Exercise-induced fatigue:
Exploring mechanisms
and counter strategies.

By

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to fulfil the requirements for the degree of
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in the discipline of
Exercise Science

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2017
Statement of Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Faizal Abdul Manaf
Statement of Contributors

The work presented herein is my own work. Specifically, I was involved in the concept development of each chapter, reviewed the literature of each chapter, wrote the first draft of each chapter, and helped interpret the data in each chapter. I was solely responsible for all data collection, including the blood sampling in each chapter. With respect to the metabolomics data in Chapter 3, I learned the techniques and processing steps, however, the final samples analysed for this chapter, were analysed at the Centre for Integrative Metabolomics & Computational Biology, Edith Cowan University.

I would like to acknowledge the contribution of Prof. David Broadhurst and Mr. Nathan Lawler who conducted this work.
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Abstract

Mechanisms leading to fatigue have been the subject of immense research investigation. However, the exact mechanisms causing the development of fatigue, remain largely unknown and fiercely debated. Herein we identify plasma metabolites and neuromuscular factors associated with exercise-induced fatigue, and assess potential nutritional strategies which may help delay the onset of fatigue.

The role of individual metabolites in the development of fatigue were assessed using metabolomic profiling of plasma during a constant endurance cycling bout in Chapter 3. Analyses revealed important associations between fatigue-onset with free fatty acids, tryptophan and glucose. The contribution of central- and peripheral-fatigue mechanisms during isoenergetic constant and self-paced cycling trials was assessed in Chapter 4. Central fatigue was similar following each protocol, but total fatigue was shown to be greater following self-paced cycling.

Importantly, the serotonin precursor tryptophan has been implicated in the onset of fatigue, and tryptophan is displaced from albumin when lipid concentrations increase; this may explain the association between increased lipid concentration and fatigue observed in Chapter 3. Therefore, using a branched-chain amino acids (BCAA) supplement, we manipulated the free-tryptophan (unbound) passage across the blood brain barrier (Chapter 5). We observed greater endurance capacity with the BCAA compared to a placebo, although this was independent of changes in central fatigue.

In an attempt to mitigate the decrease in blood glucose concentrations in endurance cycling (observed in Chapter 3), a mixed-carbohydrate comprising starch and disaccharides was ingested before and immediately after a prolonged cycling bout (Chapter 6). We hypothesised that this mixed-carbohydrate beverage would minimise hypoglycaemia when compared to an isocaloric glucose beverage and improve overall performance. Although blood glucose and insulin were significantly different between conditions, there were no differences in exercise performance or fatigue markers between conditions. The significance of these findings and future directions of research are discussed in Chapter 7.
Acknowledgements

*Alhamdulilah*, all praises to Allah and Him alone for endowing me the strength and courage to endure and complete my thesis, after all the extreme challenges and difficulties that were beyond my expectations.

First and foremost, I would like to express my greatest gratitude to Dr. Timothy Fairchild, as my advisor for his guidance in academic and real life through this dissertation process, and for being a great example as a supervisor, lecturer, researcher, academician, and leader. I am deeply indebted to my co-advisors, Dr. Jeremiah Peiffer and Dr. Garth Maker for their constructive guidance, valuable advice, and continuous support.

My sincere appreciation to my friendly and cheerful colleagues within the School of Psychology and Exercise Science for being there for me through thick and thin. I would like to acknowledge all those who were directly and indirectly involved in my thesis completion. For those whose names I did not mention – you know who you are, and your contribution means a lot to me.

Last but not least, my deepest gratitude goes to my family, especially my mother, Maimon Abdullah, for her unconditional love, continuous support, sacrifice and *du’a* to see me reaching this point. I wish to dedicate this thesis to my mother and my late father, Assoc. Prof. Dr. Abdul Manaf Haji Hamid for leading me into the life of an academician. Not to forget, my parents-in-law, who always have faith in me and for sending endless prayers to give me strength.

My sincere thanks to my wife (Nor Najihah Ismail) and kids, and I am grateful to have you there for me, through good times and bad. Without your understanding, I am sure this thesis would never have been completed. Thanks all, and may Allah repay all the support and help given to me.
Dedications

This thesis is dedicated to my parents especially to my 'super mum' who showered me with an unconditional love as well as my parents-in-law for their invaluable advice and constant prayer during this journey.

“Behind every young child who believes in himself, is a parent who believed first”.

Matthew Jacobson

I would also like to dedicate this thesis to my wife for her love, advice, support and patience because she always understood.

“The greatest wealth a man can have is an understanding wife”.

Euripides
Table of Contents

Statement of Declaration............................................................... iii
Statement of Contributors.......................................................... v
Publication and Award.................................................................... vii
Abstract........................................................................................ ix
Acknowledgements........................................................................ xi
Dedications ................................................................................... xiii
Table of Contents.......................................................................... xv
List of Figures ............................................................................... xix
List of Tables .............................................................................. xxiii
List of Abbreviations .................................................................... xxv

Chapter 1
Introduction................................................................................... 1
  1.1 Specific Aims of the Studies.................................................. 2
  1.2 Significance of the Thesis.................................................... 4
  1.3 References........................................................................... 5

Chapter 2
Literature Review ......................................................................... 9
  2.1 Fatigue .................................................................................. 9
    2.1.1 Energetics of Peripheral Fatigue....................................... 10
    2.1.2 Electrolytes and Ions in Peripheral fatigue ...................... 12
    2.1.3 Central Fatigue............................................................... 14
      2.1.3.1 Calculations for Central Fatigue Assessment............. 14
        2.1.3.1.1 Central Activation Ratio (CAR)......................... 15
        2.1.3.1.2 Voluntary Activation Level (VAL).................... 15
      2.1.3.2 Central Fatigue Assessment in Dynamic Exercise......... 16
        2.1.3.2.1 Before and After Exercise ............................... 16
        2.1.3.2.2 During Exercise............................................. 19
        2.1.3.2.3 Recovery....................................................... 20
      2.1.3.3 Central Fatigue Hypothesis...................................... 21
      2.1.3.4 Summary............................................................ 23
  2.2 Nutritional Manipulation..................................................... 25
    2.2.1 Branched-chain Amino Acids (BCAA) and Tryptophan (TRP)...... 25
    2.2.2 Summary......................................................................... 29
    2.2.3 Carbohydrate Supplementation...................................... 30
      2.2.3.1 Carbohydrate Supplementation Before Exercise........ 31
List of Figures

Figure 2.1 Proposed mechanisms of Central Fatigue Hypothesis during prolonged exercise. .................................................................................................................. 22

Figure 2.2 Proposed mechanisms of nutritional manipulation when BCAA is consumed, elevated plasma BCAA concentration which competes with f-TRP for entry into the brain. ...................................................... 26

Figure 2.3 A few analytical techniques to analyse variety group of metabolites....37

Figure 3.1 Scores plot from the Principal Component Analysis (PCA). The QC samples clearly cluster to the right and demonstrate large homogeneity while samples (four plasma samples per participant) are clustered to the left and are clearly separated from the QC samples. The analysis was conducted on all (identified and non-identified) metabolites present in the plasma sample. ......................... 78

Figure 3.2 Principal Component projection followed by Canonical Variate Analysis (PC-CVA) of identified metabolites. The mean (x) and 95% CI (dashed circle around x) are presented. Significant differences are determined where 95% CI does not overlap. Baseline (20-min steady state) is strongly separated along PC-1 from all time points. Pre-fatigue and post-fatigue (at fatigue) are clustered closer although significant separation was achieved. The 20-min post-fatigue samples are separated from the fatigue (pre-fatigue, post-fatigue) samples along PC2. ........................................................................ 80

Figure 3.3 Loadings illustrating metabolites contributing to PC1 (left) and PC2 (right). Data are presented as mean-loading values ±95% CI. Red-coloured CIs identify metabolites that significantly contribute along the respective PC; blue-coloured CIs identify metabolites that do not contribute significantly. ................................................................... 81

Figure 3.4 Circular clustergram classifying individual metabolites into seven associated clusters (A-G). ........................................................................ 82

Figure 3.5 Marginal means (±95% confidence interval) plots of each cluster relative to time. Bottom right panel provides a relative-indication of the timing for each blood sample to provide some reference. ........ 83

Figure 4.1 Experimental design scheme of the two modes of prolonged exercise. Small arrow, single supramaximal electrical stimulation; large arrow, doublet supramaximal electrical stimulation. ...................... 115

Figure 4.2 Total duration of exercise between TTE and TT trials; white and black columns, respectively. Mean ± SE..................... 118

Figure 4.3 Variations in a) power output (PWR) b) cadence (CDE) c) heart rate (HR) and d) ratings of perceived exertion (RPE) during TTE and TT trials. These variables were recorded after the total duration of exercise was divided into 10 blocks and each of the ten periods (10–100%) data were averaged. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p<0.001), ***Significant interaction effect (time x trial: p<0.05)................................. 119
Figure 4.4 Changes in maximal voluntary contraction (MVC) torque b) muscle Voluntary Activation Level (VAL) c) Peak Twitch (PT) d) Potentiated peak doublet (PD) for TTE and TT at baseline (PRE), post-exercise (POST), and 20-min post-exercise (POST 20). Mean ± SE. *Significant time effect (p<0.01), **Significant trial effect (p<0.01). ................................................................. 120

Figure 5.1 Time to completion between PLA and BCAA trials; white and black columns, respectively. Mean ± SE ................................................................. 148

Figure 5.2 Changes in a) power output (PWR), b) cadence (CDE), c) heart rate (HR) and d) ratings of perceived exertions (RPE) between PLA and BCAA trials. These variables were recorded after the total duration of exercise was divided into 10 blocks and each of the ten periods (10–100%) were data-averaged. *Significant time effect (p<0.01). **Significant trial effect (p<0.05). ................................................. 150

Figure 5.3 a) Maximal voluntary contraction (MVC) torque b) muscle Voluntary Activation Level (VAL) c) peak twitch (PT) d) peak doublet (PD) for PLA and BCAA at baseline (PRE), immediately post-exercise (POST), and 20-min post-exercise (POST 20). *Significant time effect (p<0.01). ................................................................. 151

Figure 6.1 Exercise duration (top panel) in the 16.1-km TT with EnergySmart (ES) and glucose (GLU) trials. Power output (bottom panel) during 16.1-km TT cycling performance between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE ............................................................... 172

Figure 6.2 a) Blood glucose and b) blood insulin concentration between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p<0.01), ***Significant interaction effect (time x trial: p<0.05). Significantly different between trials: + p<0.05, ++ p<0.01, +++ p<0.001. ......................................................................................... 173

Figure 6.3 Difference in blood glucose (mmol/L; GLU subtract ES) at each time point during trials. Solid line represents the mean difference while dotted lines provide the 95% CI of the mean. Significant difference is observed where all three lines are above or below the zero line. Arrows indicate the time of beverage ingestion .............................................. 174

Figure 6.4 a) Partial pressure of oxygen (pO₂) between EnergySmart (ES) and glucose (GLU) trials. b) Partial pressure of carbon dioxide (pCO₂) between EnergySmart (ES) and glucose (GLU) trials. c) Blood lactate concentration between EnergySmart (ES) and glucose (GLU) trials. d) Blood pH between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p<0.001). ................................................................. 175

Figure 6.5 a) Blood sodium ions (Na⁺) concentration between EnergySmart (ES) and glucose (GLU) trials. b) Blood potassium ions (K⁺) concentration between EnergySmart (ES) and glucose (GLU) trials. c) Blood calcium ions (Ca²⁺) concentration between EnergySmart (ES) and glucose (GLU) trials. d) Haematocrit level (Hct) between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p<0.001). ................................................................. 176
Figure 6.6 a) Respiratory exchange ratio (RER) between EnergySmart (ES) and glucose (GLU) trials. b) Heart rate (HR) between EnergySmart (ES) and glucose (GLU) trials. c) Rated perceived exertion (RPE) between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p<0.001)................................................................. 177

Figure 6.7 Cycling economy (CE) between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05).......................... 178
List of Tables

Table 3.1 Characteristics and statistical table of each identified metabolite. Significance was based on q-value (FDR-adjusted p-values) and significance set at q≤0.05. All significant metabolites are bolded. . 84

Table 4.1 Mean (± SE) of muscle Voluntary Activation Level with reference to the resting doublet (VALr), Central Activation Ratio (CAR), root mean square normalized to the amplitude of M-wave (RMS.M^-1 ratio) and M-wave peak-to-peak amplitude. * p<0.05, indicates a difference between PRE and POST. ............................................................... 122

Table 5.1 Physical characteristics and physiological capacities of the participants. ....................................................................................................... 142

Table 5.2 Mean (± SD) values of total duration of exercise (s) and mean (± SE) of power output (PWR), cadence (CDE), heart rate (HR) and ratings of perceived exertion (RPE) for PLA and BCAA trials. * p<0.05, ** p<0.01, *** p<0.001 between trials................................................................. 149

Table 6.1 Differences in subjective sensations of thirst, nausea, fullness and stomach upset during each trial. P represents differences (p-values) pre and post trials; Between represents differences in the change scores (post-pre) between trials. ................................................................. 179
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ATP-PCr</td>
<td>Phosphagen</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BAN</td>
<td>Banana</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CAR</td>
<td>Central Activation Ratio</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>Cl(^{-})</td>
<td>Chloride ions</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DEX</td>
<td>Dextrose</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>ES</td>
<td>EnergySmart</td>
</tr>
<tr>
<td>ESSA</td>
<td>Exercise and Sport Science Australia</td>
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<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>f-TRP</td>
<td>Free tryptophan</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GI</td>
<td>Glycemic index</td>
</tr>
<tr>
<td>GTE</td>
<td>Green tea extracts</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hydrogen ions</td>
</tr>
<tr>
<td>[H⁺]</td>
<td>Concentration of hydrogen ions</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HIIT</td>
<td>High-intensity interval exercise</td>
</tr>
<tr>
<td>HMS</td>
<td>Hydrothermally modified waxy maize</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma-mass spectrometry</td>
</tr>
<tr>
<td>IMP</td>
<td>Inosine monophosphate</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ions</td>
</tr>
<tr>
<td>[K⁺]</td>
<td>Concentration of potassium ions</td>
</tr>
<tr>
<td>KE</td>
<td>Knee extensor</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LNAA</td>
<td>Large neutral amino acids</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significance difference</td>
</tr>
<tr>
<td>MAL</td>
<td>Maltodextrin</td>
</tr>
<tr>
<td>MAMS</td>
<td>Acid/alcohol modified starches</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MAP</td>
<td>Maximal aerobic power</td>
</tr>
<tr>
<td>MAV</td>
<td>Maximal aerobic velocity</td>
</tr>
<tr>
<td>MOD</td>
<td>Moderate exercise</td>
</tr>
<tr>
<td>MVC</td>
<td>Maximal voluntary contraction</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ions</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>[Na⁺]</td>
<td>Concentration of sodium ions</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH:NAD⁺ ratio</td>
<td>Ratio of reduced form to oxidized form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonia</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>PF</td>
<td>Plantar flexor</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>P_i</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>[P_i]</td>
<td>Concentration of inorganic phosphate</td>
</tr>
<tr>
<td>PLA</td>
<td>Placebo</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RPE</td>
<td>Ratings of perceived exertion</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviations</td>
</tr>
<tr>
<td>SPE-LC-MS/MS</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
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<tr>
<td>TRP</td>
<td>Tryptophan</td>
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<tr>
<td>TT</td>
<td>Time-trial</td>
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<tr>
<td>TTE</td>
<td>Time-to-exhaustion</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>UAMS</td>
<td>Unmodified corn starches</td>
</tr>
<tr>
<td>UCCS</td>
<td>Uncooked corn starch</td>
</tr>
<tr>
<td>VAL</td>
<td>Voluntary Activation Level</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$</td>
<td>Maximal oxygen uptake</td>
</tr>
<tr>
<td>VO$_{2\text{peak}}$</td>
<td>Peak oxygen uptake</td>
</tr>
<tr>
<td>VT</td>
<td>Ventilatory threshold</td>
</tr>
<tr>
<td>W</td>
<td>Watts</td>
</tr>
<tr>
<td>W$_{\text{max}}$</td>
<td>Maximal power output</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine/serotonin</td>
</tr>
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Chapter 1  Introduction

Exercise-induced fatigue is characterised by sensations of tiredness and decrements in muscle performance and function [1]. Fatigue that is associated with alterations at or the distal of neuromuscular junction (NMJ) may be broadly categorised as peripheral fatigue [2], while changes within the central nervous system (CNS) that are associated with the onset of fatigue may broadly be classified as ‘central fatigue’. Peripheral fatigue is characterised by significant changes in metabolites, ions and electrolytes within both the intra-cellular and inter-cellular compartments of skeletal muscle [1, 3, 4]. Central fatigue on the other hand, is characterised by a progressive reduction in voluntary activation of the skeletal muscle during exercise [5]. Performing a maximal voluntary contraction (MVC) before and after exhaustive exercise provides a reliable measure to detect exercise-induced fatigue [6]. The contribution of central fatigue in turn, can then be assessed by systematically combining the MVC with an electrical stimulation technique known as twitch interpolation [7].

Despite significant advances in the field, considerable debate regarding the underlying mechanisms and origins of exercise-induced fatigue continues (i.e., [8-15]). The overarching aim of this dissertation is to assess and identify factors associated with the onset of fatigue during prolonged cycle-exercise, using techniques designed to assess both peripheral (i.e., metabolomics) and central (i.e., neuromuscular assessments) parameters of fatigue.
1.1 Specific Aims of the Studies

*Study one (Chapter 3): Changes in the plasma metabolome during and following constant cycling to exhaustion*

This chapter will adopt a targeted metabolomics approach, which will enable the simultaneous assessment of >100 metabolites prior to and at the point of fatigue-onset. This chapter is specifically focused on assessing metabolites associated with the ‘Central Fatigue Hypothesis’ [16]. This theory is of particular interest since it provides a possible feedback mechanism between metabolites associated with peripheral fatigue, and the decreased voluntary activation associated with central fatigue. Specifically, the theory proposes that an increase in free fatty acids displaces the amino acid tryptophan from its transporter albumin, and in so doing, increases the transport of tryptophan into the CNS. Tryptophan is the precursor to serotonin which has been long-recognised as playing a role in exercise-induced fatigue [1, 3, 16]. We hypothesise that the free fatty acids, tryptophan and 5-methoxy-3-indoleacetic acid (the end product of serotonin catabolism) will demonstrate significant increases with exercise and fatigue onset.

*Study two (Chapter 4): Neuromuscular fatigue following isoenergetic prolonged constant versus self-paced cycling exercise.***

The primary purpose of this study is to investigate the changes in neuromuscular fatigue following two modes of isoenergetic prolonged exercise at constant and self-paced cycling. Constant power time-to-exhaustion (TTE) protocols and self-paced time-trials (TT) are widely used to assess cycling performance and fatigue [1]. The validity of the TTE protocols has however been questioned on the basis that cyclists rarely maintain constant power output during competition [17]. Additionally, the TT has demonstrated higher repeatability and lower variability than compared to TTE trials [17] although this may be dependent on an appropriate pacing strategy during the TT [18]. We hypothesise that performance will be superior (higher power output; shorter time to completion) in the TT, and this will be associated with greater peripheral fatigue, but not central fatigue.
Study Three (Chapter 5): The effect of acute branched-chain amino acids (BCAA) supplementation during isoenergetic prolonged cycling exercise on cycling performance and neuromuscular fatigue.

The primary purpose of this study is to investigate the effects of acute branched-chain amino acids (BCAA) supplementation on prolonged cycling time trial (TT) performance and neuromuscular changes associated with the BCAA and TT performance. Supplementation with BCAA allows the manipulation of serotonin concentration in the CNS, since the BCAA compete with TRP for entry into the CNS. Specifically, the BCAA and TRP use the same transporter for entry into the CNS and will outcompete the TRP, therefore resulting in reduced CNS serotonin levels. In accordance with the Central Fatigue Hypothesis above therefore, this should result in delayed fatigue and improved performance. Supplementation with BCAA has to date been shown to either improve performance [19-21] or have no effect on performance [22, 23], and these contradicting results may be associated with large heterogeneity in methodology and small samples. We hypothesise that BCAA will improve performance during the TT, and be associated with reduced levels of central fatigue.

Study four (Chapter 6): Metabolic and performance responses to the ingestion of different carbohydrates during prolonged exercise.

The primary purpose of this study was to investigate the metabolic and performance effects of a mixed-carbohydrate (CHO; starch and sugars) product on prolonged endurance performance compared to a glucose control solution (GLU). Despite the intense research conducted on the role of CHO supplementation and performance, the benefits of ingesting CHO within the final hour of exercise is still unclear. Some studies have demonstrated an ergogenic benefit [24, 25] while others have shown no improvement [26-28], or even an impairment in endurance performance [29, 30]. Such impaired exercise performance has been attributed to the existence of rebound hypoglycemia, reduced fat oxidation and inhibition of lipolysis by elevated plasma insulin concentrations [31]. While the ingestion of complex CHO (or more slowly
digested CHO) has been explored as a potential ergogenic aid [32] due to the more prolonged nature of glucose release, the combination of complex CHO with simple CHO on prolonged exercise performance has not been explored. We hypothesised that a mixture of complex and simple CHO would result in significant improvements in TT performance as compared to ingestion of an isocaloric mixture of only simple CHO.

1.2 Significance of the Thesis

The mechanisms underpinning exercise-induced fatigue are varied, complex and incompletely understood. While significant advances have been made and much knowledge generated within this field, important questions remain. Much work in the field has focused on either a few metabolites implicated in peripheral fatigue or on neuromuscular assessment of central fatigue, but limited research exists which has attempted to bridge these fields in an attempt to answer the remaining questions. By adopting techniques which enable the assessment of the plasma metabolome and neuromuscular parameters associated with the onset of fatigue, we will attempt to, in part, bridge these disparate fields.
1.3 References


Chapter 2  Literature Review

Exercise-induced fatigue is characterised by sensations of tiredness and decrements in muscle performance and function [1]. The fatigue experienced during exercise is distinct from the general fatigue experienced by the estimated 7-45% of the population [2-6], because of its transient and replicable nature. A unique feature of exercise-induced fatigue is that both the perception and duration of fatigue may be manipulated by altering intrinsic components (i.e., exercise mode, exercise intensity, exercise duration) of the exercise bout. Despite our advanced understanding of exercise-induced fatigue, the mechanisms underpinning fatigue, and the interaction of these mechanisms in inducing fatigue, remain largely unknown and fiercely debated [7-24]; although it is generally understood to involve some complex interaction between central and peripheral factors [25, 26].

2.1 Fatigue

Early studies attempting to identify causes of fatigue commonly focused on peripheral factors [27]. These factors are associated with alterations at or below the neuromuscular junction (NMJ) [28] and are thought to be initiated within the muscle itself [29], or involve factors (i.e., neurotransmitters, membrane associated electrolyte imbalance) which may affect muscle function [30-32]. Fatigue induced by so-called peripheral factors is named “peripheral fatigue” and typically involves factors which are quantifiable via biochemical assessment of muscle or blood tissue.
2.1.1 Energetics of Peripheral Fatigue

As a consequence to the early identification of adenosine triphosphate (ATP) as the body’s energy currency in combination with the rapid depletion of these stores (reviewed in [33]), resulted in much of the early work in fatigue focusing on the depletion of ATP and mechanisms associated with repletion of these stores [27, 33-36]. The phosphagen (ATP-PCr) system does not require oxygen to replenish ATP and provides near-instantaneous energy to maintain ATP stores [37], but is limited in capacity (~5-7 seconds at high-intensity). Specifically, phosphocreatine (PCr) levels are significantly reduced in the first 5-6 seconds of maximal intensity exercise [38, 39] and this is more pronounced in type 2 muscle fibres [40-42]. While a clear temporal relationship between PCr and fatigue exists, the importance of PCr is further supported by the improvement in sprint performance and delay in the onset of fatigue during repeated sprint exercise [43, 44].

Muscle glycogen depletion has long been implicated in the onset of fatigue during exercise [35, 45, 46], and the ingestion of glucose unequivocally improves performance during prolonged (>90 minutes) exercise by reducing the rate of glycogen utilisation [47-49]. Indeed, the ratio of blood-borne glucose and glucosyl-moieties derived from glycogen as substrates for glycolysis are largely influenced by (i) the intensity of exercise, wherein higher intensity exercise relies more greatly on muscle glycogen [50, 51]; (ii) muscle fibre type, wherein type 2 fibres rely more heavily on muscle glycogen [50-52]; (iii) duration of exercise, wherein the contribution of muscle glycogen declines in parallel with a decrease in muscle glycogen availability [51]. While the stores of muscle glycogen are much more substantive than the PCr stores, muscle glycogen may be “depleted” (note that these stores and other energetic substrates do not reach below ~10 %) within a few minutes of intense exercise [51, 53-55].

Muscle glycolysis can also be supported by blood borne glucose and indeed the uptake of glucose may rise to 20- to 35-fold after 40-60 minutes of work [56]. Despite constituting a small store per se (~5 g total), blood glucose may contribute as much as 30-50 % of the total energetics used during prolonged exercise [57]. In order to maintain these demands and avoid hypoglycaemia, hepatic glucose output increases 2- to 3-fold during moderate intensity and hepatic gluconeogenesis.
concomitantly increases by 20-25% [58]. Irrespective of these additional processes, liver glycogen, as with muscle glycogen, is depleted during prolonged exercise [59] and at this stage, the onset of fatigue is inevitable [60]. This depletion is associated with “hitting the wall” which generally occurs around the 32 kilometres mark during a marathon [61], although this speculated “cause-and-effect” is based only on theory and temporal associations.

Importantly, mitochondrial respiration plays the dominant role during prolonged exercise, and as a result of the increased availability of pyruvate (along with other substrates, amino acids and free fatty acids (FFA), reduced form of nicotinamide adenine dinucleotide (NADH) and oxygen, muscle pyruvate is almost exclusively oxidised during prolonged exercise [1, 34]. Unsurprisingly, mitochondrial respiration and more broadly, muscle oxidative capacity is increased with training and may therefore explain at least part of the improved performance during prolonged exercise after a period of training [62-64]. Likewise, a hypoxic environment which is sufficient to induce hypoxemia, will reduce mitochondrial capacity as a result of the reduced availability of oxygen and results in reduced performance acutely [1, 65]. This will directly increase the accumulation of so-called “waste products” which the early researchers in the field of fatigue describe [66] and specifically “lactic acid” [67]. Lactate accumulation occurs when the rate of pyruvate formation exceeds the rate of pyruvate entry and utilisation in mitochondrial respiration and is associated with an elevated ratio of reduced form to oxidized form of nicotinamide adenine dinucleotide (NADH:NAD\(^+\)) and hydrogen ions (H\(^+\)) accumulation [68]. The temporal association of lactate accumulation and H\(^+\) formation led to the erroneous theory of “lactic acid” as being the H\(^+\) donor, however other mechanisms including rapid catabolism of ATP to adenosine diphosphate (ADP) contribute to the increased H\(^+\) concentration [69] and only lactate (salt) is formed, not lactic acid. The accumulation of H\(^+\) in excess of the buffering capacity of the tissue has detrimental effects on oxygen delivery [70], inhibition of glycolysis and glycogenolysis by inhibiting phosphofructokinase (PFK) [63] and displaces calcium ions (Ca\(^{2+}\)) from troponin, thus directly reducing the ability of the muscle to contract [71]. In concert with the increase in the concentration of hydrogen ions ([H\(^+\)]), there is an increase in the concentration of inorganic phosphate ([Pi]) associated with the increased rates of glycolysis and the
catabolism of ATP. The increase in \([P_i]\) deteriorates force capacity and is associated with fatigue [72, 73], possibly due to inorganic phosphate (\(P_i\)) inhibiting cross-bridge detachment and, along with \(H^+\), reducing myofibrillar \(Ca^{2+}\) sensitivity [74] and pH that forms acid phosphate, \(H_2PO_4\) [28].

2.1.2 Electrolytes and Ions in Peripheral fatigue

During intense exercise, there may be an increase in ammonia (\(NH_4^+\)) which is related to the deamination of adenosine monophosphate (AMP) to inosine monophosphate (IMP) by AMP deaminase [28, 34, 75]. The increase in \(NH_4^+\) inhibits pyruvate dehydrogenase and isocitrate dehydrogenase enzymes, thus, attenuating oxidative metabolism [76]. Ammonia therefore needs to be neutralised (i.e., conversion of pyruvate to alanine) and/or eliminated swiftly [77]. This occurs by the formation and subsequent excretion of urea via the urea cycle [34]. However, this cycle requires the liver and kidneys and therefore multiple steps including the transport of \(NH_4^+\) from the muscle in the form of alanine and glutamine are required prior to the elimination of \(NH_4^+\). Importantly, \(NH_4^+\) is also produced when the energetic contribution of amino acids is increased, such as during the latter stages of endurance events, wherein the deamination of amino acids is required for the oxidation of these compounds [34].

Excitation-contraction coupling (ECC) is the process linking the muscle contraction (mechanical) to the propagation of the action potential (AP; electrical signal) [78]. The AP has been the focus of many previous reviews (i.e., [79]) which have concluded that muscle fibre excitability is dependent on the AP threshold produced when sodium ions (\(Na^+\)) rushes into (influx) the fibre during depolarization of the membrane (in response to acetylcholine receptor activation) and potassium ions (\(K^+\)) subsequently rushes out (efflux) [80, 81]. When sodium influx occurs and the depolarization threshold is reached, the sodium-gated channel deactivates quickly (~1-2 milliseconds) followed by the activation of the potassium-gated channel [79]. Besides \(Na^+\) and \(K^+\), chloride ions (\(Cl^-\)) are also involved as a repolarizing agent during the repolarization phase [82]. These electrolytes have long been investigated as potential mediators of fatigue [30-32]. More recent studies on isolated muscles
of animals have confirmed an important role for the balance between intracellular and extracellular; concentration of sodium ions ([Na+]]) and concentration of potassium ions ([K+])), respectively, in the development of fatigue during high-frequency stimulation [83]. The influx of Na+ increases rapidly during exercise and is fibre-type specific leading to a more rapid rise in K+ efflux in the fast twitch fibres (i.e., extensor digitorum longus) than slow twitch muscles (i.e., soleus) and an associated increase in interstitial and plasma [K+] [83, 84]. The intensity of work plays an important role in this process, with the loss of K+ progressively increasing during intense exercise [85]. This leads to a reduced capacity for excitability due to the decrease of chemical gradient and slow inactivation of sodium channels [86]. Chloride ions plays an important role (along with K+) in re-establishing the membrane potential and enabling a subsequent AP; at rest, Cl− contributes nearly 80 % of this “inhibitory current” [79].

A critical component of the ECC is the Ca^{2+} release from the sarcoplasmic reticulum (SR) which occurs in response to the AP and the associated reuptake of Ca^{2+}, which has itself been the subject of much scrutiny relating to its role in muscle fatigue [87]. The rate of Ca^{2+} release is fibre-dependent, wherein fast-twitch fibres (i) release Ca^{2+} three times faster [88]; (ii) contain twice as many SR and 4- to 8-fold more calcium ATPase compared to slow-twitch muscle fibres [89, 90]. Exercise at moderate intensity can depress Ca^{2+} release by ~15-20 %, while intense activity results in ~35% depression in Ca^{2+} release [91-93]. Concurrent with these changes in Ca^{2+} release, intense muscular contraction increases [P_i] which has been proposed to enter the SR and bind with Ca^{2+}, leading to calcium phosphate (CaP_i) precipitation and reduction in available Ca^{2+} for SR release [40, 74, 78].
2.1.3 Central Fatigue

Alterations within the central nervous system (CNS) that are associated with the onset of fatigue are broadly classified as ‘central fatigue’. Central fatigue can be defined as a progressive reduction in voluntary activation of muscle during exercise [26]. Central fatigue may occur at various levels and for several reasons including: (i) loss of recruitment of high threshold motor units [94, 95]; (ii) reduced central drive [96]; (iii) blocked central conduction from demyelination or motor neuron dropout [97]; and (iv) increased negative feedback from muscle afferent types iii and iv sensory neurons [98]. As a consequence of the many proposed levels and reasons for central fatigue, the measurement of central fatigue is equally challenging.

2.1.3.1 Calculations for Central Fatigue Assessment

Maximal voluntary contraction (MVC) is considered the “gold standard” for measuring muscle force and fatigue, as it is highly reproducible in both non-fatigued and fatigued conditions [99]. By systematically combining MVC with electrical stimulation, the occurrence of central fatigue can be detected and quantified using burst superimposition and twitch interpolation techniques. In this case, the definition of central fatigue is specific to the progressive exercise-induced reduction in maximal voluntary activation of a muscle [100]. There have been a number of protocols which have been adopted using twitch interpolation, and there are distinct differences in these protocols especially the number of interpolated stimuli. Merton (1954) developed and introduced the twitch interpolation technique which utilized the single twitch stimuli. This method has been widely adopted by other researchers. Subsequently, train stimulations have been adopted using a burst superimposition technique as it produces a larger plateau torque, is more sensitive [101] and valid [102]. However, these repeated stimulations can be painful [103, 104] and unbearable to participants [105].

It has been suggested that doublet, triplet and quadruplet stimulations are more reliable compared to single twitch stimulations as it reduces variability [106] and is less painful compared to train stimulation protocols. Increasing the number of
interpolated stimuli, increases the superimposed torque [107, 108], signal-to-noise ratio [109], and thus, makes it more readily detected [110]. The most often used calculations to determine central fatigue based on burst superimposition technique and twitch interpolation technique are Central Activation Ratio (CAR) and Voluntary Activation Level (VAL), respectively.

2.1.3.1.1 Central Activation Ratio (CAR)

CAR is calculated using the following equation:

\[ CAR = \frac{MVC}{MVC + \text{Superimposed stimulation amplitude}} \]

Thus, if CAR equals 1.0, it indicates a full activation of the stimulated muscle [101], while any extra torque resulting from the supramaximal electrical stimulation (i.e., a CAR value of less than 1.0) indicates a failure of full activation by the brain [111]. This method has been questioned as it compares superimposed torque with voluntary torque, and additional torque may possibly occur from synergistic muscles that are not recruited during electrical stimulation [112].

2.1.3.1.2 Voluntary Activation Level (VAL)

VAL has been reported to be more sensitive than the CAR [113] and can be calculated as follows:

\[ VAL = \left[ 1 - \left( \frac{\text{superimposed stimulation amplitude}}{\text{resting stimulation amplitude}} \right) \right] \times 100 \]

Further studies showed that using the potentiated doublet as a reference rather than the resting doublet was more valid and reproducible [99] since it can be assumed that the potentiated doublet (occurring within approximately 5 seconds after the MVC) and the MVC will be equally potentiated. For cases where superimposed stimulation is being elicited slightly before or after MVC, a correction is applied, as superimposed amplitude tends to increase at torques which are below maximal [114]. This relationship has been proposed to be linear [115] although this is not universally accepted (reviewed in [116]). Accordingly, a correction has been
proposed to this technique [114] and appears to be more valid and reliable [99], such that VAL is calculated as:

\[
= [1 – (\text{superimposed stimulation amplitude} \times \text{voluntary torque level just before the superimposed doublet/maximal voluntary torque})/\text{potentiated doublet amplitude}] \times 100
\]

2.1.3.2 Central Fatigue Assessment in Dynamic Exercise

Classically, studies on exercise-induced fatigue of quadriceps muscles were done on isokinetic dynamometers [96, 117-120] or modified chairs [102, 112, 121-123] where the assessments of fatigue were conducted. Additionally, the mode of exercise to induce fatigue were typically either isometric [99, 119, 120, 124] and/or isokinetic [125-130] muscle contractions. Recently, twitch interpolation technique has been used extensively to assess fatigue induced by dynamic exercise such as cycling [96, 117, 118, 131, 132] and running [133-138] since they represent greater ecological validity to sports and exercise.

2.1.3.2.1 Before and After Exercise

Neuromuscular changes can also be evaluated by assessing fatigue before and after exercise. Instead of performing exercise to exhaustion in the laboratory, Millet et al. (2002) preferred the use of an actual race-event, because each subject would be highly motivated to perform during the race. Ultra-marathon was chosen to induce the exercise-associated fatigue, because limited studies had focused on the neuromuscular changes after an ultra-endurance event, wherein significant central and peripheral fatigue are expected. In this study, neuromuscular fatigue of the knee extensor (KE) and plantar flexor (PF) muscles were measured one week before and immediately after the race. The femoral and posterior tibial nerves were stimulated and the maximal voluntary activation was estimated by using the twitch interpolation technique (VAL; resting twitch). There was 30% reduction in MVC torque and 28% reduction in voluntary activation after the race, demonstrating significant fatigue and this fatigue was attributed to central processes (i.e., central
fatigue) [135]. In a follow-up study, Millet et al. (2003) assessed central fatigue contributions during intermediate-duration running exercise (30-km running race). Single twitch and 0.5 seconds’ tetanus (20- and 80-Hz) were applied to the femoral nerve and handgrip force measured to assess supra-spinal fatigue. There was a 23.5 % reduction in MVC torque and a 7.5 % reduction in VAL (potentiated twitch) indicating that the reduction in MVC was due to central and peripheral fatigue processes after this type of event.

The same research group used a similar tetanus stimulation (20- and 80-Hz) protocol [136] to study road cyclists following a 140-km race [105]. The 140-km race took an average of 278 minutes to complete and the neuromuscular assessments completed between 15 and 30 minutes after the race. The MVC loss was small (9 %) but significant compared to the two aforementioned studies and there was a lack of central fatigue with no significant changes in the VAL (using both twitch and train stimulations). Given that the neuromuscular fatigue assessment was completed between 15 and 30 minutes after the race, the effects of metabolic (peripheral) fatigue were expected to be low. However, the twitch parameters of the muscle were also unchanged as evidenced by no significant changes in high-frequency tetanic forces. Importantly, using evoked tetanus on relaxed muscles is not suitable as they were too painful which only resulted in four participants agreeing and completing this method, thus it is not statistically comparable to MVC and maximal voluntary activation [105].

High-intensity exercise is associated with significant fatigue [94, 139] but can also be associated with higher eccentric loads leading to muscle damage [140]. To investigate the behaviour of fatigued muscles after a high-intensity exercise but without significant eccentric loads, an intermittent high-intensity uphill running protocol was designed [134]. The protocol consisted of 10 bouts of running at 120 % of maximal aerobic velocity (MAV) with an 18 % gradient on a treadmill using a 1:2 minutes of work to rest ratio. This protocol produced expected metabolic changes with the blood lactate concentration reaching 18.4±3.6 mmol/L at the end of the exercise. However, there were no significant changes in VAL but greatly altered twitch contractile properties and the presence of low-frequency fatigue. This
indicated that fatigue following this type of exercise was mainly due to ECC failure and the contributions from central factors were insignificant [134].

The effect of constant versus variable-intensity endurance exercise on neuromuscular fatigue in cycling exercise is an important consideration and has been studied by Lepers and colleagues. This effect is important given that a race, even when conducted on flat terrain, will not result in constant power output but rather will result in a J-shaped power curve [141]. To study this, neuromuscular assessments were performed before and after each trial using a doublet/paired stimulation instead of tetanus stimulations as the use of superimposed paired stimulations at 100-Hz has been shown to be as sensitive as quintuplets for detecting muscle inactivation [112]. The first study recruited male triathletes who were asked to cycle for 30 minutes at self-selected cadence to achieve 75% of maximal aerobic power (MAP), one at a constant-power output and one with alternating power output. There were significant reductions in MVC torque and VAL, but no significant difference in neuromuscular fatigue between trials which was attributed to the low-variations of power output in alternating power output trial [118]. In a follow-up study using 10 well-trained cyclists completing a similar protocol (33 minutes corresponding to 70% MAP) with the exception of greater variance in the cycling intensities during variable power output trial. The MVC torque and VAL reduced significantly in variable intensity trial compared to constant intensity trial. Unfortunately, by using the twitch interpolation technique, the study was unable to verify if the central fatigue was produced from supraspinal and/or spinal mechanisms [26]. This study concluded that high-variations of power output including efforts above the MAP altered neuromuscular fatigue and cardiovascular responses compared to constant power cycling exercise [132].

While laboratory-based tasks are generally considered to have reduced ecological validity, cycling time-trials (TT) are generally considered to be more closely aligned with race characteristics and result in similar power characteristics to races when compared to constant-power outputs [1]. As expected, a 4-km TT (short high-intensity) showed a higher contribution of peripheral fatigue while in 20- and 40-km TT (long low-intensity), a higher degree of central fatigue contributed to the exercise-induced fatigue [142]. Central fatigue was measured with twitch
interpolation technique (VAL) which indicated a reduction in firing frequency (-11 %, -10 %, -7 % for 20-, 40- and 4-km TT, respectively) and transcranial magnetic stimulation to assess corticospinal function (-12 %, -10 %, -6 % for 20-, 40- and 4-km TT, respectively). The overall results indicated that the duration and distance of an exercise protocol produces different neuromuscular alterations.

Finally, while exercise characteristics may alter neuromuscular activation, environmental factors can clearly also play a role. Exposure to heat is a common phenomenon experienced by endurance athletes and is associated with fatigue and hyperthermia [1]. Periard, Caillaud and Thompson (2011) conducted a study to compare central and peripheral fatigue during exercise with and without hyperthermia. To isolate the effect of the exercise, eight male participants immersed their body in a warm water bath until the rectal temperature reached 39.5 °C. On separate days, the participants performed cycling time-to-exhaustion (TTE) at 60 % maximal oxygen and neuromuscular assessments were conducted before and after the hyperthermia inducing phase. It was demonstrated that VAL reduced similarly in both trials but the force production showed a greater decrease in the exercise protocol with hyperthermia and was associated with an increase in cardiovascular strain. However, in this study, the tetanus stimulation was delivered to the muscle directly instead of femoral nerve stimulation [143] and the use of electrical muscle stimulation may lead to partial or different spatial recruitment [123, 144, 145]. The reason is that the electrical muscle stimulation uses surface electrodes placed over the targeted muscle to directly depolarise muscle terminal axonal branches while electrical nerve stimulation directly stimulates nerve trunk of targeted muscle [146].

2.1.3.2.2 During Exercise

Neuromuscular fatigue assessment during exercising requires the participants to stop cycling for specific time intervals in order to conduct the neuromuscular assessment [96]. Nine endurance-trained cyclist or triathletes cycled for 5 hours at 55 % of MAP and neuromuscular assessments were conducted before, each hour during cycling, immediately at the end of cycling and 30 minutes post-exercise. The neuromuscular assessment took about 10 minutes to complete. During the 5 hours
of prolonged cycling exercise, ECC properties were altered as early as the first hour of cycling, as reflected by significant reductions in MVC. However, the muscle excitability was reduced only after 4 hours of cycling as indicated through a significantly lower M-wave peak compared to the resting condition. The VAL also showed a significant fall in the final hour of cycling phase reflecting the decrease in neural drive and possibly a result of the accumulation of brain serotonin and spinal or supraspinal mechanisms [96]. Similar to the aforementioned study, Place, Lepers, Deley and Millet (2004) used running to assess exercise-induced fatigue and evoked tetanic contractions (20-Hz, 80-Hz) together with single twitch and doublet stimulations. In Place et al. (2004)’s study, MVC loss was highly correlated with VAL, and similar to Lepers et al. (2002), significant reductions occurred after 4 hours of exercise. Potentiation effects occurred after exercise, however, tetanic stimulations at 80-Hz were not affected, suggesting that the MVC loss was from central activation and/or muscle excitability and not at the cross bridge-level [147].

### 2.1.3.2.3 Recovery

Presland, Dowson, and Cairns (2005) have applied MVC and tetanus interpolation technique to quantify muscle fatigue and the occurrence of central fatigue, respectively. Neuromuscular fatigue assessments were performed immediately after and during recovery following prolonged constant cycling TTE at 70 % of peak oxygen uptake (VO$_{2\text{peak}}$) exercise every 10 minutes, for up to 30 minutes. The participants were healthy men and physically active but not well-trained. The MVC torque declined about 44-82 % at post-exercise compared to the resting value, remained unchanged at 10 minutes post-exercise, and only partially recovered after 30 minutes while central fatigue persisted [148]. These findings suggested that the total fatigue processes persisted for the 30 minutes, despite partial recovery of MVC, but the central fatigue had not changed. This study used 3 methods to quantify central fatigue with varying results; tetanus interpolation technique (33 %), the relative decline of MVC and peak tetanic force (54 %) and extrapolating linear regression line of central fatigue data (58 %). Peripheral fatigue was demonstrated by the decrement in peak stimulated MVC torque, which fell by about 20 %.
Interestingly, exhaustion was closely linked with subjective ratings of perceived exertion (RPE).

Another study investigated the effect of altering the recovery strategy from exercise on fatigue characteristics. In this study, the investigators used an intermittent high-intensity exercise as the mode of fatiguing exercise before applying recovery interventions to the neuromuscular function which were passive (remain seated for 20 minutes), active (running at 50 % MAV) and low-frequency electromyostimulation on both lower limbs. Participants performed an all-out running test (running TTE at 90 % MAV), to see if any interventions would delay fatigue and improve subsequent performance. There were no significant differences among the three recovery interventions. Surprisingly, MVC torque 45 minutes after exercise (after applying the recovery interventions) was lower compared to immediately after exercise and the pattern was not associated with the changes in VAL, which may be due to an incomplete restoration of ECC [133]. These results (lack of effect of recovery strategies) may have arisen since this type of exercise is likely to be associated with high eccentric loads and may therefore have induced significant eccentric-damage; therefore, the recovery mode is unlikely to have an immediate effect and the MVC remained depressed for the whole recovery duration.

2.1.3.3 Central Fatigue Hypothesis

Previously, Eric Newsholme and colleagues proposed the ‘Central Fatigue Hypothesis’ [149], which has been at the core of a large body of the fatigue-related research literature. The authors suggested that an increased serotonergic activity in the CNS, specifically 5-hydroxytryptamine (5-HT or serotonin), during physical activity reduces exercise capacity, consequently, leading to a decrement in performance [149]. Serotonin (5-HT) is well-known for its effects on sleepiness, mood, arousal and lethargy [150] but it has been shown that an increased concentration of 5-HT in the brain during prolonged exercise is associated with fatigue [151] in both rats [152, 153] and human studies [154, 155]; although the latter is much more complicated to study and requires sampling of cerebrospinal fluid (CSF).
The production of 5-HT is directly related to the concentration of its amino acid precursor, tryptophan (TRP). When free tryptophan (f-TRP) is transported into the CNS through the blood-brain barrier (BBB) via large neutral amino acid (LNAA) transporter [156], this may result in an increased production of 5-HT following two reactions catalysed by TRP hydroxylase and aromatic amino acid carboxylase respectively [157]. While most TRP circulates in the plasma lightly bound to albumin, it is the f-TRP which can bind to the LNAA for entry into the CNS [150]. The increased concentration of FFA during prolonged exercise however, may displace TRP from albumin, resulting in a parallel increase in f-TRP in plasma [158] as shown in Figure 2.1. Therefore, with progressive increase of FFA contribution to exercise energetics (i.e., latter stages of endurance exercise; ~30-40 minutes) there is an increased ratio of f-TRP:BCAA which allows for more f-TRP to be transported across the BBB, resulting in potentially increased synthesis of 5-HT (Figure 2.1) [159].

Figure 2.1 Proposed mechanisms of Central Fatigue Hypothesis during prolonged exercise.
Serotonin-containing neurons are widely distributed throughout the brain. The enzyme TRP hydroxylase is not saturated by its substrate TRP under normal resting conditions [160], the increase of TRP centrally therefore, leads to an increase in both the synthesis and release of 5-HT which has been shown in both animal [161-163] and human [164] studies. Although, under non-homeostatic conditions such as exercise the contribution of brain 5-HT to exercise-induced fatigue is complicated, and can only be directly assessed in animal studies which has been elegantly reviewed by Meeusen and De Meirleir (1995). Catabolism of 5-HT occurs via monoamine oxidase (MAO) and aldehyde dehydrogenase and results in the formation of 5-hydroxyindoleacetic acid (5-HIAA), which is ultimately excreted in the urine [165]. It is the ratio of f-TRP and 5-HIAA which may be used to assess 5-HT synthesis and catabolism, respectively, however, accurate measures require CSF and or direct measurement of the neurons within the brain. However, since total TRP, f-TRP and 5-HIAA are also present in plasma, the measurements are typically conducted in humans although their validity may be questioned [164] and the biochemical processing of f-TRP to 5-HT and other products is not exclusive to neurons nor specific to central processes.

2.1.3.4 Summary

Maximal voluntary contraction is considered the “gold standard” in measuring muscle fatigue. The burst superimposition technique and twitch interpolation technique are the most widespread techniques used to quantify central fatigue with the two most commonly used formula being the CAR and VAL, respectively. VAL has been reported to be more reliable compared to CAR. Neuromuscular fatigue assessment has traditionally been conducted during isometric exercise, but has increasingly been applied to assess neuromuscular changes associated with dynamic exercise (especially cycling and running). Additionally, during post-exercise recovery the assessment has been done at multiple time points starting immediately after the termination of exercise. Although important to determine, high eccentric loads and the associated muscle damage may compromise interpretation of fatigue-related data, since the techniques used to assess central and peripheral fatigue cannot always tease out the effect of damage. Therefore,
comparisons between exercises associated with high- and low-eccentric loads need to be carefully considered.

It is apparent that a greater level of fatigue is demonstrated in athletes following race conditions when compared to laboratory conditions, although this may be related to the type of exercise protocol chosen (i.e., constant-power versus TT). For instance, while only a few studies have focused on differences in the neuromuscular characteristics associated with constant-power output versus alternating-power output, it appears that when matched for time and work, small variances in the power output fail to show significant differences in fatigue but when the variance in power output is increased (i.e., greater separation between high-power loads and low-power loads) then the fatigue characteristics between modes vary. The use of TT in laboratory-based testing may therefore be superior, as they more closely resemble the power characteristics of races than constant-power tests and externally applied variations in power (i.e., pre-determined changes in power).

Finally, the idea of a peripheral “feedback” loop in fatigue which exists to protect muscle integrity is generally acknowledged [18, 166, 167] although not by all [17]. The hypothesis posited by Newsholme et al is therefore attractive, since it integrates metabolic signatures from the periphery, with the down-regulation in central drive experienced with fatigue. It is noteworthy that much of the literature in fatigue is focussed on either peripheral (metabolic) or central (using a variety of stimulation techniques) mechanisms, with the longer exercise duration being associated with a higher contribution of central factors [96] but there is limited research which has explored the combination of these factors despite the well acknowledged multi-factorial nature of fatigue.
2.2 Nutritional Manipulation

2.2.1 Branched-chain Amino Acids (BCAA) and Tryptophan (TRP)

The central fatigue theory is based on the idea that increased concentration of 5-HT in the CNS and specifically the brain is not only associated with fatigue, but indeed the cause of the fatigue [151-155]. In order to assess this theory, a number of research studies have attempted to manipulate the 5-HT concentration in the CNS using amino acid supplementations. Supplementation of BCAA in particular, leads to a reduced ratio in f-TRP to BCAA and should theoretically reduce the available f-TRP for conversion to 5-HT. This is because the rate of f-TRP entry into the brain is dependent on both the concentration of f-TRP as well as the concentration of BCAA which competes with f-TRP for entry into the brain [159] as shown in Figure 2.2. A number of studies shown improved performance during prolonged exercise with BCAA supplementation [168-170], while several studies have shown no effect of BCAA consumption on performance during prolonged bouts of exercise [171, 172]. The reason for these discrepancies are unknown, although differences in studies will be briefly discussed with a focus on studies which identified no significant difference.
The landmark study on BCAA supplementation during exercise was conducted in the early 1990s, using a cross country (30-km) and full-marathon (42.2-km) running event. The primary finding was that after dichotomising runners into a slow- and fast-group, BCAA consumption appeared to be effective for the “slower” runners but not the faster runners [159]. Since runners were allowed to consume beverages including carbohydrate (CHO) beverages *ad-libitum* during the race, the findings of the study have been questioned [172]. Subsequently, van Hall et al. (1995) mixed amino acids into the CHO beverages, to avoid the effect of different quantities of CHO on fatigue and replicated the aforementioned study. This study was conducted under laboratory conditions; TTE using 4 types of supplements consisting of 6 % sucrose as a control and three mixtures comprising 6 % sucrose mixed with i) 3 g/L of TRP, or; ii) 6 g/L of BCAA (low BCAA), or; iii) 18 g/L of BCAA (high-BCAA). Despite each supplement increasing the concentration of respective plasma concentration of amino acids, there were no significant differences in TTE at 70-75 % of maximal power output ($W_{\text{max}}$) between trials. Importantly, the BCAA supplementation was estimated to reduce TRP uptake (measured by using kinetic parameters of transport of human brain capillaries) by ~10 % (with no difference between doses) while TRP ingestion increased uptake by 7- to 20-fold.

**Figure 2.2** Proposed mechanisms of nutritional manipulation when BCAA is consumed, elevated plasma BCAA concentration which competes with f-TRP for entry into the brain.
However, as previously mentioned, cycling TTE (open-loop) involves numerous motivational factors and does not represent the nature of a competition. A closed-loop experimental design, where participants are set targets (i.e., assigned distance covered, work performed, calories burned) has been shown to be more reproducible compared to open-loop design such as cycling TTE [1]. The closed-loop design enables participants to plan and control the power required to complete the task [173], while open-loop designs may be confounded by psychological factors such as boredom and motivation [1]. A closed-loop study design has been used to assess efficacy of BCAA on exercise performance by Blomstrand et al. (1997). Eight endurance-trained cyclists were required to exercise for 60 minutes, at approximately 70% of their maximal oxygen consumption (VO$_{2\text{max}}$), followed by maximal work capacity for an additional 20 minutes (primary outcome measure). Participants consumed 150-200 ml of liquid either with BCAA (7 g/L) or placebo (PLA) every 15 minutes throughout the first 60 minutes-bout. While RPE was lower in the BCAA group compared to the PLA during sustained exercise, there was no significant difference in amount work done during the last 20 minutes final effort [174]. As outlined earlier however, the contribution of central fatigue to total fatigue increases with duration, therefore a 20 minutes effort is unlikely to have triggered significant central fatigue and may have explained the lack of effect of BCAA supplementation on exercise performance. Two additional studies assessing BCAA supplementation in longer duration events have been assessed; one study in a 100-km cycling TT [175] and one study during a 100-km ultra-marathon [176]. Despite numerous methodological differences, there were no benefits of the BCAA supplements on performance in either study. It is noteworthy that the participants in these studies consumed BCAA in tablet form before and during exercise and were allowed an ad-libitum access to food and fluids at the aid stations. Furthermore, while the BCAA tablets comprised mainly BCAA (40 % BCAA), the tablets contained all amino acids including TRP (~2 % of amino acids) [176].

To remove the confounding effects of variable gastric uptake rates, BCAA have also been infused during incremental exercise in individuals who previously completed a glycogen depleting bout of exercise [177]. Although higher blood lactate concentration was demonstrated at maximal power output during the BCAA
condition, there were no significant differences in performance during the BCAA infusion compared to control condition.

The effect of longer-term feeding (six weeks) of BCAA on exercise performance has also been assessed in Wistar rats using three different types of diet (n=12 per group): control diet (C), a diet supplemented with 3.57% of BCAA (S1), and 4.76% of BCAA (S2) for six weeks. Results showed that TTE was significantly longer (37%) in the S1 group compared to C group, while the S2 group showed a significantly shorter TTE compared to other two groups. This study highlighted the importance of dose, and concluded that the chronic diet with a high BCAA dose caused hyperammonaemia and this was theorised as being the main factor leading to early fatigue during prolonged exercise in the high dose group [178].

Acute supplementation of BCAA before and during exercise has also been assessed during warm conditions and under conditions where individuals are glycogen depleted [170, 179, 180]. The rationale for this assessments is based on the idea that these conditions increase reliance and plasma concentration of FFA [158, 181], which are known to compete with TRP for binding sites on albumin. Hence, when FFA increases in the plasma, f-TRP concentrations increase relative to total TRP. Consumption of BCAA supplements were found to improve exercise performance when exercise was initiated in a glycogen depleted state, while concurrently enhancing lipid oxidation and reducing respiratory exchange ratio (RER) [179]. Moreover, the original study conducted to investigate the effect of BCAA administration on fatigue during a TTE exercise bout (40% VO_{2peak}) following heat exposure (n=7 males, 6 females; passive heat: 34.4±1.8 °C, relative humidity: 39±14%; duration = 2 hours) found that the consumption of 5 mL/kg body weight of beverages containing either 5.88 g/L of BCAA (54% leucine, 19% isoleucine, 27% valine) or 5.88 g/L of polydextrose as PLA every hour prior to exercise and every half an hour while cycling improved (14±5%) exercise TTE [170]. However, there was no effect on RPE scores, plasma FFA or NH\textsubscript{4}\textsuperscript{+} concentration in the BCAA trial. However, these results could not be replicated in a subsequent study assessing the combined effects of glycogen depletion and exercise performed in a warm environment [180]. The TTE trial was at a slightly higher workload (50% VO_{2peak})
and interestingly, NH₄⁺ concentrations were significantly elevated in the BCAA trial under these conditions.

An alternative approach to assessing the role of f-TRP on exercise performance is to assess the role of f-TRP depletion which will equally result in a reduction in the BBB crossing of f-TRP, and should result in a reduction in the serotonergic activity. A comparison between an amino acid load (CON) containing alanine (5.5 g), arginine (4.9 g), cysteine (2.7 g), glycine (3.2 g), histidine (3.2 g), isoleucine (8.0 g), leucine (13.5 g), lysine monohydrochloride (11.0 g), methionine (3.0 g), phenylalanine (5.7 g), proline (12.2 g), serine (6.9 g), threonine (6.5 g), tyrosine (6.9), valine (8.9 g) and trp (2.3 g) compared with an alternate identical load without TRP (TD) has been investigated in cycling TTE at 55 % VO₂peak in a warm environment; 30.1±0.5 °C, 30±7 % of relative humidity [182]. In response to the supplements, the ratio of f-TRP to BCAA the day prior, pre-exercise and post-exercise were all lower in TD compared to CON trial, but there was no significant difference in TTE between these two trials. This suggests that the availability of TRP as a metabolic precursor for serotonergic activity was not a significant factor that led to fatigue during prolonged exercise in a warm environment [182].

2.2.2 Summary

The mechanisms of fatigue are complex due to the presence of both central and peripheral factors. Changes which occur at the proximal area to the NMJ are associated with central fatigue while peripheral fatigue is related to changes at the distal area to the NMJ. The ‘Central Fatigue Hypothesis’ highlighted that the accumulation of 5-HT in the brain during prolonged exercise is associated with central fatigue. Numerous studies have been conducted based on this theory by focusing on nutritional manipulation through the supplementation of BCAA to delay the accumulation of 5-HT in the brain in an attempt to delay the onset of fatigue. Plausible reasons for the level of inconsistency in the aforementioned findings may be due to methodological variations, differences in BCAA concentrations in the supplemented beverages, forms of supplemented BCAA, supplement administration protocols, frequency of supplement provision and
differences in exercise protocols particularly with respect to the environmental temperature and the availability of both exogenous CHO and endogenous CHO (liver and muscle glycogen stores).

The adopted exercise protocols to assess fatigue may also contribute to the inconsistent findings. In a study investigating various protocols, including i) cycling TTE at 75 % \( W_{\text{max}} \); ii) constant cycling at 70 % \( W_{\text{max}} \) for 45 minutes followed by 15 minutes TT (mixed protocol), and iii) 60 minutes TT; the coefficient of variation (CV) was shown to be highest in TTE trial (26.6 %), the lowest in TT protocol (3.35 %) while mixed protocol was as reliable as the TT (3.49 %) [183]. The reliability of mixed protocols was also confirmed in recreationally active individuals (but non-cyclists) [184]. To date, no study has assessed metabolic responses and quantified central fatigue at the point of exhaustion between isoenergetic closed-loop and open-loop cycling performance and the effect of BCAA supplementation in isoenergetic closed-loop cycling performance.

2.2.3 Carbohydrate Supplementation

Hypoglycemia and glycogen depletion have long been associated with fatigue [45] and decrements in performance during prolonged exercise [35, 46, 185]. Accordingly, the ingestion of CHO during prolonged exercise has been shown to delay fatigue and enhance work output [47, 48, 60, 186] while post-exercise CHO ingestion replenishes muscle glycogen and ensures the stores are replete prior to undertaking exercise [187-190] and may indeed lead to glycogen supercompensation [191]. These strategies may be separated into pre-exercise, during exercise and post-exercise CHO strategies. There are some excellent reviews discussing the role of CHO ingestion on exercise performance [49, 185, 192] and while it appears that CHO consumption during exercise is most efficacious (i.e., least controversial regarding improved performance with CHO consumption during exercise), this review will focus on the consumption of CHO in the pre-exercise period.
Carbohydrate Supplementation Before Exercise

The performance benefits of ingesting CHO within an hour prior to the onset of exercise remain unclear. Some studies have demonstrated an ergogenic benefit [193, 194] while others have shown no improvement [195-197], or even an impairment in endurance performance [198, 199]. Such impaired exercise performance has been attributed to the existence of rebound hypoglycemia, reduced fat oxidation and inhibition of lipolysis by elevated plasma insulin concentrations [192]. However, well-trained cyclists who developed rebound hypoglycaemia were shown [200] to have no difference in power output when compared to cyclists who did not develop rebound hypoglycaemia. Participants (n=20) in this study ingested 75 g of glucose 45 minutes prior to performing 20 minutes of constant-intensity cycling at 40 % VO$_{2\max}$ followed by a TT to complete work output equal to 80 % $W_{\max}$ for 40 minutes. Moreover, consuming 75 g of glucose 45 minutes prior to exercising at different intensities (either at 40 %, 65 % or 80 % or at maximal power output) showed there were no significant differences in plasma glucose and insulin responses between conditions [201]. Specifically, glucose concentrations decreased in the first 5 minutes of exercise, the glucose concentrations stabilised throughout the remainder of the exercise trials resulting a non-significant between-condition difference. Moreover, when the timing of glucose ingestion was altered (15 minutes pre-; 45 minutes pre-; 75 minutes pre-exercise) relative to the exercise bout and performance of a TT [202], there were no significant performance differences in the ~42 minutes TT. The effects of glucose load have also been explored by this group, using 0 g (control), 25 g, 75 g or 200 g consumed 45 minutes prior to an exercise bout consisting of 20 minutes of steady state exercise followed by a TT (pre-determined workload based on $W_{\max}$; 0.8 x $W_{\max}$ x 2400 seconds) lasting approximately 43 minutes. Interestingly, the overall plasma glucose responses were similar across the glucose conditions, wherein hypoglycaemia was attained in the first 20 minutes of exercise in 6 participants, but this did not affect exercise performance [196]. Finally, since different types of CHO produce different types of plasma glucose and insulin responses due to their different glycaemic index/load (GI), the effect of different CHO types have also been examined by this group. Galactose and trehalose for example have lower GI, and these saccharides were compared to glucose. As expected, these saccharides showed more stable changes
in plasma glucose and insulin response with only one subject demonstrating hypoglycaemia earlier in the exercise bout, while 4 subjects had hypoglycaemia following the glucose supplementation [197]. Despite the differences in insulin and glucose concentrations however, there were no performance based differences across conditions, which is in agreement with a previous study [203].

Based on these well designed and systematic assessments of CHO ingestion prior to exercise, it can be concluded that i) rebound hypoglycaemia does occur when glucose is ingested 15 minutes to 45 minutes prior to exercise, ii) the hypoglycaemia is independent of dose, iii) CHO type, and specifically GI alters the risk of hypoglycaemia such that lower GI foods reduce the risk of hypoglycaemia, iv) glycaemia however, appears to stabilise during exercise of at least moderate intensity and does not affect TT performance [192]. It is however important to note that i) the hypoglycaemia observed in the initial 20-minutes steady state exercise condition returned to normoglycaemia prior to TT performance; ii) a TT lasting less than 60 minutes may not be sufficient in duration to realise the ergogenic effect of CHO supplementation. For these reasons, alternate sources of CHO supplements have been investigated and continue to be of interest with a particular focus on slow digesting CHO sources in longer-duration events.

2.2.3.2 Corn Starch

2.2.3.2.1 Glucose and Insulin Response at Rest

Carbon storage in plants is mainly in the form of starch which exist as semi-crystalline granules composed of the two polymers of glucose known as amylose and amylopectin [204]. Amylose comprises straight chains connected by $\alpha$1-4 bonds while amylopectin has a more branching structure, connected by $\alpha$1-4 bonds and branches of $\alpha$1-6 bonds approximately every 24-30 glucose residues [205]. The digestibility of starch therefore is predominantly impacted by the percentage of amylose and amylopectin where a higher percentage of amylose content results in reduced plasma glucose and insulin peak in the first hour after consumption and a more prolonged or sustained release [206]. Typically, the ratio of amylose:amylopectin is usually about 20:80 [206] but in the most common
commercially available corn starch mixtures, the ratio of amylose:amylopectin is 70:30 [207]. This ratio in turn, will alter the plasma glucose and insulin response, with less fluctuation in peak glucose levels (reduced glycaemic variability) observed in meals with higher amylose compared to amylopectin content, although the total glucose area under the curve (AUC) which is reported as mean glucose levels, were not significantly different between the two meals [207]. Specifically, the initial (first 30 minutes) plasma glucose and insulin responses were significantly lower in the meals with higher amylose content. Uncooked corn starch (UCCS) is a particularly slow-digested starch, shows a blunted response in both plasma glucose and insulin when compared to glucose consumption in young men [208]. Remarkably, the incremental AUC of plasma glucose and insulin produced in UCCS remain stable up to 120 minutes and has been shown to maintain normoglycaemia in individuals with glycogen storage disease for up to 9 hours.

2.2.3.2.2 Supplementation of Corn Starch Before Exercise

While supplementation with starchy foods prior to exercise has not been as extensively studied as other forms of CHO [49, 192], the experimentation with these slower-absorbed CHO as a pre-exercise supplement has been considered for a long-time due to its slow rate of absorption [209]. In one of the earlier assessments of corn starch as a pre-exercise supplement, the corn starch was consumed with 400 ml of water and compared with glucose and fructose supplements [210]. The CHO supplements were enriched with Carbon-13 ($^{13}$C) to assess oxidation rates. Fructose oxidation was significantly lower (54±6 %) compared to corn starch (67±9 %) and glucose (73±7 %) supplementation, but there was no significant difference between corn starch and glucose supplementation group. Plasma glucose and insulin response were also similar in glucose and corn starch group throughout the 60 minutes pre-exercise period and during the prolonged (120 minutes at 60 % $\text{VO}_{2\text{max}}$) exercise bout. Thus, the researchers concluded that corn starch did not offer an additional advantage over glucose as a pre-exercise meal [210]. However, the corn starch adopted within that study had an amylose percentage of ~8 %. It has been suggested that in order to see the beneficial effect of complex CHO on the
postprandial insulinaemic response, the tested CHO should contain at least 30 % of amylose [211].

More recently, techniques have emerged which allow the modification of starches (e.g. hydrothermally or acid/alcohol modified starches) and enables the manipulation of gastric-emptying rates and CHO availability independent of the amylose:amylopectin ratio [212]. The metabolic responses to supplementation with acid/alcohol-modified starches (MAMS), unmodified corn starches (UAMS), dextrose (DEX) and PLA were assessed by Johannsen and Sharp (2007). Endurance-trained cyclists consumed 1 g/kg body weight of supplement and cycled at 66.4 % VO$_{2peak}$ for 2 hours. Plasma glucose and insulin in DEX spiked significantly higher compared to other 3 trials and CHO oxidation rate was high in DEX and MAMS but only MAMS sustained the CHO oxidation for the 120 minutes, while DEX sustained the oxidation for 90 minutes before falling to the same extent as PLA at 120 minutes [213].

Unmodified amylo maize-7 (AMY-7) contains high percentage of amylose (70 %) [214] but is not recommended for use as CHO supplementation because it is insoluble in cold water [214] and only around 73 % digestible [210]. However, after undergoing partial acid and alcohol modification, amylo maize becomes soluble in water and up to 92 % digestible [214]. Consequently, randomised trial using AMY-7 was conducted by Bell (2011) wherein participants ingested either AMY-7, DEX or PLA 45 minutes prior to exercise. Participants then cycled at 60 % W$_{max}$ for 15 minutes followed by a TT (a workload equivalent to 15 minutes at 80 % W$_{max}$). The results showed that ingestion of AMY-7 resulted in less variation in serum glucose and insulin concentrations compared to DEX. However, there were no differences in TT performance between treatments, although there was a tendency for participants to complete the trials faster in the AMY-7 condition [215].

While UCCS is associated with problems in terms of normoglycaemia maintenance, palatability, digestion [216] and poor absorption [217], heat modified WM was shown to improve the maintenance of normoglycaemia [218, 219] and better tolerated [220]. The first study investigating the effect of modified WM supplementation on endurance exercise was therefore recently conducted using hydrothermally modified WM (HMS). The effect of WM was compared to
maltodextrin (MAL). Participants consumed the first dose of supplement 30 minutes prior to 150 minutes cycling at 70 % \( \text{VO}_{2\text{peak}} \) followed by a TTE trial at 100 % \( \text{VO}_{2\text{peak}} \); participants then consumed the second dose immediately after exercise. As expected, plasma glucose and insulin spiked higher in MAL while HMS showed a slow and steady increased after the consumption of supplement, respectively, however, there were no differences in term of performance between HMS and MAL [221].

2.2.3.3 Summary

Hypoglycaemia and glycogen depletion has long been associated with fatigue and a decrement in performance during exercise of a prolonged (i.e., >60-90 minutes) nature. The intake of CHO during exercise, has consistently been shown to improve performance. However, the benefit of CHO intake immediately (i.e., within the last two hours prior to exercise) prior to exercise has been controversial. Researchers have typically speculated that CHO ingested immediately prior to exercise leads to hypoglycaemia, and this in turn leads to reduced exercise performance. While rebound hypoglycaemia does occur in response to CHO ingestion, it appears the hypoglycaemia is not associated with reduced performance; at least in exercise trials less than 60 minutes in duration.

Ingestion of complex CHO (i.e., slower digestible CHO) has also been explored as a potential ergogenic aid, due to the more prolonged nature of glucose release. Specifically, it may be theorised that when participants are able to ingest CHO only prior to an event and/or have access only to limited CHO sources during an endurance to ultra-endurance event, then consumption of a slower release CHO (or low GI) may lead to improved performance. While the relative percentage of amylose and amylopectin in starch can affect the digestibility and absorption rate of starch this may also be achieved using acid/alcohol-modified and hydrothermally modified starches. While research to date has been limited, investigation regarding the benefits of mix CHO (starch and sugars) supplementation on prolonged exercise performance is somewhat requires attention.
2.3 Metabolomics

While only a handful of metabolites are often described or assessed, biological tissues including blood are characterised by a vast array of metabolites which are associated with multiple interconnected metabolic pathways. Metabolomics is the technique used to not only assess a broad range of these metabolites, using a combination of different instrumentation, but to also identify and map these metabolites to corresponding pathways. These metabolites may be identified and quantified using sophisticated analytical technologies such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) [222] and inductively coupled plasma-mass spectrometry (ICP-MS; highly sensitive for the detection of metals and some non-metals) [223] using a targeted (examining specific markers of interest) or untargeted approaches (attempting to profile all of the compounds present with any given technique) as shown in Figure 2.3. The major advantages of metabolomics analysis are the substantial information which can be obtained from very small biological sample volumes. It is important to note that each platform, has the capacity to measure many metabolites; however, no one platform can measure all metabolites. For this reason, multi-platform approaches are favoured for metabolomics analysis, or using a single platform but in a different mode (i.e., acquisition of data in both negative and positive ion modes).
2.3.1 Metabolomics and Exercise

Considering the substantial changes associated with blood metabolites during exercise, metabolomics analysis has the potential of becoming a powerful tool to map these exercise-based changes [224]. One of the first studies to utilise a metabolomics approach in blood samples collected at pre-, post-, 3 and 24-hour post-exercise (two conditions: running for 60 minutes at ~75 % VO$_{2\text{max}}$ max; and 120 minutes at ~55 % VO$_{2\text{max}}$) identified the medium and long-chain acylcarnitines as the most discriminating exercise-related metabolites in continuous endurance running [225]. In arguably one of the most comprehensive studies conducted in blood samples relative to exercise, Lewis et al. (2010) mapped the metabolic changes in response to acute maximal and prolonged exercise involving multiple pathways such as tricarboxylic acid cycle, glycogenolysis, fatty acid oxidation and lipolysis concurrently. An acute exercise treadmill test (ETT; Bruce protocol) was used [226] and the changes at the point of exhaustion (±10 minutes after termination of exercise) assessed. The study demonstrated an increase in glycolytic intermediates and products (lactate and pyruvate), lipolysis product (glycerol), amino acids (alanine and glutamine) and sequential products of adenine nucleotide catabolism such as AMP, inosine, hypoxanthine and xanthine. Conversely, there
was a reduction in plasma ketone body acetoacetate at the point of exhaustion in acute maximal exercise. The participants were divided into two different groups according to their fitness level based on median peak percentage of oxygen consumption. A greater increase in glycerol and pantothenate was found in the fitter participants while in less-fit participants, methionine excursions and glutamine concentrations were greater but lactate concentrations did not differ between both groups. In the same study, twenty-five participants who completed the Boston Marathon were randomly recruited by open e-mail invitation to local clubs and were used as the platform for a field study in prolonged exercise. Peripheral blood samples were collected before and after completing the marathon. There was a significant reduction in gluconeogenic amino acids (alanine, threonine, serine, proline, valine, histidine, glutamine, and asparagine) and unexpected increased in TRP metabolites (kynuremate, quinolate and anthranilate). The participants were also divided into two groups (fast- and slow-runner) based on their median finish time (240 minutes). Fumarate which correlates with fitness level in the acute maximal exercise was correlated with running speed during a marathon. Greater excursions in span two tricarboxylic acid (TCA) cycle intermediates including succinate and malate were associated with the fast-runner group. This study showed that i) metabolites from multiple pathways may be mapped simultaneously; ii) based on metabolomics, there was a clear and significant distinction between the acute exercise changes and the prolonged exercise changes; iii) metabolomics analysis was able to separate the fit and unfit individuals.

The difference in metabolic responses to high-intensity interval exercise (HIIT) and work-matched (isoenergetic) continuous moderate-intensity exercise has also been investigated using a targeted GC-MS [139]. Ten well-trained athletes were given a standard breakfast before HIIT or moderate exercise (MOD) trial. The total amount of work used in this study was obtained from a previously completed familiarisation trial where participants completed 10 repetitions of cycling at 80 % VO2max for 4 minutes (work), interspersed with cycling bouts at 50 Watts for 2 minutes (recovery). The MOD trial consisted of cycling at 65 % VO2max. In this study, the metabolic changes were focused on TCA cycle, amino acids and specific non-esterified fatty acids (NEFA). The concentration of plasma glucose, lactate, and rate of CHO oxidation increased significantly with HIIT only, while fat oxidation, total
serum NEFA showed an increase but with no significant differences between HIIT and MOD. With respect to TCA intermediates, malonic acid, citric acid, aconitic acid and succinic acid were shown to demonstrate 1.6-, 1.8-, 2- and 3-fold increments with HIIT, respectively. The specific NEFA changes included an increase in myristic acid, decanoic acid, dodecanoic acid, hentadecanoic acid and palmitoleic acid in both trials. Of particular note, there were unique changes associated with the HIIT trial, which included an increase in myristic acid and oleic acid; while γ-linolenic acid increased in the MOD trial only [139]. Recently, Bassini and Cameron (2014) coined the term sportomics and suggested sportomics may be used as a tool in helping athletes to manage their training and performance by providing a deeper understanding of metabolic changes associated with performance during training and competition [227].

2.3.2 Metabolomics: Nutrition and Exercise

A metabolomics approach was adopted to assess metabolite and performance changes following ingestion of a banana (BAN) versus a 6 % CHO prior to completing a 75-km cycling test [228] in fourteen well-trained cyclists. The BAN or CHO drink (0.2 g CHO per kg body weight) were consumed every 15 minutes during exercise. Blood samples were collected immediately before and after exercising, and one hour after completing the time trial. The untargeted GC-MS approach showed 103 metabolites, of which 56 demonstrated significant differences across time, while only one (dopamine) was significantly different between the BAN and CHO trial. There were 15 metabolites which strongly contributed to the time-based differences, and these were related to glutathione production (5 metabolites); CHO, lipid and amino acid metabolism (8 metabolites); and TCA cycle (2 metabolites). However, the CHO drink and BAN ingestion during 75-km cycling resulted in similar changes in blood glucose, markers of inflammation and oxidative stress, and innate immune levels, while performance remained largely unchanged [228].

A series of studies have also been conducted to investigate the effects of green tea extracts (GTE) on metabolic responses to exercise [229, 230]. The first study
investigated the effects of GTE consumed as a supplement for seven days and containing 1200 mg total catechins and 240 mg caffeine/day on human body metabolism at rest and during constant, moderate-intensity exercise (56±4 % VO$_{2\text{max}}$). Twenty-seven physically active males involved in a PLA-controlled, double-blind, randomised, parallel study, were divided into two groups; GTE (n=13) or PLA (n=14). The participants consumed GTE or PLA for 7 days (twice a day) and performed cycling exercise for 2 hours a day before consuming the supplementations on day 8. In this study, both targeted solid phase extraction (SPE-LC-MS/MS) and untargeted (GC-MS AND LC-MS/MS) approaches were used. Metabolite profiling showed enhanced lipolysis, fat oxidation, glycolysis, activated TCA cycle, reduced amino acids catabolism and Cori cycle intermediates, suggesting that it has potential to induce aerobic energy and fat metabolism at rest. Surprisingly, GTE reduced fat oxidation during exercise, possibly as a result of the exercise response being far greater than the response to the GTE. In addition, the timing for supplementation affected metabolic responses at rest (blood samples collected a day after 8 days of supplementation) and during exercise (blood samples collected 2 hours and longer after supplementation). GTE also reduced the concentration of metabolites from the TRP pathway at rest [229]. The same researchers conducted a subsequent study, in an attempt to separate the chronic (GTE for 8 days) and acute (single dose of GTE 2 hours prior to exercise) responses [230]. However, the researchers were clearly able to separate the metabolic changes from the single GTE bolus, compared to the metabolic response to 7-day supplementation with GTE.
2.3.3 Summary

The application of metabolomics in exercise-based research is relatively new, and has demonstrated significant potential. Considering the powerful influence of exercise on the blood metabolome, it is unsurprising that altered metabolic profiles exist within and between different exercise conditions. Within the exercise-related research, only very few studies have utilized multiple platforms or multiple modes within any given platform to provide a more comprehensive metabolic profile. Moreover, no study to date has attempted to identify metabolites specifically associated with fatigue. Considering the multifaceted nature of fatigue, metabolomics presents as a unique technique to investigate the metabolic signature associated with fatigue.
2.4 References


Opportunities in Dual Process Theory and Process Tracing Methods. 


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Chapter 3 Changes in the plasma metabolome during and following constant cycling to exhaustion: A metabolomics approach
### 3.1 List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AABA</td>
<td>$\alpha$-aminobutyric acid</td>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<td>AdNT</td>
<td>Adenine nucleotide transferase</td>
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<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
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<tr>
<td>BABA</td>
<td>$\beta$-aminobutyric acid</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>CHCL</td>
<td>Chloroform</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CVA</td>
<td>Canonical Variate Analysis</td>
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<tr>
<td>ESI+/-</td>
<td>Electrospray ionisation positive and negative</td>
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<td>ESSA</td>
<td>Exercise and Sport Science Australia</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<td>f-TRP</td>
<td>Free tryptophan</td>
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<tr>
<td>GABA</td>
<td>$\gamma$- aminobutyric acid</td>
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<td>HR</td>
<td>Heart rate</td>
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<td>HVA</td>
<td>Homovanilic acid</td>
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<tr>
<td>LC-HRMS</td>
<td>Liquid Chromatography-High Resolution Mass Spectrometry</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<td>PC</td>
<td>Principal component</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PCA</td>
<td>Principal components analysis</td>
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<td>PC-CVA</td>
<td>Principal component projection followed by Canonical Variate Analysis</td>
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<tr>
<td>QC</td>
<td>Quality control</td>
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<td>QC-RSC</td>
<td>Quality Control-Robust Spline Correction</td>
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<td>RM-ANOVA</td>
<td>Repeated measures analysis of variance</td>
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<td>RPE</td>
<td>Ratings of perceived exertion</td>
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<tr>
<td>RSDQC</td>
<td>Relative standard deviations for pooled QC injections</td>
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<td>RSDSample</td>
<td>Relative standard deviations for sample injections</td>
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<td>TRP</td>
<td>Tryptophan</td>
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<td>TTE</td>
<td>Time-to-exhaustion</td>
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<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatograph</td>
</tr>
<tr>
<td>VO2peak</td>
<td>Peak oxygen uptake</td>
</tr>
</tbody>
</table>
Abstract

**Purpose:** The purpose of this study was to investigate the metabolic profile of plasma during prolonged constant cycling time-to-exhaustion (TTE), with the specific aim of identifying the metabolic profile associated with the onset of fatigue and ‘Central Fatigue Hypothesis’.

**Methods:** Eighteen healthy and recreationally active men with an age range from 19 to 44 years old (mean±SD age: 24.7±4.8 years old; mass 67.1±6.1 kg; height: 171.7±4.9 cm) were recruited to this study. Participants performed prolonged cycling TTE at an intensity corresponding to a fixed blood lactate concentration (3 mmol.L\(^{-1}\)). Plasma samples collected at 10-min of exercise, prior to fatigue (last sample prior to fatigue; <10 min prior to fatigue), immediately post-fatigue (point of exhaustion) and 20-min post-fatigue were assessed using a liquid chromatography–mass spectrometry based metabolomics approach (positive and negative mode).

**Results:** Participants completed ~80.9±13.6 min of cycling TTE and maintained an average heart rate of 143.8±8.2 bpm. Of the 1847 reproducible features, 105 metabolites were putatively identified and confirmed, with 87 metabolites demonstrating a significant main effect of time. Seven clusters of associated metabolites were identified with four clusters: C (n=17 metabolites), D (n=13 metabolites), F (n=11 metabolites) and G (n=17 metabolites) contributing to the separation between the during-exercise samples; while three clusters: E (n=18 metabolites), F and G, contributed strongly to the separation between pre-fatigue and post-fatigue samples. Four clusters: A (n=20 metabolites), B (n=9 metabolites), D and F; demonstrated a strong downward shift in their trajectory between the post-fatigue and 20-min post-fatigue sample.

**Conclusion:** The current study identified a unique metabolic profile of plasma samples collected during (10-min of cycling; pre-fatigue; at fatigue) and after (20-min post) exercise. In accordance with our hypothesis and the ‘Central Fatigue Hypothesis’, free-fatty acids (FFA) and tryptophan (TRP) played important roles in discriminating between the plasma metabolome at these time-points.
### 3.3 Introduction

Exercise-induced fatigue is associated with transient reductions in force and power, culminating in reduced performance [1-6]. The magnitude and duration of exercise-induced fatigue may be altered by manipulating intrinsic factors of exercise, such as the duration, type, and intensity of the exercise [7-10]. The intrinsic factors may in turn be modulated by factors such as sex, age, environmental conditions and the nutritional status and training status of the individual [5, 8, 11-18]. In response to the complex and interactive nature of these factors, numerous theories and models of exercise-induced fatigue have been proposed [7, 19-21]. Although general consensus indicates an underlying cause of fatigue which is likely multi-factorial in nature, large discrepancies between proposed models exist. Considering the relative ease of blood collection and the exercise-associated changes in the metabolic milieu of the blood [22], the identification of fatigue based biomarkers in blood presents as an attractive option. Indeed, a number of blood-borne biomarkers have previously been identified as being associated with fatigue including un-buffered protons, electrolytes, glucose, lactate, cytokines and amino acids (reviewed in: [23]). The measurement of these biomarkers has typically been restricted to methodologies which are highly targeted, limiting the measurement to specific metabolites. However, through the application of metabolomics, a global view of the blood metabolome may be achieved and has been applied in the exercise science field [22, 24-27]. Adoption of this technique led to the identification of medium and long-chain acylcarnitines as being the most discriminating exercise-related metabolites associated with continuous endurance running [22], while ketone bodies, argininosuccinate, niacinamide, and citrulline distinguished marathon running and acute short-term exercise [25].

The application of metabolomics to assess the blood-metabolome at the onset of fatigue may lead to novel biomarkers or groupings of metabolites, which relate to the onset of fatigue. Therefore, the primary aim of this study was to assess and compare the blood metabolome at the point of fatigue, with the blood metabolome prior to fatigue, and following 20-min of recovery. Whilst this study was designed to be hypothesis-generating, we sought to examine the ‘Central Fatigue Hypothesis’ as proposed by Newsholme and colleagues [28, 29]. Specifically, this hypothesis is
based on an increased concentration of free fatty acids (FFA) with prolonged exercise, leading to displacement of tryptophan (TRP) from albumin resulting in the increased availability of free TRP (f-TRP) for entry into the central nervous system (CNS). Tryptophan is the precursor to serotonin, an increase of which in the CNS has associated with fatigue in both rats [30, 31] and human studies [32, 33]. We, therefore, established the following hypotheses; i) that blood sampled at fatigue will be distinct from blood sampled prior to and subsequent to fatigue; ii) free fatty-acids, 5-methoxy-3-indoleacetic acid (the end product of serotonin catabolism), and tryptophan will significantly contribute to the differences observed in the metabolic profile of blood, at the point of fatigue.
3.4 Methods and procedures

Participants

Eighteen healthy and recreationally active men between the ages of 18 and 44 years old (mean±SD age: 24.7±4.8 years old; mass 67.1±6.1 kg; height: 171.7±4.9 cm) were recruited for this study. Participants were initially screened for eligibility via email and follow-up telephone conversation. Exclusion criteria for this study included: diagnosis of diabetes mellitus; uncontrolled hypertension; acute (i.e., viral infection) or chronic inflammatory disease; currently taking medication for chronic health conditions. Additionally, participants were excluded (or referred to their General Practitioner to obtain exercise clearance) if they presented with more than two risk factors in stage two or checked ‘Yes’ at stage one of the Exercise and Sport Science Australia (ESSA) risk assessment questionnaire, or if they were identified as having any condition which may be exacerbated through exercise participation. All participants were informed about the experimental procedures and provided written informed consent before testing. The protocol has been approved by the Murdoch University Human Ethics Committee (Project number 2013/206).

Research protocol design

Participants attended the laboratory at Murdoch University on two separate occasions. The first visit comprised preliminary testing and familiarisation, while the second visit comprised the experimental session. The experimental protocol for this study was a single-arm trial, consisting of participants cycling at a constant pace at an intensity corresponding to a blood lactate concentration of 3 mmol.L\(^{-1}\). The experimental trial was performed at least 48 hours after the preliminary and familiarisation trials. All testing was conducted in the Exercise Physiology Laboratory at Murdoch University.
Preliminary and familiarisation trials

Anthropometric data (height and weight; Scales Plus, Western Australia, Australia) was measured prior to participant’s peak oxygen uptake (VO_{2peak}; ParvoMedics, Utah, USA) being determined using a continuous incremental cycle protocol. The protocol for the VO_{2peak} test consisted of an initial workload of 50 W, which was increased 35 W in a step-wise fashion every 3 min. All testing was conducted on the same electromagnetically braked cycle ergometer (Velotron, Racermate Inc., Seattle, USA). Finger prick blood samples were collected at the end of each stage for measurement of blood lactate (Lactate Plus, Nova Biomedical, Massachusetts, USA) until the lactate concentration exceeded 4 mmol.L\(^{-1}\). Thereafter, the workload increased 35 W every minute until exhaustion (defined as not being able to maintain cadence above 60 rpm despite verbal encouragement). The workload corresponding to a blood lactate concentration of 3 mmol.L\(^{-1}\) (W_{3mM}) was then interpolated from this test and recorded. The ratings of perceived exertion (RPE) and heart rate (HR) (Polar, Kempele, Finland) were measured in the last 10 s of each stage and data from the metabolic cart collected continuously and assessed in 15 s epochs for determination of VO_{2peak}. Participants were then familiarised with the TTE cycling test. The workload during the TTE familiarisation was set at the predetermined W_{3mM}, and where necessary, the workload was adjusted to ensure the blood lactate concentration was fixed at 3 mmol.L\(^{-1}\). The familiarisation TTE was 20 min in duration. The cycle ergometer setting was recorded and remained consistent throughout the experimental trials.

Experimental trial

Participants reported to the laboratory in the morning following a \(\geq 10\) h overnight fast. A HR monitor (Polar, Kempele, Finland) was placed on the chest of the participants. For repeated blood sampling, a venous catheter (VasoFix Safety, B.Braun, Melsungen, Germany) was placed into a forearm vein. A baseline blood sample (4 ml) was drawn 30 min before the initiation of exercise. Participants were provided with 7 ml.kg\(^{-1}\) body weight (BW) of fluid (low caloric lime cordial mixed with water; 1:4 ratio of cordial:water) 30 min prior to testing and 5 ml.kg\(^{-1}\) BW 10
min prior to the testing. Participants were asked to empty their bladder before the initiation of exercise and were provided with 2 ml.kg\(^{-1}\) BW every 15 min of the same fluid as well as *ad libitum* access to water. Participants performed a non-fatiguing warm-up for 2 min on the electromagnetically braked cycle ergometer (Velotron, Racermate Inc., Seattle, USA) to ensure they were comfortable. Participants then performed the cycling TTE at an intensity corresponding to 3 mmol.L\(^{-1}\). The HR, RPE, power output and cadence were recorded continuously throughout the exercise. Blood samples were drawn every 10 min during cycling, then, at the point of exhaustion (post-fatigue sample) and 20 min after the termination of exercise (20-min post-fatigue). The point of exhaustion was determined at participant’s own volition or when their cadence dropped below 60 RPM and could not increase cadence even after receiving verbal encouragement. The RPE (scale 6-20 [34]) were collected throughout the trial.

**Blood sampling**

Approximately 4 ml of venous blood was drawn at every time point and transferred into 4 ml blood collection tubes containing lithium heparin (BD Vacutainer, Plymouth, UK) for the metabolomics analysis. These blood samples were immediately centrifuged at 1800 g x 10 min to collect blood plasma. These plasma samples were transferred into Eppendorf tubes and stored in the freezer at -80 °C before being analysed in the Centre for Integrative Metabolomics & Computational Biology, Edith Cowan University.

**Sample preparation for metabolomics analyses**

Four time-points were chosen to assess the metabolic changes associated with fatigue. The 10-min exercise sample (i.e., participants cycling for 10 min) served as the baseline sample for comparisons; the pre-fatigue sample, was the final sample drawn prior to volitional exhaustion (pre-fatigue); the blood sample drawn immediately after the point of fatigue was identified as the post-fatigue sample;
while the final sample drawn 20 min following the exercise bout served as the 20-min post-fatigue sample.

Plasma samples were prepared in randomised batches according to the Bligh and Dyer method [35]. On the day of analysis, samples were thawed on ice, and protein precipitation was performed by either adding 150 µL of ice-cold LC-MS grade methanol containing two internal standards; D5 Tryptophan (Cambridge Isotopes Laboratories) and Taurocholate Sigma Aldrich (St Louis, MO, USA) to 50 µL of plasma for reverse phase chromatography (RP), or for hydrophilic interaction liquid chromatography (HILIC) by adding 150 µL of ice-cold LC-MS grade acetonitrile containing two internal standards; C (13) Leucine, D8 Valine. Samples were thermomixed at 4 °C for 60 s at 1400 rpm before centrifugation (14000 rpm) for 20 min at 4 °C. An aliquot (50 µL) of each supernatant was transferred to separate LC vials and placed into the auto sampler tray at 6 °C ready for metabolomics analysis. Samples were analysed within 24 h. Quality control (QC) samples were prepared by pooling 20 µL of each plasma sample; 50 µL aliquots were then prepared as study samples. Quality control samples were prepared and analysed throughout the LC/MS runs to assess instrument drift and ‘batch-batch’ variability. Prior to data acquisition, the analytical sequence was randomised by the participant, and then time point. To monitor analytical drift and assess precision, QC samples were injected after every 5th sample [36].

**LC-high mass spectrometry**

Experiments were analysed using a Dionex UltiMate 3000 RS comprising an Ultra High-Pressure Liquid Chromatography Pump (UHPLC) coupled to Orbitrap Exactive (Thermo Fisher Scientific, CA, USA) fitted with a heated electrospray ionisation probe (HESI). Non-polar metabolites were separated on a RP Hypersil GOLD column (100 x 2.1 mm, 1.9 μm particle size; ThermoFisher Scientific, Runcorn, UK) with an in-line filter. Separation was performed as described by Dunn, Broadhurst [37]. Briefly, the eluent gradient for RP analysis, in both positive and negative mode, comprising water with 0.1 % formic acid (solvent A) and methanol with 0.1 % formic acid (solvent B) was: isocratic at 100 % A for 1.0 min,
followed by an increase to 100 % B in 15 min, maintained at 100 % B for 4 min. Initial conditions were returned over 2 mins and then held at 100 % A to equilibrate for 3 min. The flow rate was 0.4 mL/min for positive and 0.36 mL/min for negative; injection volume was 2 µL, and column oven temperature 50 °C.

Polar metabolites were separated on HILIC Syncronis (100 x 2.1 mm, 1.9 µm particle size; ThermoFisher Scientific, Runcorn, UK) column with an in-line filter. Sample analysis in positive mode was performed using water with 0.1 % formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B). For negative mode, water with 10 mM ammonium acetate (solvent A) and acetonitrile (solvent B). Eluent gradient for HILIC was as follows: isocratic at 95 % B for 1.5 min, 95 to 40 % B in 10.5 min, maintained at 40 % B for 2 min, returned to initial conditions over 1 min, and then held for 5 min for column equilibration. The flow rate was 0.3 mL/min; injection volume 2 µL, and column oven temperature 50 °C. To avoid bias due to the instrument drift, the injection sequence were block randomised by participant and within each block by time point (baseline, pre-fatigue, post-fatigue and 20-min post-fatigue). To assess analytical precision, 24 QCs were injected throughout the sequence; four samples bracketed by one QC.

Full scan and ddMS² were acquired on the Orbitrap mass analyser. Full scan was set at 70,000 resolution and a scan range of 70 – 1000 m/z with the source and ion transfer conditions as follows; source heater = 350 °C, sheath gas = 35 (arbitrary units), aux gas = 10 (arbitrary units), capillary temperature = 350 °C, ion spray voltage = 3.0 kV (positive ion mode) and 2.5 kV (negative ion mode), slens = 50 % and AGC = 1 x 10⁻⁶. MS/MS experiments were performed at a resolution 17,500 on each sample with the HCD energy set at 30 eV. Data acquisition was carried out using Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA). Before analysis, the Exactive Orbitrap was externally calibrated using ready-made calibration solutions (ESI negative ion calibration and ESI positive ion calibration solution), obtained from Thermo Fisher Scientific. San Jose, CA, USA.
Data pre-processing

Data from each MS ionisation mode were grouped and pre-processed separately using Compound Discoverer 2.0™ (Thermo Fisher Scientific, San Jose, CA, USA), using an untargeted metabolomics workflow. Briefly, Compound Discoverer was used to align total ion chromatograms along retention time using an adaptive curve, with a maximum shift of 0.5 min and 5 ppm mass tolerance. Detected features with an intensity of no less than 1,000,000 and a S/N greater than 5 in each set of data were extracted and merged into components according to ion adducts. Resulting compounds were searched for in online databases such as mzCloud, HMDB and KEGG for verification and annotation. Resulting data matrices were exported and corrected using the Quality Control-Robust Spline Correction (QC-RSC) algorithm [38]. Relative standard deviations were calculated for the pooled QC injections (RSDQC) and the sample injections (RSDSample), and the ratio of RSDSample to RSDQC was also calculated. To suppress the mathematical confounding effect of highly collinear data (and to roughly group chemically similar features, such as adducts and isotopes) metabolite features were combined into a single peak using a simple hierarchical-clustering algorithm. First, a Pearson’s correlation matrix was calculated, mapping the pair-wise correlation between each metabolite feature. Second, a retention time difference matrix was calculated, mapping the pair-wise difference in retention time between each metabolite feature. Features that had a pair-wise correlation >0.8 and pair-wise retention time difference < ± one-second were grouped into a single metabolite “cluster”. For each cluster, the feature with the largest peak-area was then used for quantification. Orphan features were considered as clusters with N=1.
**Metabolite identification**

Metabolite identifications were established prior to statistical analysis, by matching against an in-house MS/MS spectral library and searching online mzCloud spectral libraries (mzCloud, Metlin, HMDB and Massbank). This process corresponds with the reporting standards for chemical analysis proposed by the Metabolomics Standards Initiative [39]. Identified metabolites are tabulated (Table 3.1), including information of most probable identity and m/z.

**Statistical analysis**

The metabolite cluster data from both the positive and negative ionisation modes were combined into a single data matrix. Missing values were imputed using the k-nearest-neighbour methodology (k=3) [40]. Data were log transformed, both to stabilise variance, and to approximate the multivariate normal distribution needed for parametric univariate and multivariate statistical modelling.

Before any formal models were tested, principal components analysis (PCA) was performed on the complete data set (all identified and non-identified features), with scores plot labelled QC and Sample (Figure 3.1). Here the aim was to assess the natural multivariate variance of the data as well as identify and remove sample outliers resulting from either poor sample collection or instrument error. For each identified metabolite in turn, the null hypothesis that there were no differences in population means across the four-consecutive time-points was tested using repeated measures analysis of variance (RM-ANOVA). The method described by Benjamini and Hochberg [41] was used to control for the probability of false discovery, which is unavoidably inflated with multiple parallel statistical comparisons. A false discovery rate (FDR) of 0.1 was considered appropriately conservative to avoids false positives [42], whilst at the same time avoiding false negative results. Identified metabolites (name; formula; retention time) were tabulated with F-scores, p-values, FDR-adjusted p-values (i.e., q-value) in Table 3.1.

The identified peaks were then combined into a single multivariate discriminant model using Principal Component projection followed by Canonical Variate
Analysis (PC-CVA) [43]. PC-CVA is performed to visualise the multivariate covariance in the data and uncover multivariate latent structure therein. The means and 95% confidence interval (CI) of the mean for each subject trajectory scaled to unit variance are presented in the PC-CVA; time-points (clusters) are considered significantly different when 95% CI bands show no overlap. Since univariately weak- but correlated- variables can become significant when combined into a multifactorial biomarker signature. The number of PCs to be projected into CVA space was determined by identifying the inflection point in the PCA screen plot. Bootstrap resampling/remodelling was used (n=500) to determine which metabolites significantly contributed to the optimal model (P<0.05). The loadings plot for PC1 and PC2 (mean±95% CI; significance identified by 95% CI bands not including absolute zero and indicated in red) of identified metabolites are presented in Figure 3.3.

Cluster analysis was used to divide the multivariate datasets into clusters and presented as a circular clustergram (Figure 3.4). Association of continuous variables was calculated using Euclidean distance and Ward’s method, which results in each observation being forced into a cluster. Resultant clusters are presented as plots of marginal means (±95% CI) relative to time (Figure 3.5).
3.5 Results

Exercise performance

Workload for the time-to-exhaustion trial was set at 110.3±23.0 W. Participants rode for 4853.7±813.8 sec (~80.9±13.6 min) and maintained an average HR of 143.8±8.2 bpm. The RPE at the completion of the exercise trial was 17.9±2.8.

![PCA Scores Plot](image)

**Figure 3.1** Scores plot from the Principal Component Analysis (PCA). The QC samples clearly cluster to the right and demonstrate large homogeneity while samples (four plasma samples per participant) are clustered to the left and are clearly separated from the QC samples. The analysis was conducted on all (identified and non-identified) metabolites present in the plasma sample.

Metabolomics Analysis

After data cleaning and similarity clustering, Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) analysis of plasma resulted in the reproducible detection of 1847 features (with RSD <20%). Principal components analysis (PCA) showed (Figure 3.1) tight clustering of the QC samples and much
smaller QC-variance than sample-variance. There were no outliers detected in the PCA.

Of the reproducible features, 105 metabolites were putatively identified and confirmed, with 87 metabolites identified as demonstrating a significant main effect of time (Table 3.1). The results of the PC-CVA (Figure 3.2), showed significant multivariate discrimination between group means. Principal Component 1 (PC1) explained 35% of total dataset variance and demonstrated a non-recovery trajectory which progressively increased (demonstrated by rightward shift) from baseline to pre-fatigue, at fatigue and then 20-min post-fatigue. Principal component 2 (PC2) explained 12% of the total dataset variance and demonstrated a recovery trajectory, wherein pre-fatigue and post-fatigue samples are elevated along PC2, while the 20-min post-fatigue samples are below the baseline sample (Figure 3.2).

The contribution of individual metabolites to the PCA plot loadings are presented in Figure 3.3, with seven clusters of associated metabolites (based on trajectories) being identified in the cluster diagram (Figure 3.4). The trajectories for each identified cluster are in turn plotted to provide a visual representation of the observed changes for these groups of metabolites. It is apparent that clusters C (n=17 metabolites), D (n=13 metabolites), F (n=11 metabolites) and G (n=17 metabolites) contribute strongly to the separation between the baseline sample and the pre-fatigue sample; while clusters E (n=18 metabolites), F and G contributed strongly to the separation between pre-fatigue and post-fatigue samples. Clusters A (n=20 metabolites), B (n=9 metabolites), D and F demonstrated a strong downward shift in their trajectory between the post-fatigue and 20-min post-fatigue sample, while cluster G demonstrated a continued notable upward trajectory.
Figure 3.2 Principal Component projection followed by Canonical Variate Analysis (PC-CVA) of identified metabolites. The mean (x) and 95% CI (dashed circle around x) are presented. Significant differences are determined where 95% CI does not overlap. Baseline (20-min steady state) is strongly separated along PC-1 from all time points. Pre-fatigue and post-fatigue (at fatigue) are clustered closer although significant separation was achieved. The 20-min post-fatigue samples are separated from the fatigue (pre-fatigue, post-fatigue) samples along PC2.
Figure 3.3 Loadings illustrating metabolites contributing to PC1 (left) and PC2 (right). Data are presented as mean-loading values ±95% CI. Red-coloured CIs identify metabolites that significantly contribute along the respective PC; blue-coloured CIs identify metabolites that do not contribute significantly.
Figure 3.4 Circular clustergram classifying individual metabolites into seven associated clusters (A-G).
Figure 3.5 Marginal means (±95 % confidence interval) plots of each cluster relative to time. Bottom right panel provides a relative-indication of the timing for each blood sample to provide some reference.
Table 3.1 Characteristics and statistical table of each identified metabolite. Significance was based on q-value (FDR-adjusted p-values) and significance set at q\(\leq0.05\). All significant metabolites are bolded.

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<th>Label</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>Rt (min)</th>
<th>QC-RSD</th>
<th>F(3,51)</th>
<th>p-value</th>
<th>q-value</th>
<th>Cluster</th>
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<td>453.28472</td>
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**G** = Protein-derived; **E** = Endogenous; **F** = Foreign.
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3.6 Discussion

The primary aim of this study was to examine the metabolic profile of plasma at the point of fatigue, and contrast this profile with that occurring prior to fatigue (two time-points: 10 min into cycling task and immediately prior to fatigue) and after a 20-min period of recovery. The findings of the current study were i) there was a unique metabolic profile at fatigue, pre-fatigue (both time-points) and after 20 min of recovery; ii) there were 87 metabolites identified as significantly contributing to the unique metabolic profile associated with each time-point; iii) when associated metabolites were clustered together, significant trends over time were identified. In accordance with our hypothesis, free-fatty acids (FFA), 5-methoxy-3-indoleacetic acid, and TRP played important roles in discriminating between the time-points in the current study.

The PC-CVA score plot (Figure 3.2) corresponding to the first two principal components showed that PC1 and PC2 explained 35% and 12% of the total dataset variance, respectively. Relationships between the location of the samples in the scores plots with the respective time-points were assessed by Canonical Variate Analysis (CVA) and visually presented using colour-coded data-points. The CVA revealed the plasma metabolome was significantly altered during the course of the study which resulted in perceptible clustering of the plasma samples from respective time-points. Specifically, the approach adopted herein, clearly enabled the discrete separation of plasma samples according to the metabolic profile between sampling time-points. To provide some context, the average cycle time in this study was 81 min; a participant cycling for 81 min, had a blood sample taken at 10 min (baseline), 80 min (pre-fatigue), ~82 min (post-fatigue) and 101 min (20-min post-fatigue). Therefore, the greatest time-difference (71 min on average) occurred between the baseline sample and the pre-fatigue sample, while the shortest time-difference occurred between the post-fatigue and pre-fatigue samples which did not exceed 10 min on any occasion. As a consequence, the baseline samples are clustered further away from the other samples since their metabolic profile was more distinct from others, and unsurprisingly, the pre-fatigue and post-fatigue samples demonstrate similar metabolite profiles. It is interesting to note the
separation between the pre-fatigue and post-fatigue samples along PC1 only, while the 20-min post-fatigue samples are clearly separated along PC2. The contribution of each metabolite to PC1 and PC2 are illustrated in Figure 3.3 and highlight the influential role of lipids and metabolites associated with lipid metabolism to PC1 in particular.

**Fatigue associated metabolites: Central Fatigue Hypothesis**

The relative importance of serotonin to the onset of fatigue in prolonged exercise has been a matter of great debate [7] since the original work by Newsholme *et al.* [28, 29]. Central to this theory is the progressive increase in the concentration of long chain fatty acids with exercise duration. Specifically, this hypothesis is based on an increased concentration of FFA with prolonged exercise, leading to displacement of tryptophan (TRP) from albumin resulting in the increased availability of free TRP (f-TRP) for entry into the central nervous system (CNS). Tryptophan is the precursor to serotonin, an increase of which in the CNS has associated with fatigue in both rats [30, 31] and human studies [32, 33].

In the current study, the concentration of FFA progressively increased and were the strongest contributors (All $q \leq 0.001$) to the observed significant increase over time in Cluster G (Figure 3.5). Accordingly, glycerol (Cluster F) demonstrated significant increases ($q \leq 0.001$) reflecting an increased adipose tissue triglyceride catabolism; in this vein, oleic acid, the most abundant fatty acid in human adipose tissue [44], was one of the fatty acids which demonstrated the largest increase in the current study, along with linolenic acid and palmitic acid. The increase in palmitic acid and linoleic acid are unsurprising since palmitic acid is the most abundant saturated acid in human tissues [45] while linoleic acid is the most abundant fatty acid in plasma [45]. Concomitant with these changes in FFA, TRP demonstrated a significant decrease in concentration over time (Table 3.1; Cluster A, Figure 3.5) which may be indicative of increased CNS uptake of TRP during exercise as previously suggested [28, 46] and possible conversion to serotonin within the CNS. While speculative only, this speculation is strengthened by the observed increase ($q \leq 0.001$) in 5-methoxy-3-indoleacetic acid, the end product of
serotonin catabolism. Specifically, the major catabolic pathway of serotonin in humans is conversion to 5-hydroxyindole-acetaldehyde [47], which is in turn catabolised to 5-methoxy-3-indoleacetic acid via enzymatic conversion by aldehyde dehydrogenase and acetylserotonin N-methyltransferase (the same enzyme associated with the synthesis of melatonin), respectively. However, this is based on temporal associations in changes in a plasma concentration in metabolites in the current study only and requires confirmation in subsequent experimental studies.

**Fatigue associated metabolites: indole metabolism**

Tryptophan is a unique amino acid due to the indole ring comprising its structure [47]. Indeed, while conversion of TRP to serotonin is most commonly discussed in relation to fatigue [1, 7, 19, 28-30], the study herein identified significant increases in the concentration of indole (q≤0.001), indole-3-lactic acid (q≤0.001), methyl indole-3-acetate (q=0.003) and a decrease in indole-3-acetic acid (q≤0.001). These metabolites relate to alternate pathways of tryptophan metabolism and are unrelated to the more well-known and researched pathways of serotonin, melatonin, and kynurenine [47]. Tryptophan metabolites and indole have more recently [48] been the subject of interest due to their possible activation of the aryl hydrocarbon receptor (AHR). Within this, indole and indole 3-acetic acid have been identified as confirmed ligands of AHR, which does not exclude the possibility of the other metabolites playing a possible role in AHR activation. The physiological significance of this is that AHR activation has now been identified as playing an important and multifaceted role in immune regulation which involves regulation of both innate and adaptive immunity [48]. Future research may be warranted to explore whether these ‘alternate’ tryptophan metabolic pathways are responsible for the modulation of the various immune pathways observed after exercise [49, 50].
Fatigue associated metabolites: free fatty acids

Notwithstanding the significance of the Central Fatigue Hypothesis discussed above, there are other possible links between FFA and the onset of fatigue. Specifically, oleic acid and oleoylcarnitine have demonstrated significant inhibition (up to 30% inhibition) of the mitochondrial transporter adenine nucleotide translocase (AdNT) [51]. This transporter is responsible for transporting ATP derived from oxidative phosphorylation, to the energy-requiring reactions in the cytoplasm of the cell. Palmitoylcarnitine and palmitic acid demonstrated similar capacity of inhibition (up to 60% inhibition) of AdNT. In the current study, oleic acid, palmitic acid, oleoylcarnitine, palmitoylcarnitine and acetylcarnitine all demonstrated significant increase (All q≤0.001; Table 3.1; Cluster F and G, Figure 3.5). While this downregulation of AdNT by FFA and carnitine esters has long been acknowledged (i.e., [51]), the possible implication of this pathway to exercise-induced fatigue has to our knowledge, not previously been explored. Free fatty acids, and in particular palmitoylcarnitine has also been implicated in modulating the activity of additional proteins of interest in the fatigue literature including the sodium/potassium and calcium pumps [52, 53]; albeit that much of this work has previously been conducted in myocardial tissues. The role of electrolytes and intracellular calcium concentrations in fatigue-onset has been acknowledged since the classical work of Bergstrom et al. [1, 7, 54, 55].

The role of FFA in cell signalling, and in particular the role of FFA in regulating inflammatory pathways and pathways associated with oxidative stress has gained increasing interest [56-58]. Palmitoylcarnitine has been shown to increase reactive oxygen species [52] as well as stimulate the release of pro-inflammatory cytokines (interleukin-1; interleukin-6; tumour-necrosis factor alpha) [59]. Whether the increase in palmitoylcarnitine during exercise is involved in these previously reported increased concentrations of cytokines or the previously reported imbalance in redox status post-exercise [60] may be an important area of future research.
Fatigue associated metabolites: neurotransmitters

A number of neurotransmitters have understandably been under investigation with respect to their involvement in fatigue. Aminobutyric acid, which may comprise any of three isomeric compounds including, α-aminobutyric acid (AABA), β-aminobutyric acid (BABA), γ-aminobutyric acid (GABA) has been implicated in possibly playing a role in the onset of fatigue. In the current study, GABA was increased significantly (q=0.014) and is considered the chief inhibitory neurotransmitter in the mammalian central nervous system, given its general role is to reduce neuronal excitability [61]. It is noteworthy that 2-aminobutyric acid demonstrated a significant increase (q≤0.001) and has recently been shown [62] to increase the activity of glutathione, which plays an essential role in the maintenance of intracellular redox balance.

Two additional metabolites are of particular interest in the current study, homovanillic acid (HVA) and acetylcholine (ACh). Homovanillic acid is the major metabolite of catecholamine degradation and specifically, dopamine degradation [63]. Dopamine, in turn, is known to play significant roles in glucose homeostasis, immune regulation and mood regulation, with a specific role in the reward circuitry of the brain [64]. Homovanillic acid demonstrated a significant increase (q≤0.001) in the current study. Acetylcholine, on the other hand, is the primary neurotransmitter associated with the motor nerve and induces the muscle action potential, however, the concentration of ACh was not significantly changed (q=0.09).
Fatigue associated metabolites: energy yielding pathways

The energetics of muscular fatigue have been of particular interest for a number of years, particularly as it relates to “peripheral fatigue” [1, 7, 11, 19, 54, 55]. Since metabolites are relatively small and transporters for metabolites largely expressed ubiquitously, the plasma metabolome provides an interesting snapshot of intracellular metabolomics [25]. The contribution of amino acids to support the energetics of prolonged exercise is relatively small albeit not trivial [1, 19, 55]. The process of transamination is initiated within the muscle, and as a consequence, the release of alanine and glutamine from muscle far exceeds the release of other amino acids as these two act to transport the ammonia to the liver and kidneys. These two amino acids were identified as contributing to cluster A, and they demonstrated a slight albeit significant decrease in concentration as the exercise progressed (Table 3.1; Figure 3.5), which likely reflects increased hepatic and renal uptake beyond the increased net muscle release [1]. The remaining amino acids showed variable responses, with some demonstrating an increase (proline, valine, glutamic acid) and while others a decrease (tryptophan, threonine, citrulline, methionine, proline, arginine). The increase in phenylacetylglutamine (q ≤ 0.001; Cluster D) which is a conjugate of phenylacetate and glutamine, is noteworthy given the previously identified role of phenylacetate-containing medications of having a side-effect of fatigue, although this has not been explored in exercise-induced fatigue.

Acetylcarnitine is synthesised in a reversible reaction involving the combination of carnitine and acetyl-CoA, to form acetylcarnitine and free CoA. Since acetyl-CoA is the primary substrate for the citric acid cycle, the decrease in carnitine (q ≤ 0.001) along with the increase in acetylcarnitine (q ≤ 0.001; forward reaction), suggests a reduced substrate pool for the citric acid cycle. In considering the above, it is important to note that citric acid, the ‘first step’ in the citric acid cycle, demonstrated a progressive increase with exercise (Cluster E; q ≤ 0.001) which may suggest oxidative phosphorylation was indeed downregulated at a site distal to the citric acid cycle. Unsurprisingly, creatine showed a progressive and significant increase in response to the progressive exercise bout (q ≤ 0.001; Cluster D).
3.7 Conclusion

The adopted metabolomics approach enabled the distinction of plasma samples obtained at the point of fatigue (post-fatigue) from samples obtained prior to and following fatigue. The specific metabolites contributing to this distinct profile were then explored. In accordance with our hypothesis, FFA and TRP played influential roles in contributing to these distinct metabolic profiles and in so doing, lent support to the ‘Central Fatigue Hypothesis’.

Additionally, the 87 identified metabolites which demonstrated significant changes over time generated potentially interesting avenues for future investigation, including, for example, some novel roles for FFA in exercise-induced fatigue onset. It is important to note that while the approach adopted herein was able to capture a large number of metabolites within the plasma samples, not every metabolite was captured and identified. Moreover, it is important to note that a change in the concentration of metabolites across time-points, and in particularly a change in the concentration of intermediary metabolites, does not provide information on the rate of appearance or disappearance of metabolites within a pathway (i.e., metabolic flux). Nevertheless, despite the relatively short time-frame between sampling points in the current study, the metabolic profile of plasma demonstrated a significant shift during and after exercise and included a distinct metabolic profile at the point of fatigue.
3.8 References


Chapter 4  Neuromuscular fatigue following isoenergetic prolonged constant versus self-paced cycling exercise
## 4.1 List of abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>A/D</td>
<td>Analog-to-digital</td>
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<tr>
<td>ATP:ADP</td>
<td>Ratio of adenosine triphosphate to adenosine diphosphate</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BW</td>
<td>Body weight</td>
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<td>Ca(^{2+})</td>
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<td>CAR</td>
<td>Central activation ratio</td>
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<td>CDE</td>
<td>Cadence</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>ECC</td>
<td>Excitation-contraction coupling</td>
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<td>EMG</td>
<td>Electromyography</td>
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<td>ESSA</td>
<td>Exercise and Sport Science Australia</td>
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<td>H(^+)</td>
<td>Hydrogen ions</td>
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<td>HR</td>
<td>Heart rate</td>
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<td>MAP</td>
<td>Maximal aerobic power</td>
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<td>MVC</td>
<td>Maximal voluntary contraction</td>
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<td>Peak doublet</td>
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<td>Post-exercise</td>
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<td>20-min post-exercise</td>
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<td>PRE</td>
<td>Pre-exercise</td>
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<td>PWR</td>
<td>Power output</td>
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<td>PT</td>
<td>Peak twitch</td>
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<td>RMS</td>
<td>Root mean square</td>
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<td>Acronym</td>
<td>Description</td>
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<td>RMS.M^{-1}</td>
<td>Ratio of RMS to maximal M-wave amplitude of VL</td>
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<td>RPE</td>
<td>Ratings of perceived exertion</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>Time-trials</td>
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<td>TTE</td>
<td>Time-to-exhaustion</td>
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<tr>
<td>VO_{2peak}</td>
<td>Peak oxygen uptake</td>
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<td>VL</td>
<td>Vastus lateralis</td>
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<td>VAL</td>
<td>Voluntary activation level</td>
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<td>Voluntary Activation Level with reference to the potentiated doublet</td>
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<tr>
<td>VAL_r</td>
<td>Voluntary Activation Level with reference to the resting doublet</td>
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4.2 Abstract

**Purpose:** The purpose of this study was to compare neuromuscular changes and performance of a cycling time-to-exhaustion (TTE) trial to an isoenergetic cycling time-trial (TT) in recreationally active participants.

**Methods:** Eighteen recreationally active males (mean±SD age: 24.7±4.8 years old; mass 67.1±6.1 kg; height 171.7±4.9cm) performed cycling TTE at an intensity corresponding to a blood lactate concentration of 3 mmol.L⁻¹ (trial 1). Participants then performed the same amount of work as in trial 1, using a cycling TT (trial 2). Maximal voluntary contraction (MVC) torque, muscle Voluntary Activation Level (VAL), root mean square of electromyography (EMG) signal, electrically evoked torque and M-wave amplitude of quadriceps muscle was obtained at baseline (PRE), post-exercise (POST) and 20-min post-exercise (POST20).

**Results:** Duration of exercise in TT (4266.6±192.4 s) was significantly (p=0.006) faster compared to TTE (4853.7±191.8 s) with a mean difference of 587 s. There was a significant main effect of time on neuromuscular parameters, with each being significantly lower immediately post-exercise and 20-min post-exercise compared to baseline, except in peak-to-peak M-wave amplitude (p=0.146). There was no significant interaction effect on all neuromuscular parameters (p≥0.05). Maximal voluntary contraction torque was the only neuromuscular which has shown a significant main effect of trial with greater losses of quadriceps muscle strength in TT compared to TTE trial.

**Conclusion:** Performance in cycling TT was significantly improved compared to an isocaloric cycling TTE trial in young and recreationally active males, with greater quadriceps muscle strength loss in TT compared to TTE trial.
4.3 Introduction

Exercise-induced fatigue is directly and proportionally associated with a reduction in performance and muscular function [1]. The origin of the fatigue is generally classified as being proximal (central fatigue) and/or distal (peripheral fatigue) to the neuromuscular junction [2, 3]. While fatigue may be assessed using a number of techniques, the decrement in maximal voluntary contraction (MVC) torque before and after exercise [4] enables the quantification of total fatigue. The individual contributions of central and peripheral fatigue can then be assessed by combining electrical stimulation techniques with the MVC, known as the twitch interpolation technique [5, 6]. This combination has been widely adopted to characterise and identify mechanisms of neuromuscular fatigue arising from isometric exercise [7, 8] prolonged dynamic exercise [9-11] and extreme prolonged dynamic exercise [11, 12].

In turn, constant power time-to-exhaustion (TTE) protocols and self-paced time-trials (TT) are widely used to assess cycling performance [13, 14]. The validity of the TTE protocol, however, has been questioned on the basis that cyclists rarely maintain constant power output during competition [14, 15]. Additionally, the TT has demonstrated higher repeatability and lower variability compared to TTE trials [13, 16] and the TT has the benefit of enabling the cyclist to self-regulate their workload. If a cyclist adopts an inappropriate pacing strategy during the TT, however (i.e., starting too fast), performance is likely to be less than compared to a duration-matched TTE (~14.5min) [17].

The assessment of neuromuscular fatigue in response to dynamic exercise has largely been conducted using constant-load protocols (i.e., TTE), with very few studies assessing neuromuscular fatigue following self-paced (i.e., TT) efforts [18-20]. With the exception of one study [20], these have typically involved short-duration (i.e., less than 30 min) efforts. The study by Thomas et al. [20] compared TT of different duration (4-km, 20-km, and 40-km) and found peripheral fatigue was significantly greater in short-duration TT (i.e., 4-km), while central fatigue was greater in longer-duration TT (i.e., >30-min); a finding corresponding with research adopting constant-load protocols [1]. However, direct comparison of
neuromuscular fatigue during constant-load and self-paced trials have, to the authors’ knowledge, not been conducted. While neuromuscular fatigue is significantly greater during highly variable exercise loads compared to constant-load exercise [21], this difference diminishes when the variability is reduced [22]. Thus, the aim of the present study was to investigate and compare the neuromuscular fatigue associated with an isoenergetic prolonged TT and TTE protocol. The authors hypothesised that performance would be superior (higher power output; shorter time to completion) in the TT, and this would be associated with greater peripheral fatigue, but not central fatigue.
4.4 Methods and procedures

Participants

Eighteen healthy and recreationally active (≥2 sessions per week) males, aged between 19 to 44 years old were recruited to this study via local advertisement posted on public noticeboards. Participants were initially screened for eligibility via email and follow-up telephone conversation. Exclusion criteria for this study included: diagnosis of diabetes mellitus; uncontrolled hypertension; acute (i.e., viral infection) or chronic inflammatory disease; currently taking medication for chronic health conditions. Additionally, participants were excluded (or referred to their General Practitioner to obtain exercise clearance) if they presented with more than two risk factors in stage two or checked ‘Yes’ at stage one of the Exercise and Sport Science Australia (ESSA) risk assessment questionnaire, or if they were identified as having any condition which may be exacerbated through exercise participation. The Murdoch University Human Research Ethics Committee approved the study protocol (No. 2013/206) and all experiments were performed in accordance with relevant guidelines and regulations. All participants were informed about the experimental procedures and provided written consent prior to participation.

Study design

This study comprised two phases: i) phase one comprised a single preliminary and familiarisation session, and; ii) the experimental phase which consisted of TTE trial and a TT trial. All preliminary and experimental testing was conducted on a cycle ergometer (Velotron, Racermate Inc., Seattle, USA) and all trials completed at least three days apart in sequential (non-randomised) order. The TT was always performed after the TTE because the target workload for the TT was based on the workload achieved during the TTE trial. All testing was conducted at the Murdoch University Exercise Physiology Laboratory.
### Preliminary testing and familiarisation

During this session, participant’s peak oxygen uptake ($\text{VO}_2\text{peak}$; ParvoMedics, Utah, USA) was determined using a continuous incremental cycle protocol. The protocol for the $\text{VO}_2\text{peak}$ test consisted of an initial workload of 50 W, which was increased 35 W in a step-wise fashion every 3 min. Finger prick blood samples were collected at the end of each stage for measurement of blood lactate (Lactate Plus, Nova Biomedical, Massachusetts, USA) until the lactate concentration exceeded 4 mmol.L$^{-1}$. Thereafter, the workload increased 35 W every minute until exhaustion (defined as not being able to maintain cadence above 60 rpm despite verbal encouragement). The workload corresponding to a blood lactate concentration of 3 mmol.L$^{-1}$ ($W_{3\text{mM}}$) was then interpolated from this test and recorded. The ratings of perceived exertion (RPE) and heart rate (HR; Polar, Kempele, Finland) were measured in the last 10 s of each stage and data from the metabolic cart collected continuously and assessed in 15 s of epochs for determination of $\text{VO}_2\text{peak}$.

Familiarisation with the TTE cycling test was then conducted after a 20-min period of recovery from the $\text{VO}_2\text{peak}$ test. The workload during the 20-min TTE familiarisation was set at the predetermined $W_{3\text{mM}}$, and where necessary, the workload was adjusted to ensure the blood lactate concentration was fixed at 3 mmol.L$^{-1}$. The cycle ergometer settings and workloads were recorded and remained consistent throughout the experimental trials. Preliminary testing and familiarisation with the neuromuscular assessments (MVC; supramaximal electrical stimulation) were then conducted on the isokinetic dynamometer (Humac Norm, CSMI, Massachusetts, USA) as detailed below.

### Maximal voluntary contraction (MVC)

Participants were comfortably secured in a seated position using thigh, pelvic, shoulder and torso straps, in order to reduce body movement during the test. The hip joint was positioned at an angle of 85°. The axis of the dominant knee was brought into line with the axis of the dynamometer exercise arm. The lateral femoral epicondyle was used as a bony landmark for matching the axis rotation of the
dynamometer shaft. The same radius for each participant was applied in all experimental trials. Participant’s dominant ankle was strapped and then the dominant knee joint angle was adjusted to 60° of flexion (0°=leg fully extended). Participants performed an initial warm-up using the non-fatiguing submaximal contraction of the quadriceps muscle group. Participants then performed 3x5 s MVC of the dominant knee extensor muscles, with 2-min recovery between repetitions.

**Electrical stimulations**

A self-adhesive silver chloride electrode with a circular surface (Skintact, Leonhard Lang, Florida, USA) of 0.5 mm diameter was used as the cathode while a 50 cm² (10x5 cm) rectangular electrode (ValuTrode Cloth, Axelgaard, California, USA) was used as the anode for the femoral nerve stimulation. The cathode was located on the femoral nerve of the dominant limb and anode in the gluteal fold opposite the cathode. The participant’s femoral nerve was stimulated (transcutaneous electrical stimulation) using a constant-current stimulator (model DS7AH; Digitimer, Hertfordshire, England) coupled with a train/delay generator (model DG2A; Digitimer, Hertfordshire, England) to control frequency and duration of the stimulus. The stimulation site was adjusted based on the greatest visible muscle contraction response. Voltage was set at maximal 400 V with a stimulus duration 1 ms, and a 10 ms delay was used during the doublet stimulations. The use of doublets with 10 ms delay is perceived as less painful compared to train stimulations [23]. The intensity of the stimulus was increased until there was no further increase in twitch force and the M-wave amplitude (peak-to-peak) of the vastus lateralis (VL). Supramaximal stimulus intensities were then conducted at 125% of this intensity for both the single and doublet stimulations.
**Electromyography (EMG) recordings**

Prior to electrode placement, the participant’s skin was shaved, rubbed and cleaned with alcohol to minimize skin impedance over the quadriceps region of the participant’s dominant leg. Self-adhesive circular surface silver chloride electrodes (Duo-Trode; Myotronics Inc., Seattle, WA, USA) with a 10 mm diameter (an interelectrode center-to-center distance of 21±1 mm) was then placed over the muscle belly of VL. Electromyography activity was recorded using wireless transmitters (TeleMyo DTS EMG Sensor, Noraxon Inc., USA) and the data collected by the receiver (TeleMyo DTS Belt Receiver, Noraxon Inc., USA). Another receiver (TeleMyo 2400R G2 Receiver, USA) was used collect data from the isokinetic dynamometer (CSMI, Massachusetts, USA) and stimulator (Digitimer, Hertfordshire, England), and these were then synchronized with EMG muscle activity for analysis. EMG signals were analog-to-digital (A/D) converted using a 16-bit A/D card with a ±2.5 V range and were sampled at 1500 Hz. Raw EMG signals were analog bands pass filtered with a range of 10-500 Hz and were differentially amplified (common mode rejection ratio >90 dB at 60 Hz; impedance input =2 MΩ; gain 500) to maximize signal amplification. The data were stored for analysis with Noraxon’s Myoresearch XP – Basic Edition software (Noraxon Inc., USA).

**Experimental phase**

Participants arrived at the laboratory in a fasted state and having avoided vigorous physical activity on the day prior to the trials. On the first trial, participants recalled their dietary intake from the prior day (24-h food diary/recall) and were asked to replicate the dietary intake prior to the second trial. Thirty minutes prior to testing, participants consumed 7 ml.kg$^{-1}$ body weight (BW) of artificially flavoured water, and then 5 ml.kg$^{-1}$ BW of the same drink 10 min prior to testing to minimise pre-exercise dehydration. Participants were asked to empty their bladder immediately before the initiation of exercise.

Participants then performed a warm-up which included submaximal contractions of the dominant knee extensor muscles (~50%, 60%, 70% of MVC for 5 s at each intensity with 15 s interval) followed by ~10 non-fatiguing submaximal
contractions at the participant’s own preferred intensity. Participants were then given 3 single supramaximal electrical stimulations separated by 3 s (total duration of 9 s); prior to the doublet supramaximal electrical stimulation delivered 3 s before (resting doublet), during (superimposed doublet over the isometric plateau), and ~2 s after the MVC (potentiated doublet) with total duration of 15 s; followed by 3 single supramaximal electrical stimulations separated by 3 s [4, 5].

Participants were then transferred to the cycle ergometer (Velotron, Racermate Inc., Seattle, USA) where they performed either the TTE (trial 1) or the TT (trial 2). The power for the TTE was set at $W_{3mM}$ and participants verbally encouraged to exercise until exhaustion (unable to maintain a cadence of 60 rpm). Participants were also provided with the same artificially flavoured drink at 2 ml.kg$^{-1}$ BW every 15 min during cycling and were also provided ad libitum access to plain water. The fluid consumption protocol was replicated in the subsequent experimental trial (TT). The total work performed was recorded and then used as the target in the TT. Immediately after completion of the TTE and the TT, participants were transferred back to the isokinetic dynamometer (Humac Norm, CSMI, Massachusetts, USA) for neuromuscular assessment. The experimental protocol is presented in Figure 4.1.
Figure 4.1 Experimental design scheme of the two modes of prolonged exercise. Small arrow, single supramaximal electrical stimulation; large arrow, doublet supramaximal electrical stimulation.
Data analysis

M-wave peak-to-peak amplitude of VL and peak twitch (PT) torque were obtained from the average of three trials of single supramaximal electrical stimulation, and peak doublet (PD) torque was taken from potentiated doublet stimulations. Muscle Voluntary Action Level (VAL) was quantified using muscle Voluntary Activation Level with reference to the potentiated doublet (VAL_p) and with reference to the resting doublet (VAL_r) [4]. VAL_p was calculated as: [1 - (superimposed doublet amplitude/potentiated doublet amplitude)] x 100. The VAL equation was corrected when the superimposed doublet did not correspond with the real maximal torque, and in this case, VAL was calculated as: [1 – (superimposed doublet amplitude x voluntary torque level just before or after superimposed doublet/maximal voluntary torque)/potentiated doublet amplitude] x 100. This formula was used because superimposed amplitude tends to develop at torques lower than maximal [24], and has been and proven to be in a linear relationship [5]. Central Activation Ratio (CAR) is calculated using the following equation: [MVC / (MVC + superimposed doublet over isometric plateau)]. If CAR equals 1.0, it indicates a full activation of the stimulated muscle [25]. EMG activity of the surrogate muscle of quadriceps, VL muscle was collected within 500 ms timeframe during MVC, quantified as root mean square (RMS) and normalized to M-wave peak-to-peak amplitude of VL to obtain the ratio of RMS to maximal M-wave amplitude (RMS.M^-1) [4].

Statistical analysis

Results are expressed as mean ± standard deviation (SD) unless otherwise noted; while group differences between trials or time-points are presented as mean differences with 95% Confidence Intervals (CI). Differences to assess changes in the primary outcome variables (performance; neuromuscular assessments) between the two trials (TT; TTE) over time (Performance: 10 workload-equivalent intervals; PRE, POST, POST20) were estimated using linear mixed models (LMM). The hypothesis of interest was the condition by time interaction (modelled as fixed effects; random intercept) which was examined with pairwise comparisons of the
estimated marginal means. Main effects for trial and time were explored and assessed for relevance to each outcome. Statistical significance was set at 0.05. All statistical procedures were completed using IBM SPSS Statistics 22.
4.5 Results

Participants were young (24.7±4.8 y), lean with normal body mass index (BMI: 22.8±2.2 kg.m$^{-2}$) and recreationally active (VO$_{2peak}$: 40.9±6.1 ml.kg.min$^{-1}$).

![Figure 4.2](image)

**Figure 4.2** Total duration of exercise between TTE and TT trials; white and black columns, respectively. Mean ± SE.

**Cycling performance**

Participants completed the TT (4266.6±192.4 s) significantly (p=0.003) faster than the TTE trial (4853.7±191.8 s; mean difference: 587 s; 95% CI: 235, 939 s) (Figure 4.2). Mean power output (PWR; TT: 124.5±4.5 W; TTE: 110.4±4.5 W) and cadence (CDE; TT: 80.0±1.9 RPM; TTE: 77.6±1.9 RPM) were significantly higher in TT compared to TTE (Both p$\leq$0.001; Figure 4.3). Mean heart rate was significantly (p$\leq$0.001) higher in TT (149.0±3.0 bpm) than TTE (143.8±3.0 bpm) and increased over time (10 averaged intervals based on a percentage of workload completed) in both trials (main effect of time; p$\leq$0.001; time x trial; p=0.215). There was no significant trial by time interaction effects for RPE (p=0.826) and no
difference in the main effect of trial (TT: 13.4±0.4; TTE: 13.6±0.4, p=0.087; Figure 4.3); but there was a significant main effect of time (p≤0.001; Figure 4.3d).

**Figure 4.3** Variations in a) power output (PWR) b) cadence (CDE) c) heart rate (HR) and d) ratings of perceived exertion (RPE) during TTE and TT trials. These variables were recorded after the total duration of exercise was divided into 10 blocks and each of the ten periods (10–100%) data were averaged. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p≤0.001), ***Significant interaction effect (time x trial: p<0.05).

**Neuromuscular fatigue**

There was no significant trial by time (baseline: PRE, post-exercise: POST and 20-min post-exercise: POST20) interaction in MVC (p=0.500; Figure 4.4a). There was, however, a significant main effect of trial (p=0.005), with participants producing greater force during the TTE trial (mean difference: 10.52 Nm; 95% CI: 3.3, 17.7 Nm). There was also a significant main effect of time (p≤0.001), with force decreasing 11.8% immediately post-exercise (17.8 Nm; 95% CI: 9.0, 26.6 Nm) and remaining depressed (8.6%) at 20-min post-exercise (12.9 Nm; 95% CI: 4.1, 21.7 Nm) compared to baseline.
Figure 4.4 Changes in maximal voluntary contraction (MVC) torque b) muscle Voluntary Activation Level (VAL) c) Peak Twitch (PT) d) Potentiated peak doublet (PD) for TTE and TT at baseline (PRE), post-exercise (POST), and 20-min post-exercise (POST 20). Mean ± SE. *Significant time effect (p ≤ 0.001), **Significant trial effect (p<0.01).

There was a significant main effect of time (p≤0.001) for VAL, which demonstrated an 11.1% and 7.9% decrease at post-exercise and 20-min post-exercise, respectively. There was no significant interaction effect (p=0.658) or difference observed between trials (p=0.564; Figure 4.4b). Similarly, there was a significant main effect of time (p≤0.001) for CAR, which demonstrated a 4.1% and 3.1% decrease at post-exercise and 20-min post-exercise, respectively (Table 4.1). There was no significant interaction effect (p=0.747) or difference observed between trials (p=0.505).

There was a significant (p≤0.001) decrease in the PD over time, with a 24.2% and 19.9% decrease at post-exercise and 20-min post-exercise, respectively, but no differences between trials (p=0.227) and no significant interaction (p=0.847) (Figure 4.4d). There was a significant (p≤0.001) decrease in the peak twitch over
time, with a 44.9% and 33.6% decrease at post-exercise and 20-min post-exercise, respectively, but no differences between trials (p=0.305) and no significant interaction (p=0.602) (Figure 4.4c).

There were no significant differences in M-wave amplitude between trials (p=0.915) or time (p=0.146) and no significant interaction effects (p=0.813; Table 4.1). There was a significant difference in RMS.M⁻¹ across time (p=0.029) but not trial (p=0.409) and no significant interaction (p=0.91; Table 4.1).
Table 4.1 Mean (± SE) of muscle Voluntary Activation Level with reference to the resting doublet (VAL_r), Central Activation Ratio (CAR), root mean square normalized to the amplitude of M-wave (RMS.M⁻¹ ratio) and M-wave peak-to-peak amplitude. * p<0.05, indicates a difference between PRE and POST.

<table>
<thead>
<tr>
<th>Dependent variable</th>
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<th>SP</th>
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<tr>
<td></td>
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<td>POST</td>
<td>POST20</td>
<td>PRE</td>
<td>POST</td>
<td>POST20</td>
</tr>
<tr>
<td>VAL_r (%)</td>
<td>85.8 ± 3.3</td>
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<tr>
<td>CAR (%)</td>
<td>95.3 ± 1.2</td>
<td>92.0 ± 1.2</td>
<td>92.9 ± 1.2</td>
<td>95.6 ± 1.1</td>
<td>91.2 ± 1.1</td>
<td>92 ± 1.1</td>
</tr>
<tr>
<td>RMS.M⁻¹ ratio VL</td>
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<td>0.056 ± 0.07</td>
<td>0.043 ± 0.07</td>
<td>0.056 ± 0.07</td>
<td>0.050 ± 0.07</td>
<td>0.048 ± 0.07</td>
</tr>
<tr>
<td>M-wave peak-to-peak amplitude (mV)</td>
<td>9.06 ± 0.69</td>
<td>8.80 ± 0.69</td>
<td>8.51 ± 0.69</td>
<td>9.36 ± 0.67</td>
<td>8.55 ± 0.67</td>
<td>6.92 ± 0.67</td>
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</table>
4.6 Discussion

The purpose of the current study was to compare the performance of a cycling TTE trial to an isoenergetic cycling TT in recreationally active participants, as well as assess the associated markers of neuromuscular fatigue following the trials. The authors hypothesised that performance would be superior (higher power output; shorter time to completion) in the TT, and this would be associated with greater peripheral fatigue, but not central fatigue. The main findings of the study were i) Performance (time to complete and PWR) was significantly better in the TT than compared to TTE and this was not associated with differences in RPE; ii) Both TT and TTE induced significant decreases in maximal strength capacity of the knee extensors; iii) The reduction in MVC torque was associated with central and peripheral fatigue mechanisms, but there were no significant between-trial differences; iv) The reduction in MVC torque, and the central and peripheral mechanisms of fatigue, persisted during the 20-min recovery period.

Cycling performance and physiological responses

The current study adopted a TTE protocol which was designed to exceed 30 min in duration thereby inducing both central and peripheral fatigue [21, 22]. Indeed, the total duration of exercise in the present study was 80.9 and 71.1 min in TTE and TT trial, respectively. As hypothesised, time to complete the TT was significantly lower than compared to TTE. The parabolic power curve during the TT demonstrated both positive and negative pacing strategies and while the power output for the first 10% and last 10% of the trial was above the average power output for the trial (i.e., indicative of a U-shaped curve), the nadir occurred in the latter part of the TT (80% of completed workload; [26]). This uneven distribution of work during the trial, and in particular the fast finish at the end of the trial, may relate to the relative inexperience of these participants in cycling TT. The relatively even CDE throughout the TT indicates participants were more reliant on gearing rather than CDE to alter power in the TT. It is interesting to note the similar CDE between trials (TTE and TT) from approximately 40% into each effort. In accordance with the greater work being performed, HR was significantly higher in the TT compared
to TTE. Remarkably, despite the higher workload and HR during the TT, the RPE was no different between trials. Although perception of effort likely culminates from multiple physiological and psychological inputs [1], the apparent mismatch in the RPE-HR and RPE-workload relationships in the current study suggests either i) the non-cyclist participants were insensitive to these inputs and therefore possessed a poor ability to perceive current levels of effort, or; ii) participants were more sensitive to the duration of exercise rather than the workload (i.e., duration superseded other inputs) since the TTE took approximately 10% longer than the TT.

**Total and central fatigue parameters**

There was a significant decrease in MVC torque at post-exercise (immediately post-exercise: 11.8% decrease; 20-min post-exercise: 8.6% decrease), and this decrease was similar between exercise-trials. The reduction in MVC during the TTE trial was 8%, which was comparable to other studies adopting constant load cycling where decrements in MVC were 7% [21] to 9% [22] following 30 min cycling at 70% of maximal aerobic power (MAP), 9-13% after cycling at 80% of MAP for 30 min [27], 11% and 10% after cycling at 55% of MAP for 60 and 120 min, respectively [28]. The 16% decrease in MVC during the TT was higher than that in previous studies (i.e., 12.8% loss in MVC following 30 min of variable intensity cycling at 70% of MAP; [22]), but given the duration of exercise, this is aligned with expectations. The 20 min recovery was insufficient to observe recovery in MVC, with only minor (non-significant) increases observed between the immediate post-exercise and 20-min post-exercise time points (1.5% and 6.2% in TTE and TT, respectively).

The neuromuscular assessment revealed significant central fatigue in response to the cycling trials in each parameter assessed (CAR, VALp, VALr, RMS.M−1; Figure 4.4; Table 4.1). The VALp was adopted *a priori* as the primary outcome measure for central fatigue, and this decision was based on previous research demonstrating superior reproducibility in this technique [4, 29]. There was a significant main effect of time for VALp, which demonstrated an 11.1% and 7.9% decrease in VALp.
at immediately after and 20-min after the cycling trials, respectively. The reduction in VAL\(_p\) suggests less motor units were recruited during the MVC both immediately after and 20-min after the cycling trials. There were no between-trial differences observed. Similar to the findings in previous research [4], VAL\(_p\) demonstrated to be more sensitive to fatigue than CAR as evidenced by the greater magnitude of change. The greater variance in VAL\(_r\) and RMS.M\(^{-1}\) (Table 4.1) is consistent with these being associated with a greater random error of measurement, although this was shown to be only slightly higher in VAL\(_r\) compared to VAL\(_p\) [4]. However, it is noteworthy that at POST20, VAL\(_r\) was no longer significantly different from baseline values (Table 4.1). While 30 min of cycling at highly variable power output has been shown to result in additional muscular fatigue, and in particular significantly greater reductions in VAL compared to constant and low variable cycling [21], this difference was not observed when the variability in power output was reduced [22]. Although the power-curves in the current study demonstrate some variability, the extent of this variability in the current study was not as significant as that adopted in the highly variable trial, which comprised ten repeat intervals of cycling between 50\% and up to 200\% of MAP [21]. Irrespective of the lack of significant between-trial difference, the decrements in central activation (VAL\(_p\); and supported by VAL\(_r\), CAR, RMS.M\(^{-1}\) measures; Table 4.1) implicate central fatigue in the reduction of MVC torque following the prolonged cycling trials, and this is in agreement with prior findings [21, 22, 27, 28, 30]. Whether the central fatigue originated from supra-spinal or spinal mechanisms [2], or some combination therefore, cannot be determined using the adopted techniques.

It is important to acknowledge that additional factors may also contribute to central fatigue such as environmental temperature [31], altered hydration [32] and psychological status [33]. These factors may concurrently alter neural drive and thus alter (i.e., decrease) exercise performance. In the present study, all trials were conducted in a thermoneutral environment and fluids were provided prior to and during exercise to minimise the risk of dehydration.
**Electrically evoked peripheral fatigue parameters**

The amplitude of the electrically evoked torque responses from single and paired stimulations were used in the current study to assess the level of contractile fatigue following the prolonged cycling trials [4]. In agreement with other studies adopting prolonged cycling [21, 22, 27, 28, 30], there was a significant reduction in the PT and PD after cycling, and this remained depressed at 20-min post-exercise. However, there were no significant differences between trials, which is in contrast to our hypothesis that TT would induce greater levels of contractile fatigue than TTE. The hypothesis was based on the idea that despite the total work being matched, the improvement in TT performance would necessitate significantly greater PWR during the trial. Indeed, PWR during the TT was 12.8% higher than in the TTE trial, however, this was not associated with differences in contractile fatigue.

The mechanisms contributing to a reduction in evoked torque (PT and PD) and excitation-contraction coupling (ECC) failure, are believed to involve a number of processes including reduced calcium ions (Ca$^{2+}$) release from the sarcoplasmic reticulum [34] and reduced myofibrillar cross-bridge interaction [35]. These changes may be related to the accumulation of hydrogen ions (H$^+$) and inorganic phosphate [36], although energy substrates such as the reduced ratio of adenosine triphosphate to adenosine diphosphate (ATP: ADP) and muscle glycogen depletion may also play a role [37]. In contrast to previous studies which have shown PD to be more sensitive than PT [4, 21, 22], both PT and PD responded similarly to the cycling exercise. Both PT and PD have a number of disadvantages, wherein PT is considered less sensitive due to the higher number of stimuli required to produce acceptable signal-to-noise ratio [29] and easier detection [38]; while potentiated responses may have greater variability [39]. The present study demonstrated that PD appeared to recover more slowly compared to PT, which is in agreement with a previous study [4].

M-wave peak-to-peak amplitude of VL was used to assess knee extensor muscle membrane excitability, based on prior research demonstrating that the VL muscle is representative of the quadriceps muscle [4]. The present study indicated muscle
excitability was not significantly different between trials or across time. Importantly, the lack of significant difference between time-points is likely due to the large variance in the measure. Specifically, the mean difference between baseline and immediately post-exercise was -30.7mV with a 95% CI of [-902.7, 964.1]; while the difference between post-exercise and 20-min post-exercise was -829.9 mV with a 95% CI of [-146.3, 1806.2]. Therefore, the findings indicate no significant differences in the M-wave amplitude across time (baseline vs. post-exercise measures) and therefore, neuromuscular propagation was unaffected by the cycling trials and did not contribute to changes in PT or PD across time. However, this interpretation would need to be cautiously adopted in light of the large variances observed.

**Limitations**

This study had a number of strengths including the prolonged nature of the cycling bout, the number of participants, the isocaloric matching of the cycling trials and the techniques adopted to measure each outcome variable. However, the study also had a number of limitations. In particular, the variation in the training status of the participants was diverse, based on the VO_{2peak} data, and their familiarity with performing TT which may have influenced the findings. Motivations to cycle longer and to perform better could be one of the most crucial factors in the performing TT and TTE trials other than experience in pacing and cycle-fitness. Performance in open-loop exercise such as cycling TTE is particularly reliant on psychological factors compared to closed-loop exercise [13]. However, if the motivation was lower in the TTE versus TT, then MVC torque and VAL_p would be significantly different between trials but this was not the case in the current study. The time to conduct the neuromuscular fatigue assessment after the exercise protocol may also have affected the outcomes of the current study. In particular, the total time taken to perform the neuromuscular fatigue assessment immediately post-exercise was approximately 4 min, despite the isokinetic dynamometer being located near (less than 10 m) the cycle ergometer. Neuromuscular fatigue assessment was reported in previous studies [21, 22] to occur around 1 min post-exercise and this delay may have enabled partial recovery [30]. However, this
would result in a reduction in the excitation-contraction coupling (i.e., reduced PT and PD) and consider these measures demonstrated significant differences (indeed the greatest magnitude change) in the current study, this effect would likely have been marginal, in particular when considering that the 20-min post-exercise measures were found not to be significantly different across all central and peripheral parameters assessed. Finally, the order of the trials could not be randomised since the workload for the TT was based on the performance in the TTE trial. To negate this, the current study familiarised participants with the TTE and the neuromuscular assessments prior to conducting the trial. Given the similarity in the measures of fatigue outlined above, we do not believe this would have confounded results. In addition, there were no between-trial differences in any of the neuromuscular assessments performed.
4.7 Conclusion

The present study showed that performance in a self-paced TT was significantly improved compared to an isocaloric constant-paced TTE trial in young and recreationally active males. This improvement was not associated with any differences in perceived effort (RPE), despite maintaining significantly higher power-outputs and working at a higher physiological load (HR). The neuromuscular assessments revealed no significant between-trial differences, but the MVC torque was reduced by 11.8% immediately post exercise. This reduction in MVC torque was associated with a significant decrease in central fatigue ($\text{VAL}_p$: -11.1%), demonstrating a reduced level of neural drive to the muscle [40]. Additionally, peripheral factors contributed to the reduced MVC torque, with a significant decrease in the potentiated doublet (-24.2%) demonstrating reduced excitation-contraction coupling; although neuromuscular propagation is unlikely to have significantly contributed to the fatigue. These neuromuscular changes persisted for at least 20 min after the termination of exercise. The fatigue experienced by participants in the current study were clearly associated with decrements in both central and peripheral parameters. Recreationally active males unaccustomed to cycling demonstrated superior performance when completing a self-paced TT (closed-loop) compared to a constant-pace TTE (open-loop) trial. Future studies may investigate whether trained cyclists more accustomed to these tests yield similar results.
4.8 References


Chapter 5  The effect of acute branched-chain amino acids supplementation during isoenergetic prolonged cycling exercise on cycling performance and neuromuscular fatigue.
## 5.1 List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A/D</td>
<td>Analog-to-digital</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BCAA</td>
<td>Branched-chain amino acids</td>
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<td>BW</td>
<td>Body weight</td>
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<td>Calcium ions</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>Excitation-contraction coupling</td>
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<td>Placebo</td>
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<tr>
<td>POST</td>
<td>Post-exercise</td>
</tr>
<tr>
<td>POST20</td>
<td>20-min post-exercise</td>
</tr>
<tr>
<td>PRE</td>
<td>Pre-exercise/baseline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PT</td>
<td>Peak twitch</td>
</tr>
<tr>
<td>PWR</td>
<td>Power output</td>
</tr>
<tr>
<td>RPE</td>
<td>Ratings of perceived exertion</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TRP</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TT</td>
<td>Time-trial</td>
</tr>
<tr>
<td>TTE</td>
<td>Time-to-exhaustion</td>
</tr>
<tr>
<td>VAL</td>
<td>Voluntary activation level</td>
</tr>
<tr>
<td>VL</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>VO\textsubscript{2peak}</td>
<td>Peak oxygen uptake</td>
</tr>
<tr>
<td>W\textsubscript{3mM}</td>
<td>Workload corresponding to a blood lactate concentration of 3 mmol.L\textsuperscript{-1}</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
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</tbody>
</table>
5.2 Abstract

**Purpose:** To investigate the effects of acute branched-chain amino acids (BCAA) supplementation during isoenergetic prolonged self-paced cycling time-trial (TT) on cycling performance and neuromuscular fatigue.

**Methods:** Eighteen recreationally active men (mean±SD age: 24.7±4.8 years old; mass 67.1±6.1 kg; height 171.7±4.9cm) participated in this study. Participants performed constant cycling time-to-exhaustion (TTE) at an intensity corresponding to 3 mmol.L\(^{-1}\) of blood lactate concentration. The total work completed were recorded and participants performed self-paced cycling TT (randomised and double-blinded) aimed to complete same amount of work in the shortest time possible with ingestion either BCAA (7g.L\(^{-1}\)) or a placebo (PLA) solution. Maximal voluntary contraction (MVC), muscle voluntary activation level (VAL) and electrically evoked torque using single (PT) and doublet (PD) stimulations were assessed at baseline (PRE), immediately post-exercise (POST) and 20-min post-exercise (POST20).

**Results:** Time to completion in BCAA (3978.7±166.7 s) was significantly (p=0.04) lower compared to PLA (4266.6±192.4 s). There was a significant effect of time on MVC, VAL, PT and PD which the parameters were significantly lower at point of exhaustion (MVC: -23.5±6.7 Nm; p≤0.001; VAL: -9.7±1.8 %; p≤0.001; PT: -15.7±2.0 Nm; p≤0.001; PD: -18.4±4.6 Nm; p=0.002) and 20-min post-exercise (MVC: -15.6±6.7 Nm; p=0.002; Nm; VAL: -7.4±1.8 %; p≤0.001; PT: -11.4±2.0 Nm; p≤0.001; PD: -14±4.6 Nm; p=0.015) when compared to pre-exercise values. There was no significant interaction between (trial x time) or difference between trial.

**Conclusion:** Acute BCAA supplementation significantly improved performance in prolonged cycling TT among recreationally active individuals without any notable changes in either central or peripheral factor.
5.3 Introduction

The mechanisms contributing to the onset of fatigue during prolonged exercise [1] are complex and involve both central and peripheral factors (as demonstrated in Chapter 4 and [2]). The contribution of central factors to the onset of fatigue (central fatigue) increases concomitant with the duration of exercise [3]. Central fatigue is associated with supraspinal and spinal sites connecting the brain to the working muscle via complex neural networks [4]. Although typically investigated in isolation, it appears likely that an important interplay between the peripheral and central factors occurs during the onset of fatigue. Indeed one such hypothesis, known as the ‘Central Fatigue Hypothesis’ [5] suggests that an increased concentration of a single neurotransmitter, serotonin (5-hydroxytryptamine; 5-HT) in the central nervous system (CNS), may largely explain the onset of central fatigue. The precursor to 5-HT is tryptophan (TRP), and the rate of 5-HT synthesis is proportional to the rate of TRP entry into the CNS. Tryptophan entry into the CNS in turn, is proportional to the amount of free TRP (unbound TRP; f-TRP) and the concentration of the branched-chain amino acids (BCAA; isoleucine, leucine, and valine) which compete for entry into the CNS with TRP.

The large majority of TRP circulating in the plasma is loosely bound to albumin, but TRP may be displaced from albumin by an increase in plasma free fatty acids (FFA) which outcompetes the TRP leading to increased concentration of f-TRP [6]. The increased f-TRP may now enter the CNS via specific transporters in the blood-brain barrier (BBB), which also transport the BCAA. Since increased lipolysis and the associated increased FFA concentration is not observed in exercise lasting less than 30 min, this mechanism of stimulating central fatigue is likely to only occur in prolonged exercise [7]. Importantly, studies on central fatigue have been done in both rats [8, 9] and humans [10, 11], but only the outcome of animal studies have concurred with the ‘Central Fatigue Hypothesis’ while discrepancy exists in human studies. This discrepancy is due to the complexity in measuring the changes in neurotransmitters in the CNS, and specifically in the motor cortex of the brain in humans. Despite the equivocal nature of the findings however, researchers observing diminished central activation following prolonged exercise, often implicate serotonergic activity in the reduced corticospinal impulses [3, 12, 13]. An
additional approach to assess the relative contribution or importance of the ‘Central Fatigue Hypothesis’ to exercise-induced fatigue, is to manipulate the transport of f-TRP into the CNS, by increasing the BCAA concentrations.

The effectiveness of BCAA in reducing the concentration of 5-HT synthesis in the brain and thus, delaying fatigue remains unclear. Some studies indicate that BCAA can contribute to improving performance [14-16] while others have failed to demonstrate improvements in performance [1, 7, 17-19]. Large heterogeneity in methodology may have contributed to these discrepant findings. Additionally, none of the previous studies using BCAA have adopted twitch interpolation techniques to assess for central and peripheral contributors to fatigue. The main aim of the present study therefore, was to investigate the effect of acute BCAA supplementation on performance during a prolonged cycling time-trial (TT), and to determine the relative changes in peripheral and central factors contributing to fatigue. We hypothesised that acute supplementation of BCAA will reduce neuromuscular fatigue, delay onset of fatigue and thereby improve prolonged cycling performance.
5.4 Methods and procedures

Participants

Eighteen recreationally healthy and physically active men (≥90 min per week) volunteered to participate in the study after having been informed of the experimental procedures and they provided written informed consent. Participants were recruited from the community via local advertisement posted on public noticeboards. Table 5.1 shows the participant’s physical characteristics and physiological capacities.

Table 5.1 Physical characteristics and physiological capacities of the participants.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24.7 ± 4.8</td>
</tr>
<tr>
<td>Standing height (cm)</td>
<td>171.7 ± 4.9</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>67.1 ± 6.1</td>
</tr>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>22.8 ± 2.2</td>
</tr>
<tr>
<td>VO₂peak (ml.kg.min⁻¹)</td>
<td>40.9 ± 6.1</td>
</tr>
</tbody>
</table>

Participants were pre-screened for eligibility, via email and telephone conversation using the Exercise and Sports Science Australia (ESSA). Potential participants with diabetes mellitus, uncontrolled hypertension, acute (i.e., viral infection) or chronic inflammatory disease, currently taking medicine for chronic health conditions, condition which may be exacerbated through exercise participations were excluded. Participants were also excluded if they presented with more than two risk factors in stage two or checked ‘Yes’ at stage one of the ESSA risk assessment questionnaire. The Murdoch University Human Research Ethics Committee has granted approval (No. 2013/206) and the study protocol and all experiments were performed in Murdoch University Exercise Physiology Laboratory under relevant guidelines and regulations.
Study design

The present study consisted of 2 phases: i) preliminary and familiarisation session, and ii) an experimental phase which consisted of two cycling TT, which were conducted in double-blinded, randomised order and counterbalanced at least 3 days apart. Parts of this protocol and data have been previously presented (Chapter 3 and 4).

Preliminary testing and familiarisation

Preliminary testing and familiarisation was identical to Chapter 4. Briefly, participants’ peak oxygen uptake (VO$_{2\text{peak}}$; ParvoMedics, Utah, USA) was determined using a continuous step-wise incremental cycling protocol on a cycle ergometer (Velotron, Racermate Inc., Seattle, USA). Blood lactate (Lactate Plus, Nova Biomedical, Massachusetts, USA) was measured by finger prick blood samples collected at the end of each stage to determine the workload corresponding to a blood lactate concentration of 3 mmol.L$^{-1}$ (W$_{3\text{mM}}$). The ratings of perceived exertion (RPE) and heart rate (HR; Polar, Kempele, Finland) were measured in the last 10 s of each stage and data from the metabolic cart collected continuously and assessed in 15 s of epochs for determination of VO$_{2\text{peak}}$.

Preliminary testing and familiarisation with the cycling time-to-exhaustion (TTE) and neuromuscular assessments (maximal voluntary contraction, MVC; supramaximal electrical stimulation) was then conducted on the isokinetic dynamometer (Humac Norm, CSMI, Massachusetts, USA) as briefly outlined below and detailed in Chapter 4. In a subsequent visit to the laboratory at least 24 h after this session, participants performed cycling TTE at W$_{3\text{mM}}$ until volitional exhaustion (unable to maintain a cadence of 60 rpm) despite verbal encouragement. The total work performed was recorded and used as the target in the experimental trials.
Maximal voluntary contraction (MVC)

Participants’ were secured into the isokinetic dynamometer (Humac Norm, CSMI, Massachusetts, USA) using thigh, pelvic, shoulder, and torso straps in order to minimise body movement during the test. The axis of the knee was aligned with the axis of the dynamometer arm and trials were performed on the dominant leg and participant’s ankle which the knee joint was adjusted at 60° of flexion (0° = leg fully extended) and the hip joint at an angle of 85°. Following a warm-up session using non-fatiguing submaximal contractions, participants then proceeded with 3x5 s MVC of the dominant knee extensor muscles with 2 min gap between repetitions.

Electrical stimulations

Participant’s femoral nerve was stimulated (transcutaneous electrical stimulation) using a constant-current stimulator (model DS7AH; Digitimer, Hertfordshire, England) coupled with train/delay generator (model DG2A; Digitimer, Hertfordshire, England) to control frequency and duration of stimulus. The cathode was located on the femoral nerve of dominant limb, and anode in the gluteal fold opposite the cathode. The stimulation site was adjusted based on the greatest visible muscle contraction response. Voltage was set at maximal 400 V and duration of 1 ms, and a 10 ms delay was used during doublet stimulations. The doublet with 10 ms delay are perceived as being less painful compared to train stimulations [20]. The femoral nerve was stimulated starting from low intensity and then the intensity of the stimulus was increased until there was no further increase in twitch force and peak-to-peak M-wave amplitude of vastus lateralis (VL). Supramaximal stimulus intensities were determined by adding further 25% of obtained maximal stimulus and then maintained for single and doublet stimulations throughout the trials respectively to each participant.
Electromyography (EMG) recordings

Prior to electrode placement on the quadriceps region of the participant’s dominant leg, participant’s skin was shaved, rubbed and cleaned with alcohol to minimise skin impedance. A pair of self-adhesive silver chloride electrodes with a circular surface (Duo-Trode; Myotronics Inc., Seattle, WA, USA) of 10 mm diameter (inter-electrode center-to-center distance of 21±1 mm) was placed on muscle belly of VL. Electromyography (EMG) activity was recorded from VL of every participant’s dominant limb using wireless transmitters (TeleMyo DTS EMG Sensor, Noraxon Inc., USA) which directly collected EMG activity from the transmitter and transmitted the data to the receiver (TeleMyo DTS Belt Receiver, Noraxon Inc., USA). Another receiver (TeleMyo 2400R G2 Receiver, USA) was used to receive data from isokinetic dynamometer (CSMI, Massachusetts, USA) and stimulator (Digitimer, Hertfordshire, England) which were then synchronized with EMG muscle activity for analysis. EMG signals were analog-to-digital (A/D) converted using a 16-bit A/D card with a ±2.5 V range and were sampled at 1500 Hz. Raw EMG signals were analog bands pass filtered with a range of 10-500 Hz and were differentially amplified (common mode rejection ratio >90 dB at 60 Hz; impedance input =2 MΩ; gain 500) to maximise signal amplification. The data was stored for analysis with Noraxon’s Myoresearch XP – Basic Edition software (Noraxon Inc., USA).

Experimental phase

During the experimental phase, participants arrived at the laboratory in a fasted state and having avoided vigorous physical activity on the day prior to the trials. On the first trial, participants recalled their dietary intake from the prior day (24-h food diary/recall) and were asked to replicate the dietary intake prior to the second trial. The participants were provided with 7 ml.kg⁻¹ body weight (BW) of either BCAA (leucine: valine: isoleucine; 2:1:1) or placebo (PLA; low calorie drink) 30 min prior to the initiation of exercise, and 5 ml.kg⁻¹ BW of either BCAA or placebo 10 min prior to testing, to minimise pre-exercise dehydration. Participants were also provided with either BCAA or placebo every 15 min during cycling at the rate of 2
ml.kg⁻¹ BW as well as ad libitum access to plain water. The same fluid consumption protocol was applied in every trial. Participants were asked to empty their bladder immediately before the initiation of exercise.

Participants performed a warm-up session that included a series of submaximal contractions of the dominant knee extensor muscles (at ~50%, 60%, 70% of MVC for 5 s at each intensity with 15 s recovery in between) followed by ~10 non-fatiguing submaximal contractions at participant’s own chosen intensity. Participants were then given 3 single supramaximal electrical stimulations separated by 3 s (total duration of 9 s) prior to the doublet supramaximal electrical stimulations separated by 3 s before (resting doublet), during (superimposed doublet over the MVC isometric plateau), and ~2 s after the MVC (potentiated doublet) with total duration of 15 s; followed by 3 single supramaximal electrical stimulation separated by 3 s [21, 22]. Participants were then transferred to the cycle ergometer (Velotron, Racermate Inc., Seattle, USA) where they performed self-paced cycling TT completing the same amount of total work completed in the preliminary trial (cycling TTE). Immediately after the completion of the TT, participants were transferred back to the isokinetic dynamometer (Humac Norm, CSMI, Massachusetts, USA) for neuromuscular assessment. The experimental protocol is graphically presented in Figure 4.1 of Chapter 4 and has not been replicated here to minimise duplication.

**Data analysis**

Peak twitch (PT) torque was obtained from the average of three trials of single supramaximal electrical stimulation, and peak doublet (PD) torque was taken from potentiated doublet stimulations. Muscle activation was quantified using muscle Voluntary Activation Level (VAL) [22]. VAL was calculated as: 

\[
[1 - \frac{\text{superimposed doublet amplitude}}{\text{potentiated doublet amplitude}}] \times 100.
\]

The VAL equation was corrected when the superimposed doublet did not correspond with the real maximal torque, and in this case VAL was calculated as: 

\[
[1 - \frac{\text{superimposed doublet amplitude} \times \text{voluntary torque level just before or after superimposed doublet}}{\text{maximal voluntary torque}}]/\text{potentiated doublet amplitude}] \times 100.
\]

This
formula was used because superimposed amplitude tends to develop at torques lower than maximal [23], and has been and proven to be in linear relationship [21].

**Statistical analysis**

Results are expressed as mean ± standard deviation (SD) unless otherwise noted. A mixed model analysis has been used to examine the effects of different modes of cycling across time and the interaction between the two. Least significance difference (LSD) was used as Confidence Interval (CI) Adjustment. Significance level was set at p<0.05. Paired sample *t-tests* were used to determine if there is any difference in baseline (PRE) of neuromuscular fatigue parameters between the two trials. All statistical procedures were completed using IBM SPSS Statistics 22.
5.5 Results

Time to completion and physiological responses

Time to completion was significantly (p=0.04) faster in the BCAA trial compared to PLA (Figure 5.1). The mean time to completion in PLA and BCAA was 4266.6±192.4 and 3978.7±166.7 s, respectively (Table 5.2).

![Figure 5.1](image.png)

Figure 5.1 Time to completion between PLA and BCAA trials; white and black columns, respectively. Mean ± SE.

This represents a 6.7% improvement in time with the BCAA supplementation. Accordingly, mean power output (PWR) was significantly higher (main effect for trial: p≤0.001; 5.7 W, 95% CI [2.9W, 8.5W]) in the BCAA trial, although there was no significant time x trial interaction (p=0.81; Table 5.2; Figure 5.2). This difference was not due to differences in cadence (CDE), with no significant interaction (p=0.981) or main effects between condition observed (p=0.643).
Table 5.2 Mean (± SD) values of total duration of exercise (s) and mean (± SE) of power output (PWR), cadence (CDE), heart rate (HR) and ratings of perceived exertion (RPE) for PLA and BCAA trials. * p<0.05, ** p<0.01, *** p<0.001 between trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time to Completion (s)</th>
<th>PWR (W)</th>
<th>CDE (rpm)</th>
<th>HR (bpm)</th>
<th>RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>4266.6 ± 816.2</td>
<td>124.5 ± 4.5</td>
<td>80.0 ± 1.9</td>
<td>149.0 ± 3.0</td>
<td>14.1 ± 0.4</td>
</tr>
<tr>
<td>BCAA</td>
<td>3978.7 ± 707.2</td>
<td>130.2 ± 4.5 ***</td>
<td>80.2 ± 1.9</td>
<td>151.2 ± 3.0</td>
<td>13.6 ± 0.4 **</td>
</tr>
</tbody>
</table>

The mean HR was significantly (p=0.017) higher in BCAA compared to PLA, and increased with time (main effect: p≤0.001; Figure 5.2). Despite the higher PWR and HR, the mean RPE was significantly (p=0.003) lower in BCAA than compared to PLA (no interaction effect: p=0.950) and increased in time in both trials (main effect of time: p≤0.001).
Figure 5.2 Changes in a) power output (PWR), b) cadence (CDE), c) heart rate (HR) and d) ratings of perceived exertions (RPE) between PLA and BCAA trials. These variables were recorded after the total duration of exercise was divided into 10 blocks and each of the ten periods (10–100%) were data-averaged. *Significant time effect (p≤0.001). **Significant trial effect (p<0.05).

Neuromuscular changes

There was a significant main effect for time (p≤0.001) but not condition (p=0.119) for MVC torque, and there was no significant interaction between time and trial (p=0.994; Figure 5.3). The cycling TT was associated with a 15.5% decrement in MVC torque immediately post-exercise (POST; -23.5 Nm; 95%CI [-33.2 Nm, -13.9 Nm]), and this remained 10.3% lower than baseline values at 20-min post exercise (POST20; -15.6 Nm; 95%CI [-25.2 Nm, -5.9 Nm]).

There was a significant main effect for time (p≤0.001) but not condition (p=0.429) for VAL, and there was no significant interaction between time and trial (p=0.750; Figure 5.3). The cycling TT was associated with a 9.7% decrement in VAL immediately post-exercise (95% CI [-13.7, -5.6]), and this remained 7.4% lower than baseline values at 20-min post exercise (95% CI [-11.4, -3.3]). There was a
significant main effect for time (p=0.004) but not condition (p=0.369) for PD, and there was no significant interaction between time and trial (p=0.998). The PD decreased 22.6% as a result of the cycling TT (mean [95% CI]: -18.4 Nm [-29.6, -7.2]) remained 17.3% lower than pre-exercise values at 20-min post exercise (mean [95% CI]: -14.0 Nm [-25.2, -2.8]). There was a significant main effect for time (p≤0.001) but not condition (p=0.180) for PT, and there was no significant interaction between time and trial (p=0.806). The PT decreased 53% as a result of the cycling TT (mean [95% CI]: -15.7 Nm [-18.2, -13.2]) and although the 20-min post-exercise value showed a significant (p=0.001; Figure 5.3) recovery from immediately post-exercise (mean [95% CI]: 4.34 Nm [1.82, 6.86], it remained 38.4% lower than pre-exercise values (mean [95% CI]: -11.4 Nm [-13.9, -8.9]).

Figure 5.3 a) Maximal voluntary contraction (MVC) torque b) muscle Voluntary Activation Level (VAL) c) peak twitch (PT) d) peak doublet (PD) for PLA and BCAA at baseline (PRE), immediately post-exercise (POST), and 20-min post-exercise (POST 20). *Significant time effect (p<0.01).
5.6 Discussion

This is to the authors’ knowledge, the first study to compare the effect of acute supplementation with BCAA on cycling performance and neuromuscular changes of quadriceps muscle compared to PLA. The findings showed that i) acute BCAA supplementation improved cycling performance by ~7% and this was associated with a significantly reduced RPE; ii) the MVC torque of knee extensors was significantly reduced in both trials, but there were no between-trial differences; iii) there was significant contribution of central and peripheral factors in the development of fatigue, but these were not different between trials. Overall, these findings suggest that acute supplementation of BCAA improved cycling performance and perception of effort during the cycling TT, without any notable difference in neuromuscular changes between trials.

Supplementation with BCAA resulted in a significantly improved TT performance, with participants cycling for an average 66 min when ingesting BCAA versus 71 min in the PLA trial. The power curves showed remarkably similar work distribution between trials, representing a combination of a reverse J-curve and U-shaped power curve [24] with the highest power outputs produced in the first and last 10% periods. The improved performance with acute BCAA supplementation during prolonged exercise is in accord with some findings [7, 15] but not all [1, 18, 25-27]. Our findings are in contrast to the findings of the elegant study conducted by van Hall et al. [1], who demonstrated no significant improvements in a time-to-exhaustion task with BCAA supplements mixed into CHO (6% sucrose) beverages. This null-finding was despite the supplements raising BCAA concentrations and increasing estimated TRP uptake by up to 20-fold [1]. However, prolonged cycling time-to-exhaustion trials have been criticised as they may be confounded by psychological factors such as boredom and motivation, as well as not being as reproducible as closed-loop tasks such as a cycling TT [28]. However, a study by Blomstrand et al [25] which also reported no significant performance improvements with BCAA supplementation, adopted a 20-min maximal work capacity task as the primary outcome measure of performance and this was preceded by a 60-min constant power task. It is interesting to note however, that the BCAA supplementation improved performance in these seven endurance trained cyclists.
by 9.9% (Cohen’s $d$: 0.36) and significantly reduced the RPE during the 60-min constant cycling period [25]; suggestive that the study may have been underpowered. Indeed, another study which concluded no benefits of BCAA supplementation [18], also demonstrated a 5.9% (Cohen’s $d$: 0.24) improvement in a time-to-exhaustion trial. The participants were in a glycogen depleted state and performed exercise in a warm environment (30.0°C), which was expected to enhance the effects of BCAA supplementation due to the possible reduced central drive associated with exercise in this condition [29].

Whilst multiple methodological differences between these studies exist and may help explain these discrepant findings, it is also clear that large inter-individual differences exist, which is evidenced by the large variance observed within studies. This has previously been noted [18] and may be due to individual differences in either TRP transport across the BBB, or sensitivity to serotonergic activity. It is also important to note, that the 5-HT receptor family is large, and differences in receptor expression may also largely account for the differences noted [30], particularly when dichotomising participants according to their performance characteristics (high versus low) [7].

The percentage of MVC loss was similar (15.5%) in both trials after 71 minutes (PLA) and 66 minutes (BCAA) of self-paced cycling. The percentage of MVC loss was comparable to previous studies with shorter duration of exercise (30-33 minutes); where MVC reduced by 12.8% [31] and 12.1% [32] following a variable intensity cycling at 70% of maximal aerobic power (MAP). MVC also remained significantly lower in both trials compared to the baseline following the 20 min of recovery. Interestingly, another study has also shown that MVC remains unchanged following 30 min of recovery after completing 27 min of cycling at 80% maximal power output [33].

This study adopted the twitch interpolation technique to measure VAL, which has been used to assess neuromuscular changes following prolonged cycling exercise [3, 31, 32, 34, 35]. Prolonged exercise has been shown to increase serotonergic activities [36], and in turn, this leads to the reduction of corticospinal impulses reaching the motoneurons and consequently reduced central activation [3, 12, 13]. The significant decrease in VAL following prolonged exercise in this study was in
agreement with previous studies [3, 31, 32, 34, 35] and is in accord with the stated hypothesis. However, the decrement in VAL following prolonged cycling was similar in both trials, which was not expected, given that we hypothesised BCAA supplementation to lessen ‘central fatigue’ (as measured by VAL). It is important to note that increased BCAA supplementation is also associated with increased plasma concentrations of ammonia [1, 18], which may attenuate the ergogenic effects of BCAA supplementation [6], particularly as it relates to reduced central drive. Unfortunately, the current study did not measure changes in ammonia concentration.

Electrically evoked torque was adopted in the current study to assess contractile fatigue (i.e., measure of peripheral fatigue) by using single (PT) and doublet (PD) stimulations. Similar contractile fatigue levels were observed in both trials; although PT also demonstrated significant recovery in the 20 min period following exercise. This more rapid response in PT is consistent with previous studies [22, 37], although the mechanisms for these differences are unclear, it may relate to contrasting effects of potentiation on increasing phosphorylation of myosin and the concurrent fatigue-associated reduction in intra-myocellular calcium ions (Ca\(^{2+}\)) concentration [38]. Therefore, although the adoption of PD over PT is generally recommended [22], interpretation of PD following fatiguing exercise has been cautioned [38]. The decrease in pH (or increase in hydrogen ions) is highly associated with force-reduction [39] and increases in hydrogen ions accompanied with inorganic phosphate [40-42] promotes muscle fatigue [32] and failure in excitation-contraction coupling (ECC). Excitation-contraction failure is likely due to decreased cross-bridge interactions [43] and Ca\(^{2+}\) release from sarcoplasmic reticulum [44]. Importantly, the current study also assessed M-wave characteristics which is a marker of muscle excitability [45] and there were no main-effects (time or trial) noted. Therefore, the reduced capacity of the excitation-contraction within the current study, was not related to reduced capacity to propagate the action potential.
5.7 Conclusion

The present study found that acute supplementation of BCAA during prolonged cycling exercise significantly improved performance in a cycling TT without any notable differences in neuromuscular parameters among recreationally active individuals. This finding suggests that BCAA can be used as an ergogenic aid to enhance cycle-exercise performance in non-cyclists. The current BCAA dose is recommended and higher BCAA dose should be avoided to prevent hyperammonaemia which could alter exercise performance [14]. Similar to previous findings, there were large inter-individual differences observed in performance outcomes. Further studies to distinguish central fatigue at supra-spinal or spinal level is warranted in response to BCAA supplementation, and the inter-individual differences to BCAA supplementation may yield important knowledge in this field.
5.8 References


Chapter 6  Metabolic and performance responses to the ingestion of different carbohydrates during prolonged exercise
6.1 List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ions</td>
</tr>
<tr>
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<td>Cycling economy</td>
</tr>
<tr>
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</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
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<td>Glucose/monosaccharide</td>
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<td>Respiratory exchange ratio</td>
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<td>Ratings of perceived exertion</td>
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<tr>
<td>VO_{2peak}</td>
<td>Peak oxygen uptake</td>
</tr>
<tr>
<td>VT</td>
<td>Ventilatory threshold</td>
</tr>
</tbody>
</table>
6.2 Abstract

**Purpose:** This study was undertaken to investigate the metabolic and performance effects of a mixed-carbohydrate (CHO; starch and sugars) product on prolonged endurance performance compared to a glucose control solution (GLU).

**Methods:** Sixteen male cyclists (mean±SD; age: 30.5±7.4 years old; mass 76.2±9.7 kg; height: 179.5±6.3 cm) participated in this cross-over, randomised and double blinded study. Each trial was performed at least 7 days apart and consisted of 120-min of steady state cycling at 75% of ventilatory threshold (VT) followed by a 16.1-km time-trial (TT). Participants ingested either the mixed CHO (EnergySmart; ES) or GLU in isocaloric (28.9 g of ES; 25 g GLU) and isovolumic (350 ml) portions at 45 min prior to steady state cycling and immediately following the TT. Blood samples (every 15 min) and expired gas was collected throughout the trials for measurement of blood electrolytes (K⁺, Na⁺, Ca²⁺), blood gases (pO₂, pCO₂), pH, calculation of fuel utilisation (Respiratory exchange ratio; RER) and cycling economy (CE).

**Results:** No significant performance differences were observed in TT between ES and GLU (1647.6±28.8 s vs. 1672.4±28.8 s, ES and GLU respectively; [95%CI], [-862, 366]); albeit that cyclists completed the TT 24.8 s faster with the ES. Consumption of GLU resulted in greater glycaemic variability compared to the ES. The exercise was associated with significant changes in blood gases, lactate, pH, electrolytes, but there were no significant differences in these outcomes, ratings of perceived exertion (RPE), RER between conditions or CE.

**Conclusion:** Ingestion of ES resulted in a more stable blood glucose profile but did not convey ergogenic benefits during prolonged exercise trials.
6.3 Introduction

Carbohydrate (CHO) ingestion is associated with improved performance [1-5] during prolonged (>60 min) exercise. Similarly, CHO ingestion in the days and hours prior to exercise has been associated with improved performance, specifically in exercise >90 min [6, 7]; yet, the strength of evidence supporting the ergogenic benefits of pre-exercise CHO supplementation is less convincing [8]. The influence of CHO intake within the final hours prior to exercise has been particularly controversial, with some studies demonstrating an improvement with CHO intake [6, 9], while others have shown no improvement [10-12], or even an impairment in endurance performance [13, 14].

Pre-exercise CHO ingestion caused hyperglycaemia and hyperinsulinaemia followed by reduced fat oxidation [15] and the occurrence of rebound hypoglycaemia [16] at the onset of exercise which could theoretically impair exercise performance [17]. To explore the role of pre-exercise CHO ingestion (45 min prior to exercise) on performance during a cycling time-trial (TT; ~40 min), a series of carefully designed studies were conducted in well-trained cyclists [11, 12, 18, 19]. These studies revealed (i) performance in the TT was not affected by development of hypoglycaemia [18]; there were no significant differences in either TT performance or glucose responses when the CHO load (glucose: 0 g, 25 g, 75 g, or 200 g; [11]) or type (trehalose, galactose, and glucose) was altered [12]; there was no significant difference in the TT performance when the timing of glucose ingestion was altered (15-min pre; 45-min pre; 75-min pre; [19]). These studies provide important insight into the role of pre-exercise CHO ingestion on during exercise <60 min (20 min sub-maximal cycling was performed prior to the TT). Whether acute CHO ingestion can improve performance in prolonged exercise (i.e., >90 min), remains equivocal.

Studies on slow-digested starch showed the incremental area under the curve of plasma glucose and insulin produced remain stable up to 120 min [20]. Consumption of modified starch 30 min prior to exercise (66.4 % VO_2peak) for 2 h revealed that CHO oxidation rate was high in simple sugar and modified starch but only modified starch sustained the CHO oxidation for the 120 min, while simple
sugar sustained the oxidation for 90 min only [21]. Studies on cycling performances following pre-exercise starch ingestion showed more stable blood glucose profiles without any improvement in performance [22, 23].

These studies mentioned above have typically focused on a particular type of CHO (i.e., individual monosaccharides, a mix of monosaccharides and disaccharides or group of starches). As such, the purpose of the current study was to determine whether the performance of a TT would improve in response to ingestion of a mixed CHO beverage containing starch and sugars compared to ingesting monosaccharides (i.e., glucose) alone. We hypothesised that the consumption of the mixed CHO beverage would improve performance in the prolonged exercise bout. The secondary aim was to determine differences in the glycaemic responses between the two supplements. We hypothesise that ingestion of the mixed CHO solution would demonstrate reduced glycaemic responses and minimise the risk of hypoglycaemia compared to ingestion of glucose.

6.4 Methods and procedures

Participants

Sixteen well-trained male cyclists (mean±SD; age: 30.5±7.4 years old; height 179.5±6.3 cm; mass 76.2±9.7 kg; VO2peak 61.1±6.7 ml.kg.min⁻¹) with normal body mass index (BMI; 23.6±2.1 kg.m⁻²) were recruited for this study. Participants were pre-screened via email and telephone conversation using the Exercise and Sports Science Australia (ESSA) risk assessment questionnaire and excluded from participation if they presented with more than two risk factors. Participants were informed of the risks and benefits of their participation in the study and written informed consent was obtained prior to data collection. The present study was carried out in the Murdoch University Exercise Physiology Laboratory and was approved by the Murdoch University Human Research Ethics Committee (Project No. 2015/175).
Study design

This study was conducted using a randomised and counterbalanced cross-over design. Randomisation and allocation to the experimental trials were conducted by someone not directly involved in the administration of the experimental trials (TJF), using a computer-generated number sequence of 1’s and 2’s. This study consisted of two phases. The initial phase consisted of a preliminary testing session and a familiarisation session. The second phase included two experimental trials completed seven days apart. During the experimental trials, participants consumed one of two different isocaloric beverages at two time points; 45 min before the commencement of exercise, and immediately after exercise. The drinks were pre-prepared by someone not involved in the study.

Preliminary trial (determination of peak oxygen uptake; VO\(_{2}\)peak) and familiarisation (16.1-km TT)

During the preliminary testing sessions, basic demographic data (height and body mass) were collected after which participants completed a graded exercise test for the assessment of peak oxygen uptake (VO\(_{2}\)peak). The graded exercise tests were conducted using an electromagnetically braked Velotron cycle ergometer (Racermate Inc., Seattle, USA) starting at a power output of 60 W with 30 W increments (2 min.stage\(^{-1}\)) until exhaustion. Expired ventilation was collected using a metabolic cart (TrueOne 2400, ParvoMedics, Utah, USA) from which VO\(_{2}\)peak and the ventilatory threshold (VT) were assessed [24]. The workload corresponding to 75% VT was then calculated for later use during the experimental trials [24]. Familiarisation session was applied to participants who did not have any experience participating in a competition. In the present study, only one participant involved with familiarisation trial.
**Experimental trials**

Both experimental trials were conducted in the morning with the participants in a fasted state (overnight fast; >10 h). Participants were asked to maintain their normal physical activity and avoid heavy physical activity 24 h prior to the trials as well as record their 24-h dietary intake, with the assistance of a food diary. After completing the first trial, participants were instructed to adhere to the same physical activity and dietary habits prior to the second trial.

Upon arriving at the laboratory, a venous catheter (VasoFix Safety, B.Braun, Melsungen, Germany) was placed into a forearm and kept patent for the duration of the trial with a saline solution (0.9%, B.Braun, Melsungen, Germany). Participants then consumed the first bolus of the assigned isocaloric supplement, either 350 ml beverage containing 28.9 g of the mixed CHO beverage (ES; EnergySmart® Soluble, Advanced Ingredients, LLC containing 46% of monosaccharides and 38% of mixed oligosaccharides and starches) or 25 g of monosaccharide (GLU; glucose, Glucodin) (~7.1% CHO per 100 ml of solution), after which they rested quietly while respiratory gas was collected continuously for 45 min using a calibrated metabolic measurement cart (TrueOne 2400, ParvoMedics, Utah, USA) and blood samples were collected every 15 min. Participants then completed a 120-min of steady state cycling bout at 75% VT on a Velotron cycle ergometer (Racermate Inc., Seattle, USA), while blood samples were taken at 15, 30, 60, 90 and 120 min. Expired ventilation was collected for the first 5 min after the start of exercise and subsequently for 5 min every 20 min during steady state cycling. At the completion of the 120-min of steady state cycling bout, participants immediately performed a 16.1-km TT. Blood samples were collected immediately after the completion of TT. Within 2 min of completing the TT, participants consumed the second dose of isocaloric beverage which was identical to the first dose (28.9 g of ES or 25 g of GLU) and rested quietly for 60 min. Fluid sensation scale [25] to measure subjective level of thirst, nausea, fullness and stomach upset were measured after ingesting the assigned isocaloric supplement 45 min before exercise and immediately after completing TT. While resting, expired ventilation was continuously collected and blood samples were obtained every 15
min. Water was provided ad libitum during the initial trial and matched during the second trial.

Participants wore a heart rate (HR) monitor (Polar, Kempele, Finland) throughout the experimental trials. Ratings of perceived exertion (RPE) 6-20 Borg scale [26] were measured every 15 min during resting prior to exercise, every 20 min during steady state cycling session, before and after TT and every 15 min during resting after completion of TT.

**Blood sampling and analysis**

Approximately 4 ml of venous blood was drawn at each time point and equally distributed into a lithium heparin containing tube (plasma; BD Vacutainer, Plymouth, UK) and a serum separating tub (serum; BD Vacutainer, Plymouth, UK). A small amount of blood sample (135 µl out of 2 ml) was immediately assayed using a blood gas analyzer (GEM 3500 Premier, Instrumentation Laboratory) for pH, partial pressure of oxygen (pO₂), partial pressure of carbon dioxide (pCO₂), sodium ions (Na⁺), potassium ions (K⁺), calcium ions (Ca²⁺), haematocrit (Hct), blood glucose and lactate. The remaining samples (plasma and serum) were then centrifuged at 1300 x g for 10 min before both plasma and serum samples were transferred into microcentrifuge tubes and stored in the freezer at -80 °C for subsequent insulin analysis. Plasma insulin concentrations were analysed using a commercially available Enzyme Linked Immunosorbent Assay kit (ELISA; Invitrogen Corp, MD, USA).

**Cycling economy**

The economy was obtained by dividing mean power output by mean oxygen consumption (VO₂) during the last 30 s at first 5 min and at every 20 min during steady state cycling [27].
**Statistical analysis**

Results are expressed as means ± standard deviations (SD) unless otherwise noted. A paired sample *t*-test was first used to assess differences in the time taken to complete the TT in each trial and to compare fluid sensation. Absolute differences between the trials are demonstrated using the 95% confidence interval (CI) of the difference. Linear Mixed modelling was then used to examine the effects of the supplement (condition) across time (repeated measures). Condition and time were modelled as random effects with the interaction being the primary outcome measure. Least Significance Difference (LSD) was used as Confidence Interval Adjustment. The significance level was set at $p<0.05$. All statistical procedures were completed using IBM SPSS Statistics 22.
6.5 Results

Cycling performance

No differences were observed for the time to complete (p=0.403; 95% CI [-862, 366]) or the average power output (p=0.726; 95%CI [-13.7, 19.2]) during the 16.1-km TT between the ES (1647.6±28.8 s and 229.4±61.7 W; respectively) and GLU (1672.4±28.8 s and 226.6±64.8 W; respectively) conditions (Figure 6.1).

**Figure 6.1** Exercise duration (top panel) in the 16.1-km TT with EnergySmart (ES) and glucose (GLU) trials. Power output (bottom panel) during 16.1-km TT cycling performance between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE.
Figure 6.2 a) Blood glucose and b) blood insulin concentration between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p≤0.001), ***Significant interaction effect (time x trial: p<0.05). Significantly different between trials: + p<0.05, ++ p<0.01, +++ p<0.001.
**Blood glucose and insulin**

There was a significant trial by time interaction in blood glucose and insulin response \((p \leq 0.001; \text{Figure 6.2})\). Blood glucose was lower during the ES trial at rest (pre- and post-exercise) compared to the GLU trial \((p < 0.001; 95\% \text{CI } [-0.44, -0.21])\) and demonstrated reduced variability (attenuated peaks and troughs; Figure 6.2a and 3). The blood insulin concentration (Figure 6.2b) was significantly elevated in the GLU trial compared to the ES trial \((p \leq 0.001)\).

**Figure 6.3** Difference in blood glucose (mmol/L; GLU subtract ES) at each time point during trials. Solid line represents the mean difference while dotted lines provide the 95% CI of the mean. Significant difference is observed where all three lines are above or below the zero line. Arrows indicate the time of beverage ingestion.
Blood gas, electrolytes, lactate and pH

There were no significant differences between trials and no significant interaction effect for any of the blood gases (pO$_2$, pCO$_2$), lactate or pH levels (Figure 6.4). There was, however, a significant effect of time, with each parameter demonstrating significant changes with exercise. When collapsed across time, the mean pCO$_2$ value was significantly (p=0.016) lower and pH significantly (p=0.001) higher in the ES trial compared to GLU trial, but no differences between trials observed in pO$_2$ (p=0.303) or lactate (p=0.516). There were no significant differences between trials and no significant interaction effect in blood electrolytes (Na$^+$ and Ca$^{2+}$) or Hct, but there were significant main effects of time (Figure 6.5). Only K$^+$ showed a significant trial (p=0.002) and time (p≤0.001) effect, however, without any significant interaction effect (p=0.692).

**Figure 6.4** a) Partial pressure of oxygen (pO$_2$) between EnergySmart (ES) and glucose (GLU) trials. b) Partial pressure of carbon dioxide (pCO$_2$) between EnergySmart (ES) and glucose (GLU) trials. c) Blood lactate concentration between EnergySmart (ES) and glucose (GLU) trials. d) Blood pH between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p≤0.001).
Figure 6.5 a) Blood sodium ions (Na\(^+\)) concentration between EnergySmart (ES) and glucose (GLU) trials. b) Blood potassium ions (K\(^+\)) concentration between EnergySmart (ES) and glucose (GLU) trials. c) Blood calcium ions (Ca\(^+\)) concentration between EnergySmart (ES) and glucose (GLU) trials. d) Haematocrit level (Hct) between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p≤0.001).

Physiological, economy and perceptual responses

Respiratory exchange ratio (RER) and HR differences are demonstrated in Figure 6.6, along with subjective RPE during trials. These physiological parameters showed a significant effect of time (p≤0.001) without any interaction effect (RER: p=0.947; HR: p=0.989; RPE: p=0.957).
Figure 6.6 a) Respiratory exchange ratio (RER) between EnergySmart (ES) and glucose (GLU) trials. b) Heart rate (HR) between EnergySmart (ES) and glucose (GLU) trials. c) Rated perceived exertion (RPE) between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect (p<0.05), **Significant trial effect (p≤0.001).
Similar to physiological changes, there was a significant ($p \leq 0.001$) decrease in cycling economy (CE) over time but no differences between trials ($p=0.843$) and no significant interaction ($p=0.715$) (Figure 6.7).

![Figure 6.7 Cycling economy (CE) between EnergySmart (ES) and glucose (GLU) trials. Mean ± SE. *Significant time effect ($p<0.05$).](image)

There were no significant differences in participant’s levels of feelings of nausea, fullness stomach upset at the pre- and post-exercise time points (Table 6.1). However, participants in the GLU trial were prone to feel thirstier at the completion of the GLU trial when compared to the ES trial.
Table 6.1 Differences in subjective sensations of thirst, nausea, fullness and stomach upset during each trial. P represents differences (p-values) pre and post trials; Between represents differences in the change scores (post-pre) between trials.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>EnergySmart (ES)</th>
<th>Glucose (GLU)</th>
<th>Between</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>p</td>
</tr>
<tr>
<td>Thirst</td>
<td>2.7 (1.3)</td>
<td>3.2 (1.1)</td>
<td>.057</td>
</tr>
<tr>
<td>(1=not thirsty; 5=extremely thirsty)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1.2 (0.6)</td>
<td>1.2 (0.4)</td>
<td>.669</td>
</tr>
<tr>
<td>(1=no nausea; 5=extremely nausea)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fullness</td>
<td>2.4 (1.0)</td>
<td>2.2 (1.2)</td>
<td>.632</td>
</tr>
<tr>
<td>(1=not full; 5=extremely full)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach Upset</td>
<td>1.2 (0.4)</td>
<td>1.1 (0.3)</td>
<td>.580</td>
</tr>
<tr>
<td>(1=no upset; 5=extremely upset)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.6 Discussion

The purpose of the current study was to determine whether an acute mixed CHO (ES; sugars and starch) solution consumed prior to prolonged exercise improves performance relative to consumption of a glucose (GLU) solution. The main findings in the current study were i) blood glucose concentration was significantly higher at the start of exercise in the GLU trial compared to ES, which significantly decreased with the onset of exercise (GLU: 3.7±0.7 mmol/L; ES: 4.7±0.6 mmol/L) and remained lower than ES supplement until the 30 min period (Figure 6.2a and 3); ii) while no between-trial differences were observed in ratings of perceived effort, economy, electrolyte concentrations, lactate or pH levels; these markers demonstrated significant changes in response to exercise which was consistent with previous research and iii) despite the ES supplement being associated with a slight improvement (24.8 s improvement) in TT performance, there were no significant differences in performance between trials.

Initiation of exercise in the GLU trial resulted in a significant decrease in blood glucose concentration, resulting in values of 3.7±0.7 mmol/L, wherein three participants experienced blood glucose values below 3 mmol/L (at 5 min into cycling time) without reporting any symptoms of hypoglycaemia. These results are consistent with previous research, which observed a significant elevation in blood glucose in response to supplement ingestion, but then observed a significant drop in blood glucose in the early stages of exercise [11, 12, 18]. Consistent with the findings herein, participants with hypoglycaemia did not report feelings associated with a hypoglycemic condition and hypoglycaemia did not reduce exercise performance. Blood glucose in GLU remained significantly lower compared to ES until 30 min of exercise although one participant maintained low levels throughout the exercise bout and reported mild symptoms of hypoglycaemia towards the latter stages of the trial. Accordingly, insulin concentration increased in response to increases in blood glucose (pre- and post-exercise) but was rapidly brought to baseline during exercise. Despite these differences in blood glucose, there were no significant differences in fuel utilization between trials (based on RER) as well as the cycling economy. The cycling economy was assessed by measuring oxygen
consumption per unit of power output [27, 28] to see whether consumption of ES could cause any effect to cycling efficiency compared to GLU. The present study did not show any significant differences between trials with ~7.8% decrease in average cycling economy during 120-min of steady state cycling and comparable to the previous study which has shown ~10% reduction in a 150-min of steady state cycling [29].

Electrolytes, and in particular K⁺, have long been implicated in the development of fatigue [30, 31]. Early experiments clearly demonstrated efflux of K⁺ from skeletal muscle both during electrical stimulation and voluntary contraction (swimming), with the greatest efflux of K⁺ occurring with longer swim-times (reviewed in [30, 31]). This is consistent with findings in the current study where, despite no differences being observed between trials, K⁺ concentrations increased progressively with exercise. The underlying mechanisms linking K⁺ concentration with fatigue relates to (i) alterations in the skeletal muscle membrane potential arising from a decreased ratio of intracellular:extracellular K⁺; and (ii) increased intracellular hydrogen ions (H⁺) concentration in response to the loss of K⁺ [32]. Consistent with these hypotheses, the pH in the current study demonstrated a general increase concurrent with the increase in K⁺ concentrations.

Generally, the present study showed that there were no significant differences in performance between the two trials (p=0.403), although the mean TT performance in the ES trial was 24.8 s faster (SD=115.3 s). Accordingly, there were no significant differences between trials in RPE, HR, lactate, electrolytes and cycling economy during the 120-min steady state exercise period, which immediately preceded the TT in the current study. The inter-individual variance in performance was large with nine of the 16 demonstrating an improvement in TT with the ES, and no clear trends presenting (i.e., the supplement benefiting the fastest or slowest cyclists). With respect to the supplements, it is important to note that the quantity of supplement provided was lower compared to other studies (i.e., 25 g of CHO versus 25-200 g CHO; [6, 9-11, 18, 19]). While dose (0 g, 25 g, 75 g, 200 g) has not previously been shown to have a significant effect on TT performance of shorter duration (TT: ~42 min; total exercise time: ~62 min; [11]) it may play a more
critical role in improving performance of prolonged exercise time, particularly when performed following an overnight fast.

While the study had a number of strengths, including the number of participants, the training status of the participants, the adoption of the TT at the end of a 120-min of steady state cycling protocol and the assessment of multiple markers related to fatigue, there were a number of factors which may have limited the interpretation of study findings. Firstly, while dietary records were maintained and participants reminded to repeat a similar dietary intake in the 24 h preceding the subsequent experimental trial, this may not have been adhered to. Secondly, the study was designed to compare the two supplements directly, however, the inclusion of a control condition would likely have yielded important information; in particular, whether consumption of CHO in this time-frame was superior to a no CHO condition. Thirdly, while not a limitation per se, the study was purposefully designed to provide 25g CHO, however, a higher dose (i.e., 50g-75g CHO) may have been associated with different outcomes and is worthy of future investigation.
6.7 Conclusion

In conclusion, consumption of a mixed CHO beverage (ES) resulted in a more stable blood glucose and insulin profile than consumption of an isocaloric monosaccharide beverage (GLU). However, performance during prolonged exercise was not different in response to ingestion of the ES beverage relative to the beverage containing only monosaccharides (GLU). Moreover, development of hypoglycaemia did not appear to alter performance in the subsequent exercise trial. Finally, traditionally markers associated with exercise-induced fatigue (i.e., potassium), demonstrated changes which were consistent with prior research. Considering the ES beverage resulted in a 24 s shorter TT than the GLU condition, future research may be warranted to explore the effects of increasing the CHO content on the performance of the prolonged exercise.

6.8 Acknowledgement

This study was funded (analytical costs and supplement supply) by Advanced Ingredients, LLC (USA). The authors declare no other conflict of interest.
6.9 References


Chapter 7  General discussion

7.1  Conclusions

Multiple mechanisms have been identified as contributing to fatigue-onset in exercise, yet their relative contributions and importance are still unclear and intensely debated [1-18]. Therefore, the overarching aim of this dissertation was to assess and identify factors associated with the onset of fatigue during prolonged cycle-exercise, using techniques designed to assess both peripheral (i.e., metabolomics) and central (i.e., neuromuscular assessments) parameters of fatigue onset.

The metabolomics approach adopted within Chapter 3, allowed the separation of plasma sampled at the point of fatigue (post-fatigue) from samples obtained either prior to or following fatigue. The metabolites contributing to the distinct profiles of plasma sampled at various time-points were then explored and free fatty acids (FFA) along with tryptophan (TRP) were identified as strongly influencing these distinct metabolic profiles and in so doing, lent support to the ‘Central Fatigue Hypothesis’. Additionally, the 87 identified metabolites which demonstrated significant changes over time generated alternate avenues for future investigation, including, for example, novel roles for FFA in exercise-induced fatigue onset which may be explored in subsequent studies. The ability of this technique to be able to discriminate the plasma samples obtained during exercise is somewhat surprising, given the relatively short time-frame between sampling points in the current study. When exploring the metabolites contributing to this separation, it does become apparent that multiple factors contributed to the unique plasma metabolic profile, particularly the FFA and amino acids.

Chapter 4 compared the changes in neuromuscular fatigue following two modes of isoenenergetic prolonged exercise, which were a constant paced cycling time-to-exhaustion (TTE) and a self-paced cycling time-trial (TT). Performance in a self-paced cycling TT was significantly improved compared to an isocaloric constant-paced cycling TTE trial in young and recreationally active males. This improvement was not associated with any differences in perceived effort (RPE),
Despite maintaining significantly higher power-outputs and working at a higher physiological load (HR). The neuromuscular assessments revealed no significant between-trial differences, but the MVC torque was reduced by nearly 12% immediately post-exercise. This reduction in MVC torque, was associated with a significant decrease in central fatigue (VAL\(_p\): -11%), demonstrating a reduced level of neural drive to the muscle [19]. Additionally, peripheral factors contributed to the reduced MVC torque, with a significant decrease in the potentiated doublet (-24%) demonstrating reduced excitation-contraction coupling; although neuromuscular propagation is unlikely to have significantly contributed to the fatigue. The recreationally active males recruited to the study and who were unaccustomed to cycling, demonstrated superior performance when completing a self-paced TT (closed-loop) compared to a constant-pace TTE (open-loop) trial.

Based on the findings from Chapter 3 and Chapter 4, Chapter 5 compared the effect of acute supplementation with branched-chain amino acids (BCAA) on cycling performance and fatigue assessed using neuromuscular assessments. The use of BCAA supplementation was based on the ‘Central Fatigue Hypothesis’, with the specific aim of reducing the rate at which tryptophan enters the central nervous system (CNS) thereby reducing the rate of serotonin synthesis. Therefore, we anticipated an improvement in cycling performance with BCAA supplementation. The findings of Chapter 5 were that acute BCAA supplementation improved (~7%) cycling performance and reduced RPE. Although the neuromuscular assessments demonstrated significant peripheral and central fatigue in response to exercise, there were no differences observed between the BCAA supplementation and placebo protocols. Overall, these findings suggest that acute supplementation of BCAA improved cycling performance and perception of effort during the cycling TT, without any notable difference in neuromuscular changes between trials.

Chapter 6 was designed to investigate the effectiveness of a mixed (starch and sugars) carbohydrate supplement (ES) and this was compared to an isocaloric glucose (monosaccharide) control solution (GLU). This study adopted a prolonged cycling protocol comprising a 2-h constant load cycling effort, followed by a 16.1 km TT, wherein performance in the TT was the primary outcome. The adoption of a carbohydrate supplement in the current study was based on the body of
evidence supporting a beneficial role of carbohydrates on sports performance; but with the knowledge that a gap in this evidence exists in relation to the possible ergogenic role of carbohydrates in the final 1 to 2 h prior to exercise. The findings of Chapter 6 were that consumption of the ES beverage resulted in a more stable blood glucose and insulin profile than consumption of GLU, however, performance during prolonged exercise was not significantly different. Similar to others, we found the development of hypoglycaemia did not appear to alter performance in the TT. Finally, traditionally markers associated with exercise-induced fatigue (i.e., potassium), demonstrated changes which were consistent with prior research and suggested a possible role in the onset of fatigue. Financial constraints did not enable a complete metabolomics analysis to be completed on the plasma samples obtained in this study.

7.2 Practical applications

The practical recommendations based on the studies presented herein are:

1. Individuals unaccustomed to either a time-trial (TT) or time-to-exhaustion (TTE) cycling task, demonstrate superior performance in the TT, whilst reporting no differences in their rating of perceived effort.
2. Supplementation with branched-chain amino acids (BCAA) delayed fatigue onset and improved performance in a prolonged cycling TT.
3. Supplementation with glucose in the final hour prior to exercise performance results in low blood glucose values however, this reduced blood glucose concentration does not appear to affect cycling performance even in prolonged cycling events. Large inter-individual variance in blood glucose profiles and subsequent cycling performance were identified, and carbohydrate manipulation in the final 1 to 2 h prior to competition will likely need to be tailored to individuals.
7.3 Future research directions

The adopted metabolomics approach in Chapter 3 generated multiple possible research avenues for investigators in this field. In particular, the multiple roles which free fatty acids may play in the regulation of cellular energetics is an interesting prospective research direction which appears to link with findings from earlier work conducted in the 1970’s, but which has for some reason largely been ignored in the exercise and fatigue literature more recently. The concurrent application of metabolomics with changes in neuromuscular parameters (twitch interpolation technique and EMG) would enable a richer assessment of particular metabolites in the onset of fatigue, and it may be particularly interesting to assess the metabolites associated with the neurotransmitters (i.e., acetylcholine). It is important to acknowledge that the work in Chapter 3, 4 and 5, was conducted in healthy individuals, but not athletes; future research should therefore consider whether more highly trained individuals would have similar responses, in particular to the supplementation of BCAA.

The effectiveness of mixed CHO beverage on prolonged exercise performance is worthy of further investigation, particularly with regard to the effects of a higher mixed CHO dose. This is based on the observation that hypoglycaemia does not appear to influence performance and that although insignificant, there was a tendency for improved performance with the mixed CHO beverage. This study used glucose as a comparator since glucose has previously been shown to be ergogenic, but the addition of a placebo condition and concurrent neuromuscular assessments would have been interesting; but this was not performed in the current study due to the demanding nature of the cycling task (i.e., a TT preceded by a 2 h cycling bout at the ventilatory threshold).

Despite the depth of research conducted in this field, many questions remain. How these particular markers of fatigue identified during exercise may relate to markers of fatigue observed in clinical populations, remains to be explored. Further research adopting complimentary techniques are required, in order to better understand the contribution of unique metabolites, pathways and systems to fatigue-onset.
References


Appendices
Appendix A  Ethics approvals from
Murdoch University and
Edith Cowan University
A.1 Ethics approval from Murdoch University (Chapter 3, 4 and 5)

Monday, 24 February 2014

Dr Timothy Fairchild
School of Health Professionals
Murdoch University

Dear Timothy,

Project No. 2013/206
Project Title Fatigue and metabolic responses to different types of exercises: a metabolomics approach

Thank you for addressing the conditions placed on the above application to the Murdoch University Human Research Ethics Committee. On behalf of the Committee, I am pleased to advise the application now has:

OUTRIGHT APPROVAL

Approval is granted on the understanding that research will be conducted according to the standards of the National Statement on Ethical Conduct in Human Research (2007), the Australian Code for the Responsible Conduct of Research (2007) and Murdoch University policies at all times. You must also abide by the Human Research Ethics Committee’s standard conditions of approval (see attached). All reporting forms are available on the Research Ethics web-site.

I wish you every success for your research.

Please quote your ethics project number in all correspondence.

Kind Regards,

[Signature]

Dr. Erich von Dietze
Manager of Research Ethics

cc: Dr Jeremiah Petiker, Faizal Abdal Manaf and Aaron Raman
A.2 Ethics approval from Murdoch University
(Chapter 6)

Division of Research & Development
Research Ethics and Integrity

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Wednesday, 23 September 2015

Dr Timothy Fairchild
School of Psychology and Exercise Science
Murdoch University

Dear Timothy,

Project No. 2015/175
Project Title Metabolic and performance responses to the ingestion of different carbohydrates during prolonged exercise

Thank you for addressing the conditions placed on the above application to the Murdoch University Human Research Ethics Committee. On behalf of the Committee, I am pleased to advise the application now has:

OUTRIGHT APPROVAL

Approval is granted on the understanding that research will be conducted according the standards of the National Statement on Ethical Conduct in Human Research (2007), the Australian Code for the Responsible Conduct of Research (2007) and Murdoch University policies at all times. You must also abide by the Human Research Ethics Committee’s standard conditions of approval (see attached). All reporting forms are available on the Research Ethics and Integrity web-site.

I wish you every success for your research.

Please quote your ethics project number in all correspondence.

Kind Regards,

[Signature]

Dr. Erich von Dietze
Manager
Research Ethics and Integrity

cc: Dr Jeremiah Peiffer and Faizal Abdul Manaf
A.3 Ethics approval from Edith Cowan University (Chapter 3 and 6)

HUMAN RESEARCH ETHICS COMMITTEE
For all queries, please contact:
Research Ethics Officer
Edith Cowan University
270 Joondalup Drive
JOONDALUP WA 6027
Phone: 6304 2170
Fax: 6304 9044
E-mail: research.ethics@ecu.edu.au

21 February 2017
Professor David Broadhurst
School of Science
JOONDALUP CAMPUS

Dear David

ETHICS APPROVAL – MULTICENTRE RESEARCH PROJECT

<table>
<thead>
<tr>
<th>Project Code:</th>
<th>17441</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chief Investigators:</td>
<td>Professor David Broadhurst, Mr Faizal Abdul Manaf, Dr Timothy Fairchild</td>
</tr>
<tr>
<td>Associate Investigator:</td>
<td>Mr Nathan Lawler</td>
</tr>
<tr>
<td>Project Title:</td>
<td>Metabolic and performance responses to the ingestion of different carbohydrates during prolonged exercise</td>
</tr>
<tr>
<td>Approval Dates:</td>
<td>From: 21 February 2017 To: 31 October 2018</td>
</tr>
</tbody>
</table>

Funding Source: unfunded

Thank you for your recent application for ethics approval. This proposal has been reviewed by members of the ECU Human Research Ethics Committee (HREC). The Committee noted that this project has previously been approved by the Murdoch University Human Research Ethics Committee.

I am pleased to advise that the proposal complies with the provisions contained in the University’s policy for the conduct of ethical human research and ethics approval has been granted. In granting approval, the HREC has determined that the research project meets the requirements of the National Statement on Ethical Conduct in Human Research.

All research projects are approved subject to general conditions of approval. Please see the attached document for details of these conditions, which include monitoring requirements, changes to the project and extension of ethics approval.

We wish you success with your research project.

Yours sincerely

[Signature]

Kim Gifkins
SENIOR RESEARCH ETHICS ADVISOR
Appendix B  Information Letters
B.1 Information letter (Chapter 3, 4 and 5)

Fatigue and metabolic responses to different types of exercise: A metabolomic approach

We invite you to participate in a research study which aims to quantify the level of fatigue experienced during different types of exercise. This study is part of my PhD in Exercise Science and is being supervised by Dr Timothy Fairchild and Dr Jeremiah Peiffer.

Nature and Purpose of the Study
This study has two broad aims. The first being to identify the origin of fatigue; specifically whether fatigue originates in the muscle itself or in the nervous system which stimulates the muscle to contract. The second aim is to assess changes in chemicals within the blood and determine how different chemicals may herald the onset of fatigue. As part of aim two, we will then seek to determine whether we are able to modify fatigue using a nutritional supplement (branched chain amino acids).

If you consent to take part in this research study, it is important that you understand the purpose of the study and the procedures you will be asked to undertake. Please make sure you ask any questions you may have, and that all your questions have been answered to your satisfaction before you agree to participate.

What the Study will involve
If you consent to participate in this study, you will be asked to attend the Murdoch University Exercise Physiology laboratory on four separate occasions; the first visit will involve preliminary measurements and familiarisation to the procedures, whilst the other three visits will be part of the experimental trials. The expected overall time commitment is a month where you have to attend about once a week for four weeks.

Specifically, the visits will include the following:
Visit 1: This visit will act as a baseline session in order to collect preliminary measurements and to familiarise yourself to the equipment, protocol and procedures related to the study. Any additional questions may be addressed at this stage; screening questionnaires will be completed at this stage. Preliminary measurements include anthropometric assessment, VO2max test (common fitness assessment; explained below), a 15min cycle at a fixed workload to familiarise you with the actual testing task, height and weight. During the VO2 max test, finger prick blood samples will be taken (~4-6 samples). This visit is expected to take 60min.
Visit 2: You will be asked to attend the laboratory prior to eating breakfast (overnight fast; >10 hours) and we will ask about your previous 24 hour dietary intake and will request you follow this for each of the next sessions. We will then position you on a dynamometer machine for measurement of your maximal voluntary contraction (MVC) strength. We will place four little circular pads onto your muscle belly of the anterior thigh for collection of electrical activity. You will then be asked to extend your leg as hard as possible for three seconds, which will be repeated three times. We will then give you a suitable amount of time to rest. In this next procedure, we will then ask you to maximally contract your muscle and we will apply an electrical stimulus directly to the muscle itself. We will repeat this on three different occasions to ensure we obtain the maximum reading. The muscle will contract and the force from this contraction recorded.

After that, you will be seated in a specialised chair and a catheter will be inserted into one of the veins on your forearm by a trained phlebotomist. This catheter will stay in place for the duration of the exercise session. You should inform the investigator immediately if you are experiencing any pain or undue discomfort from the catheter following its placement. Next, we will place you on the bike and we will ask you to exercise at the pre-determined workload (determined during session one testing). We will then sample 3 ml of blood every 10 min during the exercise, as well as at the point where you voluntarily stop the exercise (considered fatigue). At this point, we will transfer you immediately back to the machine where we will assess maximum voluntary contraction and the electrical stimulus will be applied as described above. Twenty minutes after cessation of exercise, one more blood sample will be taken and the muscular contraction force again measured. You will not feel anything during the blood collections; in total, we expect to sample blood on approximately 8 occasions, depending on the length of the exercise task (how long you stay on the bike). This session is expected to take ~2 hours.

Visit 3 and 4: Will be at least three days later and will resemble the procedures of the second visit. The two differences here will be (i) you will be provided with a drink (either mixed with branched chain amino acids or placebo) 30 min (~700ml) and 5 min (~400ml) before the bike test; (ii) rather than riding to fatigue, we will set you with a target workload which you must achieve as fast as possible. The drink will contain branched-chain amino acids, a commonly used supplement in sport and clinical conditions (conditions associated with muscle wasting). Only difference being the experimental test (i.e. TTP or TTB) that will be completed. Food intake for the previous 24 hours prior to these trials will again be monitored. Each of these sessions is expected to last ~2.5 hours.

VO2 max test: Is used to determine the maximum amount of oxygen that the body can consume and this is typically measured during a bout of exercise. This test will consist of cycling on a stationary bike at progressively difficult workloads whilst breathing through a mouthpiece connected to a metabolic cart. This test continues until volitional exhaustion.
The highest oxygen uptake measured will then be deemed as VO$_2$max. Heart rate will be continuously monitored throughout the test, and rating of perceived exertion (RPE: Borg scale) recorded every minute. At the same time, blood sample will be collected using finger prick to measure blood lactate concentration for calculation of power output at a set blood lactate concentration (3mmol/L).

**Anthropometric measurements:** Height and weight will be measured using a stadiometer (a device that consist of a vertical ruler with a sliding horizontal paddle which is adjusted to rest on top of head) and weighing scale, respectively, and the Body Mass Index (BMI) calculated.

**Eligibility criteria:** Since this project requires completion of an exercise bout and measurement of fatigue biomarkers, we are looking for a specific population group to participate in this study, namely those:

1. healthy male participants between 18 and 44 years old.
2. recreationally active.

You may be ineligible if you:
1. are taking any medication on a daily basis for long-term health reasons (such as Statins, blood pressure medication etc.
2. have uncontrolled high blood pressure
3. have an acute (i.e. viral infection) or chronic (i.e. asthma [moderate persistent], cancer, arthritis, atherosclerosis) inflammatory disease.

Additionally, we may request you seek medical clearance prior to participating in this study based on a pre-exercise screening tool we adopt for these type of studies.

**Voluntary Participation and Withdrawal from the Study**
Your participation in this study is entirely voluntary. You may withdraw at any time without discrimination or prejudice. All information is treated as confidential and no names or other details that might identify you will be used in any publication arising from the research. If you withdraw prior to completion of data collection, all information you have provided will be destroyed.

**Benefits of the Study**
The main benefit to participation for you include a free fitness test (the gold-standard VO$_2$max test). However, we also hope that the findings from this study provide further information related to the onset of fatigue, and ultimately, a possible marker for fatigue.

**Possible Risks**
It is possible that you may experience some discomfort during the session as a result of some of the tasks such as the exercise or the blood sampling. You will be monitored closely during the study and you are free to withdraw at anytime during the study.
There are only minor risks associated with the exercise protocol and blood sampling, although there will be some level of discomfort during the study. Some individuals may suffer light-headedness or fainting during the blood collection process, please inform the investigators if you have experienced this previously. Individuals may have bruising, numbness and pain which could last for a few days as a result of the blood collection from the finger or the catheter. Cycling time trial will be a strenuous form of exercise which may lead to light-headedness, nausea and feeling breathless. You will be constantly monitored but please inform the researchers if you experience any feeling of great discomfort during the exercise conditions. It is important for you to understand that you can ask the investigator to stop the experiment at any stage without having to provide an explanation.

You may experience some pain with the insertion of the catheter which is similar to that experienced with other forms of blood collection. Once the catheter has been placed and secured, there may be some mild-discomfort at the site of the catheter but this should not be perceived as painful.

You may also experience some pain during electrical muscle stimulations. This is expected to be at a level of discomfort to mildly painful. At all times, this is expected to remain tolerable and should not be perceived as very painful. Instruct the investigator immediately and the experiment will not be continued.

If you have any questions about this project please feel free to contact myself Mr Faizal (9360 1389; or F.AbdulManaf@murdoch.edu.au) or Dr Fairchild (9360 2959; or T.Fairchild@murdoch.edu.au) or Dr Peiffer (9360 7603; J.Peiffer@murdoch.edu.au). We will be happy to discuss with you any concerns you may have about this study.

Once we have analysed the information from this study we will publish the results of the study on the Murdoch University School of Psychology and Exercise Science website: http://www.murdoch.edu.au/School-of-Psychology-and-Exercise-Science/Research/Exercise-Science-Research/Research-findings/. You can expect to receive this feedback within 12 months.

If you are willing to consent to participation in this study, please complete the Consent Form. Thank you for your assistance with this research project.

Sincerely,
Mr Faizal Abdul Manaf

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This study has been approved by the Murdoch University Human Research Ethics Committee (Approval 2013/206). If you have any reservation or complaint about the ethical conduct of this research, and wish to talk with an independent person, you may contact Murdoch University’s Research Ethics Office (Tel. 08 9360 6677 (for overseas studies, +61 8 9360 6677) or e-mail ethics@murdoch.edu.au). Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.
B.2 Information Letter (Chapter 6)

Information Letter

Metabolic and performance responses to the ingestion of different carbohydrates during prolonged exercise

We invite you to participate in a research study which aims to determine if a mix of carbohydrate is more beneficial on cycling performance compared to a traditional supplement (comprising glucose/maltose solution). Additionally, we aim to analyses the differences in the metabolic and physiological responses following consumption of both products, which will involve measures such as blood glucose and lactate, as well as measures of heart rate and perceived effort. This study is part of my PhD in Exercise Science and is being supervised by Dr Timothy Fairchild and Dr Jeremiah Peiffer. This project is being funded by Advanced Ingredients, who manufacture one of the ingredients being tested.

Nature and Purpose of the Study
This study aims to determine whether the ingestion of waxy-maize product, specifically EnergySmart Soluble could improve performance in a prolonged cycle event to greater degree than a glucose/maltose solution. Additionally, this study seeks to determine the metabolic responses during exercise, by measuring changes in metabolites such as glucose, glycerol, non-esterified fatty acids (NEFAs), insulin and lactate concentrations. We are particularly interested in how EnergySmart Soluble could avoid the increase of plasma insulin (as insulin could lead to impairment in performance by storing fuels instead of using them) and blood glucose.

If you consent to take part in this research study, it is important that you understand the purpose of the study and the procedures you will be asked to undertake. Please make sure you ask any questions you may have, and that all your questions have been answered to your satisfaction before you agree to participate.

What the Study will involve
Eligibility criteria: Since this project requires completion of prolonged exhaustive exercise bout and measurement of concentration of blood metabolites, we are looking for a specific population group to participate in this study, namely those:

Well-trained male cyclists:
  a. Age between 18-44 years old
  b. Weekly cycling of at least 250 km
  c. VO2peak > 55 ml/kg/min
  d. BONUS – current racing experience
You may be ineligible if you:

1. Unable to achieve the minimum requirement of aerobic fitness level test prior to the experimental trial (minimum of 55 during VO2peak test)
2. Have an acute (i.e. viral infection) or chronic (i.e. asthma [moderate persistent], cancer, arthritis, atherosclerosis) inflammatory disease.
3. Are screened as ineligible from the Exercise and Sports Science screening tool.
4. Have a maize allergy

If you consent to participate in this study, you will be asked to attend the Murdoch University Exercise Physiology laboratory on three separate occasions; the first visit will involve preliminary measurements and familiarisation to the procedures, whilst the other two visits will be part of the experimental trials. The experimental trials will include completion of approximately 150 minutes exercise in each trial. Specifically, each visit will be as follows:

Visit 1: This visit will act as a baseline session in order to collect preliminary measurements and to familiarise yourself to the equipment, protocol and procedures related to the study. Any additional questions may be addressed at this stage; screening questionnaires will be completed at this stage. Preliminary measurements include anthropometric assessment, VO2 max test (common fitness assessment) and familiarisation with 16.1 km time trial. This visit is expected to last about 1 hour.

Visit 2 and 3 (first and second trials) - You will be asked to attend the laboratory prior to eating breakfast (overnight fast; >10 hours) and an indwelling cannula will be inserted into an arm vein to obtain a pre-exercise blood sample. You will then be asked to consume either the isocaloric EnergySmart product (ES) or a glucose/maltose (GLU) powder (25g) dissolved in 350 ml of water. After that, you will remain seated for 45 min, while blood is sampled every 15 min and respiratory gases are collected using the metabolic cart (ParvoMedics system). Forty five minutes after the ingestion of either ES or GLU, you will be asked to ride at the predetermined workload (75% VT2) for 120 min (you are allowed to listen to music) with blood sampled at 15, 30, 60, 90 and 120 min and respiratory gases collected every 20 min. Water will be provided ad libitum. As soon as the 120 min is completed, you will be asked to complete a 16.1km time trial to assess exercise performance and during this time you are not allowed to listen to music.
The total exercise is expected to last approximately 145 min and a blood sample collected. You will then be provided with a second dose of either isocaloric ES or GLU (25 g) dissolved in 350 ml of water. You can then then rest for a further 60 min whilst respiratory gases (continuously) and blood (every 15 min) are sampled. Heart rate and perceived exertion will be measured throughout (every 15 min) the trial. This visit is expected to last about 5 hours.

In the next visit with minimal gap of seven days, you will repeat the same procedures as previous visit, with the only difference being the supplement provided. Food intake for these trials will be monitored and you will be asked to follow similar pattern as previously.

Further information of procedures
VO2 peak test and 16.1 km time trial: VO2 peak determines the maximum amount of oxygen that the body can consume and this is typically measured during a bout of exercise. This test will consist of cycling on a cycle ergometer/stationary bike at progressively difficult workloads whilst breathing through a mouthpiece connected to a metabolic cart. This test continues until volitional exhaustion. The highest oxygen uptake measured will then be deemed as VO2peak. Heart rate will be continuously monitored throughout the test, and rating of perceived exertion (RPE; Borg scale) recorded every minute. Following the completion of VO2peak test, you will then familiarised with 16.1 km time trial. During this test, you can bring your own pedals/cleats and we can fit those to the cycle ergometer.

Anthropometric measurements: Height and weight will be measured using a stadiometer and weighing scale, respectively, and the Body Mass Index (BMI) calculated.

Collection of blood: To determine the blood metabolites, 4ml of blood will be collected from the antecubital vein (region of the arm in front of the elbow) using an indwelling cannula on 14 occasions in visit 2 and 3. This will result in 56ml of blood being collected per session. The cannula will be used to minimise the pain and discomfort associated with repeated sampling.

Voluntary Participation and Withdrawal from the Study
Your participation in this study is entirely voluntary. You may withdraw at any time without discrimination or prejudice. All information is treated as confidential and no names or other details that might identify you will be used in any publication arising from the research. If you withdraw prior to completion of data collection, all information you have provided will be destroyed.
Benefits of the Study
Benefits of participation include a free fitness test (the gold-standard VO2max test) and a bike-shop voucher to the value of $120 per person.

Possible Risks
It is possible that you may experience some discomfort during the session as a result of some of the tasks such as the exercise or the blood sampling. You will be monitored closely during the study and you are free to withdraw at any time during the study. There are only minor risks associated with the exercise protocol and blood sampling, although there will be some level of discomfort during the study.

Some individuals may suffer light-headedness or fainting during the blood collection process, please inform the investigators if you have experienced this previously. Insertion of the indwelling cannula may cause mild initial discomfort but will be minimised by following safe practices. The possible risks associated with the cannula include bruising (resulting from not properly inserting the cannula), phlebitis (inflammation of the vein), occlusion (obstruction at the catheter tip which prevents blood withdrawal), infiltration (when the catheter slips out of the vein and saline is pushed into the local tissue), embolism (if air is pushed into the catheter). However, these risks may be mitigated by following proper cannulation procedures, and these complications are rare particularly when the cannula is only placed for a short-period of time (e.g. < 12 hours). Cycling time trial will be a strenuous form of exercise which has been implemented in a plethora of previous research. Minimal risk factors include light-headedness, nausea and feeling breathless. You will be constantly monitored but please inform the researchers if you experience any feeling of great discomfort during the exercise conditions. It is important for you to understand that you can ask the investigator to stop the experiment at any stage without having to provide an explanation. The researchers in this study are trained (and current) in Senior First Aid.

Data storage
Data will not be provided to any other researchers but some data may be used for secondary analysis which will only include data already captured and may consist of an additional comparative analysis.

If you have any questions about this project please feel free to contact myself Mr Faizal (9360 1389; or F.AbdulManaf@murdoch.edu.au) or Dr Fairchild (9360 2969; or T.Fairchild@murdoch.edu.au) or Dr Peiffer (9360 7803; J.Peiffer@murdoch.edu.au). We will be happy to discuss with you any concerns you may have about this study.
Once we have analysed the information from this study we will publish the results of the study on the Murdoch University School of Psychology and Exercise Science website: http://www.murdoch.edu.au/School-of-Psychology-and-Exercise-Science/Research/Exercise-Science-Research/Research-findings/. You can expect to receive this feedback within 12 months.

If you are willing to consent to participation in this study, please complete the Consent Form. Thank you for your assistance with this research project.

Sincerely,
Mr Faizal Abdul Manaf

This study has been approved by the Murdoch University Human Research Ethics Committee (Approval 2015/175). If you have any reservation or complaint about the ethical conduct of this research, and wish to talk with an independent person, you may contact Murdoch University's Research Ethics Office (Tel. 08 9360 6677 (for overseas studies, +61 8 9360 6677) or e-mail ethics@murdoch.edu.au. Any issue you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.
Appendix C  Adult Pre-Exercise Screening Tool

ADULT PRE-EXERCISE SCREENING TOOL

This screening tool does not provide advice on a particular matter, nor does it substitute for advice from an appropriately qualified medical professional. No warranty of safety should result from its use. The screening system in no way guarantees against injury or death. No responsibility or liability whatsoever can be accepted by Exercise and Sports Science Australia, Fitness Australia or Sports Medicine Australia for any loss, damage or injury that may arise from any person acting on any statement or information contained in this tool.

Name: _____________________________  Date: _____________________________
Date of Birth: _____________________________  Male ☐  Female ☐

STAGE 1 (COMPULSORY)

Aim: to identify those individuals with a known disease, or signs or symptoms of disease, who may be at a higher risk of an adverse event during physical activity/exercise. This stage is self-administered and self-evaluated.

Please circle response

1. Has your doctor ever told you that you have a heart condition or have you ever suffered a stroke? Yes ☐ No ☐

2. Do you ever experience unexplained pains in your chest at rest or during physical activity/exercise? Yes ☐ No ☐

3. Do you ever feel faint or have spells of dizziness during physical activity/exercise that causes you to lose balance? Yes ☐ No ☐

4. Have you had an asthma attack requiring immediate medical attention at any time over the last 12 months? Yes ☐ No ☐

5. If you have diabetes (type I or type II) have you had trouble controlling your blood glucose in the last 3 months? Yes ☐ No ☐

6. Do you have any diagnosed muscle, bone or joint problems that you have been told could be made worse by participating in physical activity/exercise? Yes ☐ No ☐

7. Do you have any other medical condition(s) that may make it dangerous for you to participate in physical activity/exercise? Yes ☐ No ☐

IF YOU ANSWERED 'YES' to any of the 7 questions, please seek guidance from your GP or appropriate allied health professional prior to undertaking physical activity/exercise.

IF YOU ANSWERED 'NO' to all of the 7 questions, and you have no other concerns about your health, you may proceed to undertake light-moderate intensity physical activity/exercise.

I believe that to the best of my knowledge, all of the information I have supplied within this tool is correct.

Signature _____________________________  Date _____________________________

ESSA  Fitness Australia  Sports Medicine Australia
<table>
<thead>
<tr>
<th>INTENSITY CATEGORY</th>
<th>HEART RATE MEASURES</th>
<th>PERCEIVED EXERTION MEASURES</th>
<th>DESCRIPTIVE MEASURES</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEDENTARY</td>
<td>&lt; 40% HRmax</td>
<td>Very, very light RPE&lt; 1</td>
<td>• Activities that usually involve sitting or lying and that have little additional movement and a low energy requirement</td>
</tr>
<tr>
<td>LIGHT</td>
<td>40 to &lt;55% HRmax</td>
<td>Very light to light RPE 1-2</td>
<td>• An aerobic activity that does not cause a noticeable change in breathing rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• An intensity that can be sustained for at least 60 minutes</td>
</tr>
<tr>
<td>MODERATE</td>
<td>55 to &lt;70% HRmax</td>
<td>Moderate to somewhat hard RPE 3-4</td>
<td>• An aerobic activity that is able to be conducted whilst maintaining a conversation uninterrupted</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• An intensity that may last between 30 and 60 minutes</td>
</tr>
<tr>
<td>VIGOROUS</td>
<td>70 to &lt;90% HRmax</td>
<td>Hard RPE 5-6</td>
<td>• An aerobic activity in which a conversation generally cannot be maintained uninterrupted</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• An intensity that may last up to about 30 minutes</td>
</tr>
<tr>
<td>HIGH</td>
<td>≥ 90% HRmax</td>
<td>Very hard RPE ≥ 7</td>
<td>• An intensity that generally cannot be sustained for longer than about 10 minutes</td>
</tr>
</tbody>
</table>

# = Borg’s Rating of Perceived Exertion (RPE) scale, category scale 0-10
### ADULT PRE-EXERCISE SCREENING TOOL
#### STAGE 2 (OPTIONAL)

**Name:**

**Date of Birth:**

**Date:**

**AIM:** To identify those individuals with risk factors or other conditions to assist with appropriate exercise prescription. This stage is to be administered by a qualified exercise professional.

<table>
<thead>
<tr>
<th>1. Age</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>≥ 45yrs Males or ≥ 55yrs Females +1 risk factor</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Gender</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Family history of heart disease (e.g., stroke, heart attack) Relative</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>Age</td>
<td>If male &lt; 55yrs = +1 risk factor</td>
</tr>
<tr>
<td>Brother</td>
<td>Age</td>
<td>If female &lt; 65yrs = +1 risk factor</td>
</tr>
<tr>
<td>Son</td>
<td>Age</td>
<td>Maximum of 1 risk factor for this question</td>
</tr>
<tr>
<td>Daughter</td>
<td>Age</td>
<td>If yes, (smoke regularly or given up within the past 6 months) = +1 risk factor</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. Do you smoke cigarettes on a daily or weekly basis or have you quit smoking in the last 6 months? Yes</th>
<th>No</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>If currently smoking, how many per day or week?</td>
<td></td>
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<td></td>
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<table>
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<tr>
<th>4. Describe your current physical activity/exercise levels:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
</tr>
<tr>
<td>Seasons/week</td>
</tr>
<tr>
<td>Duration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>If physical activity level &lt; 150 min/week = +1 risk factor</td>
<td></td>
</tr>
<tr>
<td>If physical activity level &gt; 150 min/week = -1 risk factor (vigorous physical activity/exercise weighted x 2)</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>5. Please state your height (cm)</th>
<th>weight (kg)</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>If BMI ≥ 30 kg/m² = +1 risk factor</td>
<td></td>
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<table>
<thead>
<tr>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>If yes, = +1 risk factor</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6. Have you been told that you have high blood pressure? Yes</th>
<th>No</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>If yes, = +1 risk factor</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7. Have you been told that you have high cholesterol? Yes</th>
<th>No</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>If yes, = +1 risk factor</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>8. Have you been told that you have high blood sugar? Yes</th>
<th>No</th>
<th>Question</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>If yes, = +1 risk factor</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Refer over page for risk stratification.

**STAGE 2 Total Risk Factors:**
9. Have you spent time in hospital (including day admission) for any medical condition/illness/injury during the last 12 months?  
   Yes  No

10. Are you currently taking a prescribed medication(s) for any medical condition(s)?  
    Yes  No

11. Are you pregnant or have you given birth within the last 12 months?  
    Yes  No

12. Do you have any muscle, bone or joint pain or soreness that is made worse by particular types of activity?  
    Yes  No

### STAGE 3 (OPTIONAL)

**Aim:** To obtain pre-exercise baseline measurements of other recognised cardiovascular and metabolic risk factors. This stage is to be administered by a qualified exercise professional. (Measures 1, 2 & 3 - minimum qualification, Certificate III in Fitness; Measures 4 and 5 minimum level, Exercise Physiologist**+**).

<table>
<thead>
<tr>
<th>RESULTS</th>
<th>RISK FACTORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BMI (kg/m²)</td>
<td>BMI ≥ 30 kg/m² = +1 risk factor</td>
</tr>
<tr>
<td>2. Waist girth (cm)</td>
<td>Waist &gt; 94 cm for men and &gt; 80 cm for women = +1 risk factor</td>
</tr>
<tr>
<td>3. Resting BP (mmHg)</td>
<td>SBP ≥ 140 mmHg or DBP ≥ 90 mmHg = +1 risk factor</td>
</tr>
</tbody>
</table>
| 4. Fasting lipid profile* | Total cholesterol ≥ 5.20 mmol/L = +1 risk factor  
  HDL cholesterol > 1.55 mmol/L = -1 risk factor  
  Triglycerides ≥ 1.70 mmol/L = +1 risk factor  
  LDL cholesterol ≥ 3.40 mmol/L = +1 risk factor |
| 5. Fasting blood glucose* | Fasting glucose ≥ 5.50 mmol = +1 risk factor |

**STAGE 3 Total Risk Factors =**

### RISK STRATIFICATION

Total stage 2 or Total stage 3 Plus stage 2 (Q1 - Q4)

≥ 2 RISK FACTORS – MODERATE RISK CLIENTS  
Individuals at moderate risk may participate in aerobic physical activity/exercise at a light or moderate intensity (Refer to the exercise intensity table on page 2)

< 2 RISK FACTORS – LOW RISK CLIENTS  
Individuals at low risk may participate in aerobic physical activity/exercise up to a vigorous or high intensity (Refer to the exercise intensity table on page 2)

Note: If stage 3 is completed, identified risk factors from stage 2 (Q1-4) and stage 3 should be combined to indicate risk. If there are extreme or multiple risk factors, the exercise professional should use professional judgement to decide whether further medical advice is required.
## 24-h Food Diary

<table>
<thead>
<tr>
<th>Food Group</th>
<th>Food Name and Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td></td>
</tr>
<tr>
<td>Grains/Starches</td>
<td></td>
</tr>
<tr>
<td>Vegetables</td>
<td></td>
</tr>
<tr>
<td>Fruits</td>
<td></td>
</tr>
<tr>
<td>Dairy</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>Fats/Sweets</td>
<td></td>
</tr>
<tr>
<td>Beverages</td>
<td></td>
</tr>
<tr>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td><strong>Snack</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
<td></td>
</tr>
<tr>
<td>Grains/Starches</td>
<td></td>
</tr>
<tr>
<td>Vegetables</td>
<td></td>
</tr>
<tr>
<td>Fruits</td>
<td></td>
</tr>
<tr>
<td>Dairy</td>
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</tr>
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<td>Beverages</td>
<td></td>
</tr>
<tr>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td><strong>Snack</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
<td></td>
</tr>
<tr>
<td>Grains/Starches</td>
<td></td>
</tr>
<tr>
<td>Vegetables</td>
<td></td>
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<td>Fruits</td>
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<tr>
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<td>Beverages</td>
<td></td>
</tr>
<tr>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td><strong>Snack</strong></td>
<td></td>
</tr>
</tbody>
</table>
Appendix E  Borg Scale

BORG SCALE
Rating of Perceived Exertion

6
7  Very very light
8
9  Very light
10
11  Fairly light
12
13  Somewhat Hard
14
15  Hard
16
17  Very hard
18
19  Very very hard
20  Maximal exertion