 1472	6.04	0.0007	0.0025	0.2346	0.0004	0.0177	0.1378	0.7147	0.6749
2-Hydroxyvalerate	22503 ± 1549	23170 ± 1770	22694 ± 1560	21764 ± 1822	5.33	0.0017	0.0058	0.3775	0.9219	0.1276	0.7491	0.0011	0.0239
Isoleucine	16399 ± 2634	16149 ± 3854	14896 ± 2428	15348 ± 3255	5.14	0.0021	0.0069	0.8567	0.4362	0.0017	0.8967	0.0217	0.1168
Beta Alanine	18973 ± 4108	17392 ± 3243	15351 ± 2489	16027 ± 2364	4.91	0.0029	0.0088	0.3103	0.0017	0.046	0.2117	0.8176	0.6945
Myo Inositol	13607 ± 4930	14537 ± 4664	14513 ± 3380	15124 ± 4521	4.61	0.0042	0.0123	0.5559	0.1681	0.4844	0.0048	0.033	0.9145
Inosine	54120 ± 3506	53574 ± 4888	52789 ± 4008	50428 ± 3735	4.17	0.0074	0.0202	0.5744	0.8292	0.0836	0.1412	0.6972	0.0069
N-Carbamoylaspartate	19884 ± 6977	22282 ± 6713	22753 ± 5458	24328 ± 6203	3.86	0.0109	0.0286	0.1377	0.1868	0.0056	0.9979	0.6541	0.5265
Uridine	57356 ± 7094	59806 ± 7180	58212 ± 6318	55188 ± 7271	3.59	0.0154	0.0385	0.1983	0.9818	0.6026	0.3603	0.0078	0.3595
2-Deoxyinosine	4248 ± 987	4034 ± 1052	3542 ± 780	4014 ± 1197	3.37	0.0206	0.0492	0.3442	0.0099	0.3746	0.4495	0.9998	0.3887
Creatine	457249 ± 48580	477003 ± 56823	473961 ± 42889	474402 ± 41622	3.1	0.029	0.0644						

Propylene Glycol	5829 ± 1677	5894 ± 1349	5185 ± 1025	5082 ± 1156	3.09	0.0293	0.0644
O-Phosphocholine	36066 ± 5322	39095 ± 5885	38268 ± 4531	36458 ± 7275	2.8	0.0427	0.0903
Isopropanol	91378 ± 14756	84901 ± 9789	80641 ± 13664	81672 ± 12282	2.39	0.0712	0.1451
ADP	2127 ± 948	2232 ± 985	1858 ± 1004	1973 ± 925	2.33	0.0772	0.1464
Glycocholate	5392 ± 1466	5521 ± 2117	5526 ± 1262	5443 ± 1511	2.35	0.0755	0.1464
Fumarate	47924 ± 43720	31250 ± 32438	40316 ± 33833	30544 ± 24332	1.93	0.1271	0.2329
Phenylacetate	9207 ± 1438	9137 ± 1578	8723 ± 1158	8648 ± 1095	1.86	0.1394	0.2473
Dihydrothymine	24735 ± 2081	25005 ± 2407	24000 ± 2116	24142 ± 1959	1.78	0.1536	0.264
Nitrosodimethylamine	14353 ± 1378	13886 ± 1377	13713 ± 1198	13802 ± 1195	1.75	0.1603	0.2672
Valine	39519 ± 4131	38124 ± 3034	38420 ± 3073	37732 ± 2865	1.68	0.1739	0.2814
Phenylalanine	9881 ± 1400	9602 ± 1436	9260 ± 1019	9494 ± 1192	1.65	0.1807	0.284
Tryptophan	3309 ± 752	3094 ± 798	2991 ± 615	3029 ± 857	1.62	0.1883	0.2876
Acetate	403408 ± 133421	379881 ± 176659	351251 ± 106732	403215 ± 147230	1.54	0.2061	0.3063
NAD+	5237 ± 1657	5039 ± 2190	4669 ± 1195	4624 ± 2205	1.5	0.2182	0.3159

3-hydroxykynurenine	6711 ± 1897	7775 ± 2548	6454 ± 1551	6928 ± 1939	1.44	0.2352	0.3317
Hippurate	29526 ± 22305	35445 ± 21200	26346 ± 11106	41075 ± 20017	1.33	0.2661	0.3639
Formate	25985 ± 6816	25167 ± 6239	24527 ± 6077	24485 ± 5749	1.32	0.2713	0.3639
Fructose	4021 ± 1282	3583 ± 1245	3420 ± 1090	3621 ± 1614	1.19	0.3153	0.4049
Creatinine	16150 ± 7543	17648 ± 10347	14788 ± 6251	15822 ± 7087	1.19	0.3166	0.4049
Glucose	32532 ± 4371	33531 ± 3403	39108 ± 4447	37338 ± 4928	1.13	0.3377	0.4222
Tyrosine	15454 ± 1691	15110 ± 1841	14865 ± 1327	15057 ± 1530	1.11	0.3457	0.4225
Acetone	17999 ± 4508	16733 ± 4272	17040 ± 3411	17291 ± 4124	0.94	0.4229	0.5056
Creatine Phosphate	60690 ± 25287	73400 ± 37195	67304 ± 28696	72765 ± 30257	0.89	0.4458	0.5217
Allantoin	15157 ± 2334	15068 ± 2001	12960 ± 2178	13744 ± 1292	0.79	0.5036	0.5771
Acetylcysteine	4752 ± 1006	4836 ± 1173	4461 ± 727	4607 ± 1157	0.74	0.5321	0.5972
Serine	36262 ± 4792	35148 ± 4836	37221 ± 3242	36137 ± 4660	0.64	0.5921	0.6513
Adenine	2137 ± 821	1946 ± 957	1829 ± 588	1804 ± 888	0.59	0.6258	0.6749
Ethanol	87749 ± 30451	82594 ± 52864	80330 ± 24629	89292 ± 36478	0.29	0.8305	0.8784

Guanosine	4386 ± 1545	4564 ± 1499	4387 ± 990	3986 ± 1388	0.25	0.8636	0.8962
Leucine	12639 ± 2842	10993 ± 3221	10739 ± 1980	9448 ± 3171	0.22	0.8856	0.902
Threonine	139743 ± 40892	135380 ± 39612	134322 ± 29328	136847 ± 33033	0.16	0.921	0.921

Table 11.3 – All metabolite concentrations, Delta, and T-Test outputs. Expansion of Table 8.3

	Temperate			**Hot**			**Univariate Analysis (Δ)**		
Metabolite	**Pre**	**Post**	**FC**	**Pre**	**Post**	**FC**	**Raw *P*-value**	**BH adjusted *P*-value**	**Bonferroni adjusted P-value**
2-Hydroxyisovaleric Acid	143225 ± 9076	136024 ± 10978	0.083	141703 ± 11358	133276 ± 10402	0.150	0.5358	0.66976	1
2-Hydroxyvaleric Acid	452323 ± 80393	391056 ± 77260	0.061	391524 ± 57187	335827 ± 53938	0.014	0.0111	0.05017	0.27812
Acetate	9429 ± 960	11806 ± 1016	-0.173	9240 ± 717	11551 ± 846	-0.303	0.012	0.05017	0.30103
Acetoacetate	8527 ± 1362	11150 ± 1333	0.192	8762 ± 1652	10434 ± 1219	0.168	0.8103	0.92074	1
Alanine	16065 ± 2771	21912 ± 2518	0.032	13529 ± 1653	20611 ± 2741	-0.066	9.00×10^{-4}	0.00584	0.02183
Arginine	35222 ± 3248	40746 ± 4894	0.378	34348 ± 4444	41203 ± 5964	0.351	0.3366	0.49502	1
Choline	148013 ± 37522	454792 ± 111580	0.002	132241 ± 60304	458904 ± 122663	-0.025	0.9483	0.95192	1
Citrate	37203 ± 6183	34053 ± 4404	-0.040	33679 ± 4523	32694 ± 4061	-0.100	0.0407	0.12733	1
Creatine	253743 ± 14485	274997 ± 46498	0.193	253439 ± 12334	290068 ± 46055	0.185	0.5797	0.69016	1
Creatinine	115683 ± 13458	122512 ± 18808	0.030	110721 ± 11240	117402 ± 14138	0.067	0.0282	0.10085	0.70592
Glucarate	39102 ± 5421	36170 ± 4264	-0.191	38640 ± 5249	36504 ± 3812	-0.223	4.00×10^{-4}	0.0052	0.0104

Glucose	181786 ± 18485	172454 ± 15039	-0.023	180017 ± 11715	168402 ± 11969	-0.105	$9.00x10^{-4}$	0.00584	0.02335
Glutamate	120206 ± 7562	108455 ± 8798	0.287	119478 ± 6812	107021 ± 6914	0.402	0.2746	0.4576	1
Isoleucine	166388 ± 10703	166918 ± 15438	0.089	161430 ± 15175	168005 ± 14998	0.059	0.2163	0.4159	1
Isopropanol	39726 ± 3002	33589 ± 2527	0.019	39699 ± 2498	33964 ± 2370	0.070	0.4494	0.59137	1
Lactate	66870 ± 5766	69970 ± 6324	-0.337	62876 ± 3168	69572 ± 4957	-0.344	0	0.00031	0.00031
Leucine	59671 ± 14249	44366 ± 10974	-0.704	51323 ± 9253	37839 ± 7433	-0.766	0.9274	0.95192	1
Lysine	145185 ± 38289	117791 ± 31736	0.041	128494 ± 33610	102323 ± 39218	0.058	0.0492	0.13664	1
Mannose	65306 ± 6728	60602 ± 6245	0.113	62629 ± 5280	58982 ± 4895	0.122	0.0774	0.1758	1
Myo Inositol	89344 ± 19285	110548 ± 36898	-0.261	87284 ± 15429	118255 ± 46759	-0.328	0.9519	0.95192	1
NDMA	34632 ± 1721	25393 ± 2529	-0.015	34189 ± 1791	25221 ± 2265	-0.061	0.1449	0.30196	1
Phenylalanine	169318 ± 20334	172585 ± 19322	0.062	155292 ± 20011	168683 ± 16917	-0.008	0.4373	0.59137	1
Propylene Glycol	62777 ± 5767	95392 ± 7777	-0.150	61053 ± 6113	93389 ± 9489	-0.192	0.3142	0.491	1
Tyrosine	21244 ± 2360	40381 ± 4260	-0.475	18970 ± 2290	39157 ± 3770	-0.511	0.2743	0.4576	1
Valine	84867 ± 13552	90091 ± 11702	-0.246	92531 ± 16075	89073 ± 9196	-0.201	0.062	0.15504	1

Table 11.4 – Metabolite Abundance and difference pre and post exercise in during 30-minute exercise test temperate conditions (Chapter 8).

	CON			SL			SL_{Heat}			ANOVA				
Metabolite	**Pre**	**Post**	**Δ**	**Pre**	**Post**	**Δ**	**Pre**	**Post**	**Δ**	***F***	***P* Value**	**CON vs SL**	**CON vs SL_{Heat}**	**SL vs SL_{Heat}**
2-Hydroxyisovaleric Acid	238 ± 219	192 ± 127	-140 ± 185	103 ± 415	-86 ± 299	-83 ± 145	581 ± 637	225 ± 506	-309 ± 308	1.552	0.2487	0.708	0.2725	0.1112
2-Hydroxyvaleric Acid	73 ± 97	86 ± 86	-21 ± 43	-15 ± 118	-6 ± 65	25 ± 78	136 ± 225	75 ± 151	-53 ± 90	1.587	0.2417	0.368	0.524	0.0993
Acetate	-137 ± 66	-236 ± 31	-96 ± 49	120 ± 122	114 ± 189	-15 ± 201	-49 ± 62	64 ± 212	-35 ± 196	0.2662	0.7704	0.4862	0.5987	0.846
Acetoacetate	52 ± 174	59 ± 150	-75 ± 106	171 ± 238	-14 ± 154	-133 ± 107	287 ± 427	101 ± 361	-137 ± 155	0.3287	0.7256	0.4979	0.4669	0.9543
Alanine	61 ± 144	52 ± 170	-24 ± 99	-13 ± 138	47 ± 188	82 ± 195	96 ± 147	119 ± 121	-56 ± 102	1.461	0.2678	0.2761	0.7401	0.1229
Arginine	44 ± 61	-83 ± 34	-122 ± 51	35 ± 84	-40 ± 55	-79 ± 74	75 ± 86	-52 ± 67	-108 ± 106	0.3475	0.7128	0.4461	0.8075	0.5585
Choline	46 ± 135	-3 ± 125	-85 ± 25	-63 ± 191	-15 ± 197	62 ± 69	-33 ± 100	-59 ± 88	59 ± 61	9.412	0.003	0.0018	0.002	0.9319
Citrate	4 ± 37	-14 ± 60	-31 ± 72	-18 ± 95	24 ± 123	48 ± 53	-8 ± 39	-27 ± 68	-14 ± 81	2.311	0.1384	0.0511	0.2385	0.3259
Creatine	39 ± 47	-78 ± 33	-127 ± 27	5 ± 33	-45 ± 56	-59 ± 58	57 ± 28	-35 ± 41	-88 ± 50	1.93	0.1845	0.1	0.7087	0.1444
Creatinine	20 ± 89	-69 ± 112	-108 ± 54	11 ± 141	-30 ± 162	-62 ± 67	58 ± 118	-5 ± 110	-119 ± 122	0.6439	0.5412	0.449	0.8519	0.2973
Glucarate	-21 ± 60	32 ± 133	51 ± 82	-46 ± 68	24 ± 81	69 ± 113	-26 ± 53	35 ± 66	51 ± 72	0.07131	0.9315	0.7716	0.9926	0.7379
Glucose	-147 ± 63	-46 ± 89	69 ± 101	-57 ± 66	-36 ± 105	24 ± 55	-133 ± 100	-61 ± 125	140 ± 43	4.81	0.0273	0.3015	0.1165	0.0087
Glutamate	101 ± 162	83 ± 156	-82 ± 99	35 ± 172	-37 ± 130	-54 ± 60	285 ± 405	102 ± 273	-174 ± 150	1.862	0.1945	0.7064	0.2224	0.0851
Glutamine	26 ± 70	0 ± 69	-42 ± 62	-13 ± 49	-16 ± 58	-11 ± 59	45 ± 90	-59 ± 57	-52 ± 88	0.5325	0.5994	0.5154	0.8235	0.3346
Isoleucine	15 ± 123	-46 ± 151	-103 ± 50	66 ± 249	19 ± 193	-67 ± 89	156 ± 86	76 ± 125	-108 ± 85	0.443	0.6515	0.4978	0.9319	0.3963
Isopropanol	-80 ± 176	112 ± 44	242 ± 60	-6 ± 154	99 ± 46	198 ± 94	-41 ± 213	121 ± 122	266 ± 106	0.8159	0.4637	0.4769	0.6972	0.2286
Lactate	-186 ± 166	345 ± 400	523 ± 531	-194 ± 214	332 ± 300	560 ± 460	-362 ± 101	354 ± 276	544 ± 359	0.04861	0.9527	0.7842	0.7852	0.9989
Leucine	64 ± 92	-68 ± 96	-101 ± 68	95 ± 156	10 ± 75	-89 ± 104	144 ± 117	-6 ± 38	-132 ± 111	0.3042	0.7428	0.8436	0.6378	0.4585
Lysine	65 ± 43	-94 ± 75	-174 ± 53	16 ± 95	-37 ± 101	-70 ± 83	34 ± 88	-70 ± 116	-142 ± 44	3.617	0.0564	0.0253	0.4498	0.0725
Mannose	2 ± 59	89 ± 26	54 ± 45	-6 ± 80	38 ± 66	76 ± 66	53 ± 133	79 ± 117	36 ± 120	0.8412	0.4534	0.567	0.2237	0.4541
Myo Inositol	-19 ± 77	-47 ± 111	-48 ± 56	-3 ± 123	102 ± 247	141 ± 129	-56 ± 101	-116 ± 67	7 ± 122	3.839	0.049	0.0225	0.4596	0.062
NDMA	27 ± 61	-38 ± 85	-121 ± 33	7 ± 72	-13 ± 90	-27 ± 21	30 ± 86	-29 ± 49	-38 ± 44	10.2	0.0022	0.0009	0.0024	0.5924

Phenylalanine	-31 ± 45	25 ± 12	41 ± 50	-14 ± 55	31 ± 33	44 ± 34	-23 ± 34	13 ± 29	44 ± 16	0.01184	0.9882	0.8998	0.887	0.9856
Propylene Glycol	-62 ± 120	101 ± 32	144 ± 147	-25 ± 100	86 ± 39	146 ± 94	-49 ± 144	85 ± 108	219 ± 50	1.115	0.3574	0.9709	0.2454	0.2101
Tyrosine	-27 ± 30	21 ± 20	30 ± 20	-6 ± 42	37 ± 34	42 ± 31	-6 ± 29	35 ± 43	30 ± 24	0.3858	0.6874	0.4886	0.9869	0.4505
Valine	-42 ± 98	-8 ± 114	-6 ± 24	30 ± 197	14 ± 167	-20 ± 78	54 ± 82	85 ± 144	-61 ± 117	0.5666	0.5809	0.8201	0.3496	0.424

Table 11.5 – Metabolite Abundance and difference pre and post exercise during 30-minute exercise test in Hot Conditions (Chapter 8)

	CON			**SL**			**SL_{Heat}**			**ANOVA**				
Metabolite	**Pre**	**Post**	**Δ**	**Pre**	**Post**	**Δ**	**Pre**	**Post**	**Δ**	***F***	***P* Value**	**CON vs SL**	**CON vs SL_{Heat}**	**SL vs SL_{Heat}**
2-Hydroxyisovaleric Acid	162 ± 372	-86 ± 265	-248 ± 166	21 ± 555	-316 ± 552	-75 ± 155	21 ± 186	-152 ± 60	-212 ± 140	1.576	0.2468	0.1092	0.7123	0.2102
2-Hydroxyvaleric Acid	77 ± 72	19 ± 44	-58 ± 61	-17 ± 126	-68 ± 47	3 ± 59	-13 ± 39	-20 ± 26	-5 ± 50	5.762	0.0176	0.0181	0.0114	0.9511
Acetate	-177 ± 34	-211 ± 60	-34 ± 34	2 ± 97	-4 ± 241	177 ± 153	-76 ± 88	66 ± 177	122 ± 101	6.362	0.0131	0.0062	0.0226	0.4257
Acetoacetate	8 ± 188	-132 ± 136	-140 ± 86	103 ± 295	-205 ± 252	-198 ± 121	-7 ± 157	-176 ± 63	-176 ± 144	0.3141	0.7363	0.4567	0.6253	0.7784
Alanine	-8 ± 52	20 ± 76	28 ± 80	-114 ± 102	14 ± 118	-132 ± 39	-149 ± 95	-4 ± 78	162 ± 123	8.892	0.0043	0.0051	0.0031	0.9641
Arginine	74 ± 20	-71 ± 26	-145 ± 35	70 ± 74	-54 ± 24	-132 ± 39	58 ± 38	-80 ± 33	-134 ± 21	0.2336	0.7952	0.5562	0.5956	0.9295
Choline	81 ± 138	20 ± 61	-62 ± 126	-31 ± 190	-61 ± 228	-95 ± 236	-3 ± 155	16 ± 161	57 ± 76	1.346	0.2968	0.738	0.2162	0.1574
Citrate	-15 ± 49	23 ± 54	38 ± 59	-22 ± 59	64 ± 147	103 ± 67	-38 ± 92	50 ± 116	119 ± 77	4.094	0.0441	0.1075	0.0161	0.4114
Creatine	56 ± 39	-60 ± 54	-115 ± 47	43 ± 49	-49 ± 20	-90 ± 40	59 ± 47	-41 ± 37	-100 ± 87	0.2148	0.8098	0.8057	0.8057	0.8057
Creatinine	18 ± 114	-75 ± 82	-92 ± 72	73 ± 97	37 ± 205	-147 ± 160	38 ± 116	-108 ± 129	-124 ± 62	0.379	0.6924	0.4117	0.6047	0.7409
Glucarate	-42 ± 29	39 ± 114	80 ± 90	-67 ± 73	-1 ± 81	45 ± 85	-60 ± 29	29 ± 28	90 ± 39	0.4168	0.6683	0.7358	0.8337	0.7358
Glucose	-128 ± 89	61 ± 137	188 ± 191	-27 ± 162	125 ± 151	240 ± 101	-138 ± 99	42 ± 160	183 ± 151	3.911	0.0492	0.0366	0.0353	0.9041
Glutamate	90 ± 132	-48 ± 73	-138 ± 72	38 ± 228	-134 ± 96	-83 ± 38	27 ± 98	-87 ± 31	-134 ± 65	1.052	0.3795	0.2017	0.9231	0.2493
Glutamine	82 ± 50	-6 ± 79	-89 ± 53	16 ± 75	-43 ± 25	-43 ± 40	-7 ± 87	-85 ± 59	-45 ± 52	1.466	0.2694	0.1792	0.1687	0.9607
Isoleucine	39 ± 79	-51 ± 126	-90 ± 101	16 ± 236	-109 ± 138	-49 ± 50	13 ± 55	-97 ± 36	-115 ± 65	2.125	0.1621	0.0803	0.9125	0.108
Isopropanol	-139 ± 42	98 ± 34	236 ± 49	-67 ± 139	83 ± 25	212 ± 64	-124 ± 52	122 ± 54	244 ± 76	0.3029	0.7442	0.5679	0.8361	0.4642

Lactate	-423 ± 90	280 ± 383	702 ± 317	-448 ± 85	308 ± 163	802 ± 182	-462 ± 35	354 ± 136	827 ± 131	0.428	0.6614	0.7919	0.7919	0.8738
Leucine	52 ± 130	-44 ± 92	-97 ± 104	47 ± 239	-90 ± 92	-66 ± 40	37 ± 95	-74 ± 70	-104 ± 74	1.307	0.3065	0.1389	0.7044	0.2629
Lysine	91 ± 74	-68 ± 90	-159 ± 96	77 ± 53	-47 ± 37	-138 ± 49	60 ± 77	-98 ± 90	-136 ± 71	0.1432	0.868	0.6881	0.6363	0.967
Mannose	-20 ± 50	53 ± 35	73 ± 35	-40 ± 89	-10 ± 73	90 ± 40	-63 ± 19	39 ± 19	97 ± 28	0.7126	0.51	0.4519	0.2722	0.7762
Myo Inositol	-50 ± 52	-72 ± 42	-22 ± 33	-35 ± 98	146 ± 214	116 ± 63	-16 ± 93	-25 ± 101	14 ± 87	8.4	0.0052	0.0016	0.0358	0.1055
NDMA	64 ± 98	-21 ± 72	-85 ± 51	6 ± 78	6 ± 84	-26 ± 20	27 ± 105	-47 ± 71	-36 ± 26	3.695	0.0562	0.0323	0.0524	0.7067
Phenylalanine	-34 ± 17	27 ± 23	61 ± 16	-27 ± 27	9 ± 12	40 ± 28	-29 ± 27	26 ± 35	57 ± 15	1.551	0.2517	0.1133	0.7392	0.2062
Propylene Glycol	-103 ± 11	93 ± 23	197 ± 20	-26 ± 70	117 ± 68	162 ± 54	-125 ± 11	80 ± 24	205 ± 28	1.96	0.1834	0.1362	0.7125	0.085
Tyrosine	-18 ± 30	16 ± 29	33 ± 26	-24 ± 21	19 ± 16	31 ± 26	-37 ± 28	6 ± 28	36 ± 13	0.05882	0.9431	0.86	0.8547	0.738
Valine	19 ± 118	-9 ± 128	-27 ± 87	20 ± 187	-40 ± 182	23 ± 31	-3 ± 51	-53 ± 61	-62 ± 58	5.952	0.016	0.0065	0.6617	0.0174

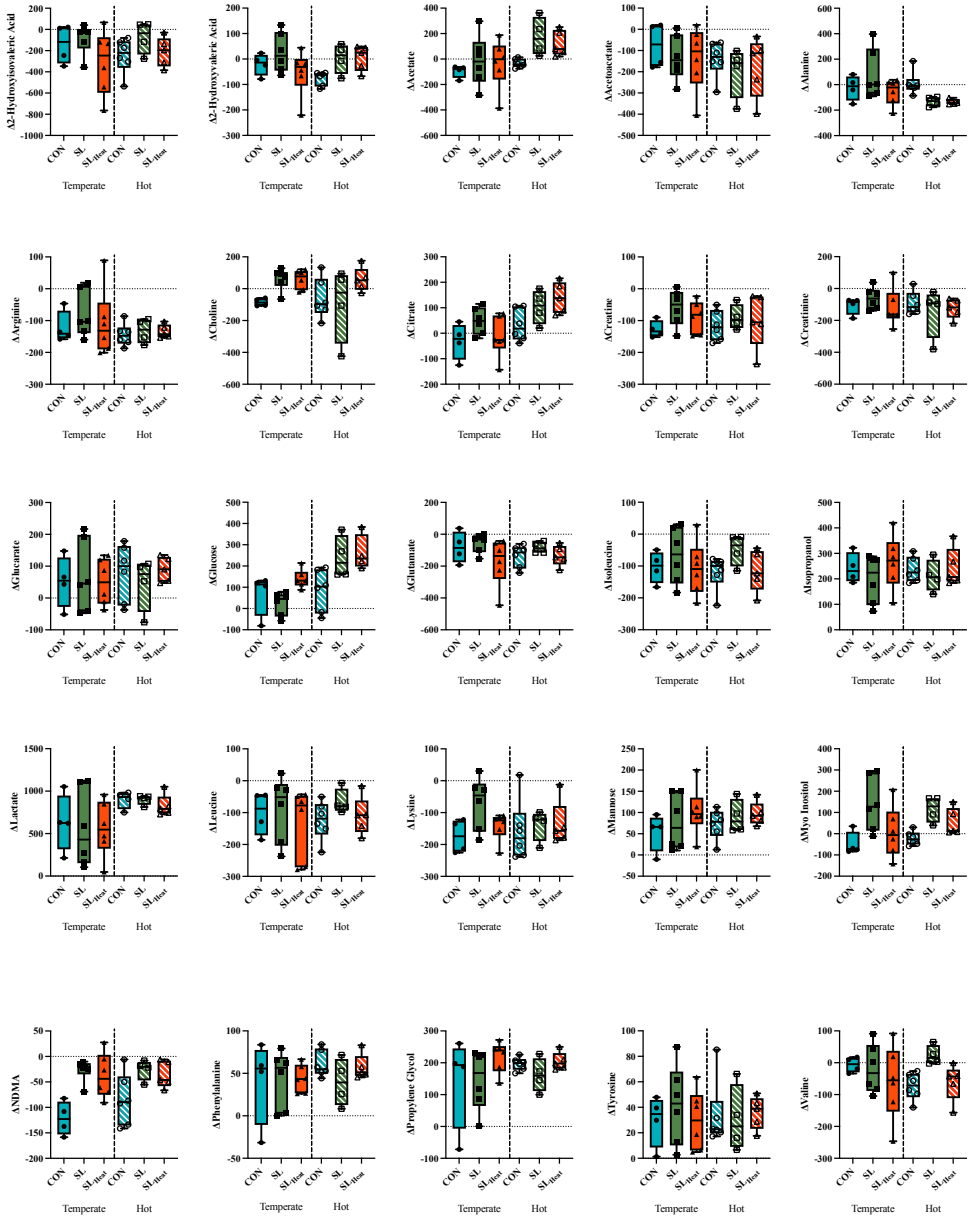

Figure 11.2 – Boxplots representing median change in CRS filtered metabolites serum metabolite concentration following exercise in Temperate (left) and Hot (right) conditions in each group. Significant difference between temperate and hot conditions denoted by *, where $P < 0.05$.