DIETARY NITRATE SUPPLEMENTATION AS AN ERGOGENIC AND THERAPEUTIC AID

JAMES KELLY

Submitted by James Kelly to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Sport & Health Sciences

November 2014

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

..................................
Abstract

Dietary nitrate (NO$_3^-$) supplementation, in the form of NO$_3^-$-rich beetroot juice, can elicit a number of biological and physiological effects within the human body, which improve exercise performance and indices of cardiovascular health. The purpose of this thesis was to investigate further the potential ergogenic and therapeutic benefits that dietary nitrate supplementation may evoke. Specific questions addressed in this thesis include whether supplementation can influence the power-duration relationship for severe-intensity exercise, and if supplementation can be effective in an older population and in varying environmental conditions. The thesis also strives to develop our understanding of the physiological mechanisms that underpin effective supplementation. Healthy, adult human subjects volunteered for all investigations presented in this thesis. A number of physiological variables were assessed in each experimental chapter, following nitrate supplementation. 

Chapter 4: Short term dietary NO$_3^-$ supplementation reduced systolic blood pressure by 4 mmHg (BR: 118 ± 5 vs. PL: 122 ± 5 mmHg) and improved exercise tolerance during exercise at 60%Δ (BR: 696 ± 120 vs. PL: 593 ± 68 s), 70%Δ (BR: 452 ± 106 vs. PL: 390 ± 86 s), 80%Δ (BR: 294 ± 50 vs. PL: 263 ± 50 s) but not 100% peak power (BR: 182 ± 37 vs. PL: 166 ± 26 s) but did not significantly alter either critical power (BR: 221 ± 27 vs. PL: 218 ± 26 W) or W’ (BR: 19.3 ± 4.6 vs. PL: 17.8 ± 3 kJ). The $\dot{V}$O$_2$ phase II time constant was significantly shorter in BR compared to PL (BR: 22.8 ± 7.4 vs. PL: 25.4 ± 7.2 s) when considered irrespective of exercise intensity. 

Chapter 5: The metabolism of [NO$_2^-$] during exercise and recovery is altered by NO$_3^-$ supplementation and, to a lesser extent, FIO$_2$. End exercise $\dot{V}$O$_2$ was significantly lower during moderate-intensity exercise in Hypoxia-BR (H-BR) compared to Hypoxia-PL (H-PL) (H-BR: 1.91 ± 0.28 vs. H-PL: 2.05 ± 0.25 L·min$^{-1}$) and Normoxia-PL (N-PL) (1.97 ± 0.25 L·min$^{-1}$). $\dot{V}$O$_2$ kinetics were faster in H-BR compared to H-PL (phase II $\tau$, H-BR: 24 ± 13 vs. H-PL: 31 ± 11 s). Tolerance to severe-intensity exercise was improved by NO$_3^-$ supplementation in hypoxia (H-PL: 197 ± 28 vs. H-BR: 214 ± 43 s), but not normoxia (N-PL: 431 ± 124 vs. N-BR: 412 ± 139 s). 

Chapter 6: In a healthy older population, NO$_3^-$ supplementation significantly reduced resting systolic (BR: 115 ± 9 vs. PL: 120 ± 6 mmHg) and diastolic (BR: 70 ± 5 vs. PL: 73 ± 5 mmHg) blood pressure. Supplementation also resulted in a speeding of the $\dot{V}$O$_2$ mean response time (BR: 25 ± 7 vs. PL: 28 ± 7 s) in the transition from standing rest to treadmill walking, although the $\dot{O}_2$ cost of exercise remained unchanged. Functional capacity (6-minute walk test), the muscle metabolic response to low-intensity exercise, brain metabolite
concentrations and cognitive function were not altered. Chapter 7: On average, muscle tissue [NO$_3^-$] across the entire exercise protocol was significantly elevated by 72% following BR. At the group level, $\dot{V}O_2$ and muscle metabolic responses during exercise were unchanged between conditions and tolerance to severe-intensity exercise was unaltered. However, further analyses revealed the existence of ‘responders’ and ‘non responders’ with the changes in steady-state $\dot{V}O_2$ and muscle [NO$_3^-$] being correlated with severe-intensity exercise tolerance. The results of this thesis demonstrate that dietary NO$_3^-$ supplementation has the potential to elicit ergogenic and therapeutic benefits in varying populations and environmental conditions. However, the presented data also clearly outline that supplementation may not always be effective. While the underlying mechanisms and parameters which may influence its effectiveness are not yet fully understood, supplementation should be carefully considered, monitored and tailored specifically for individuals and their particular requirements.
Table of contents

Abstract..............................................................................................................i
Table of contents..........................................................................................iii
List of tables..................................................................................................vi
List of figures..................................................................................................viii
Symbols and abbreviations............................................................................xii
Declaration, publications and communications............................................xv
Acknowledgements.......................................................................................xviii

Chapter 1: Introduction

History of nitric oxide physiology.................................................................1
Skeletal muscle bioenergetics..........................................................................2
Oxygen uptake kinetics...................................................................................3
Cardiovascular health.....................................................................................5

Chapter 2: Literature Review

Nitric Oxide.....................................................................................................7
   Nitric oxide production................................................................................7
   Nitrate-nitrite-nitric oxide pathway..............................................................8
   Beetroot and typical NO\textsubscript{3} intake....................................................10
   Plasma nitrate and nitrite concentrations...................................................11
   Toxicity.........................................................................................................12
      Methaemoglobinemia.............................................................................12
      Nitration of proteins..............................................................................12
      Carcinogenic properties.......................................................................12
   Summary......................................................................................................13
Dietary Nitrate Supplementation....................................................................13
   Blood pressure...........................................................................................13
   Oxygen uptake............................................................................................14
      Moderate-intensity exercise.................................................................14
      Severe-intensity exercise.....................................................................16
   Power-duration relationship....................................................................17
   Exercise performance...............................................................................20
Muscle metabolism ................................................................. 22
Hypoxia ................................................................................... 23
Senescence ............................................................................. 25
Summary .................................................................................... 27

Aims .......................................................................................... 28
Hypotheses .................................................................................... 29

Chapter 3: General Methods

Ethical approval and informed consent ........................................ 31
Health and Safety ........................................................................ 31
Subjects ...................................................................................... 31
Supplementation ........................................................................ 32
Blood pressure ........................................................................... 33
Heart rate .................................................................................... 33
Measurement of lactate, glucose, potassium and sodium .............. 33
Measurement of nitrate and nitrite concentrations ......................... 34
Pulmonary gas exchange .............................................................. 35
Normalisation of exercise intensity ................................................. 35
Mathematical modelling of VO_2 data ............................................. 37
Statistical methods ...................................................................... 39

EXPERIMENTAL CHAPTERS

Chapter 4: Effects of nitrate on the power-duration relationship for severe-intensity exercise.

Introduction .................................................................................. 40
Methods ....................................................................................... 41
Results .......................................................................................... 43
Discussion ..................................................................................... 45
References .................................................................................... 47

Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia.

Introduction .................................................................................. 49
Methods ....................................................................................... 50
Results .......................................................................................... 52
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, O\textsubscript{2} uptake kinetics, and muscle and cognitive function in older adults.

Introduction..............................................................................................................60
Methods......................................................................................................................61
Results.........................................................................................................................64
Discussion....................................................................................................................66
References....................................................................................................................68

Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O\textsubscript{2} uptake kinetics during moderate- and high-intensity cycle exercise.

Introduction..............................................................................................................72
Methods......................................................................................................................74
Results.........................................................................................................................79
Discussion....................................................................................................................91

Chapter 8: General Discussion

Research questions addressed....................................................................................96

Summary of main findings........................................................................................96
  Influence of NO\textsubscript{3}\textsuperscript{−} supplementation on the power-duration relationship....96
  Influence of NO\textsubscript{3}\textsuperscript{−} supplementation in hypoxia........................................97
  Influence of NO\textsubscript{3}\textsuperscript{−} supplementation in an older population....................97
  Influence of NO\textsubscript{3}\textsuperscript{−} supplementation on muscle metabolism.....................98
Evidence of increased NO bioavailability.................................................................99
‘Utilization’ of NO\textsubscript{3} and NO\textsubscript{2} during exercise........................................100

Ergogenic effects of NO\textsubscript{3}\textsuperscript{−} supplementation..............................................101
  Exercise tolerance.....................................................................................................101
  Exercise efficiency....................................................................................................103
  Relationship between submaximal exercise efficiency and exercise tolerance.........104
  Power-duration relationship....................................................................................104
  \textit{VO}_{2} kinetics.......................................................................................................106
  Muscle metabolism...................................................................................................108

Therapeutic effects of NO\textsubscript{3}\textsuperscript{−} supplementation........................................109
  Blood pressure..........................................................................................................109
Cerebral measures (H\textsubscript{1}MRS, ADC) and cognitive function.................110
Arterial oxygen saturation and muscle oxygenation.................................111

**Effectiveness of NO\textsubscript{3} suppletionation**..................................................112
**Ergogenic applications**..................................................................................113
**Therapeutic applications**................................................................................114

**Limitations**........................................................................................................115
Nitrate dose..........................................................................................................115
Dietary control.....................................................................................................115
Measurement of NO markers restricted to NO\textsubscript{2} and NO\textsubscript{3}..................116
Constant work rate tests to exhaustion...............................................................116
Is the 6-minute walk test a reliable and valid measure of
functional capacity?..........................................................................................117
Does pulmonary $\dot{V}O_2$ accurately reflect muscle $\dot{V}O_2$?.........................117

**Future research questions**...............................................................................118
Oral microbiome..................................................................................................118
NO\textsubscript{3} suppletionation effectiveness..........................................................118
Clinical populations............................................................................................118
Cerebral physiology and cognitive function.....................................................119

**Conclusions**.....................................................................................................119

**References**.....................................................................................................120
List of tables

Chapter 4  Effects of nitrate on the power-duration relationship for severe-intensity exercise

Table 4.1  Mean ± SD heart rate and blood [lactate], during four different severe-intensity exercise bouts…………………………………….44

Table 4.2  Mean ± SD oxygen uptake dynamics during four different severe-intensity exercise bouts…………………………………….45

Chapter 5  Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O₂ uptake dynamics during exercise in hypoxia and normoxia

Table 5.1  Arterial oxygen saturation levels during rest and in response to moderate- and severe-intensity exercise in hypoxic and normoxic conditions, following PL and BR supplementation…………………53

Table 5.2  Oxygen uptake kinetics in response to moderate- and severe-intensity exercise in hypoxic and normoxic conditions, following PL and BR supplementation……………………………………55

Table 5.3  Near infra-red spectroscopy-derived HHb, HbO₂, Hbtot and TOI dynamics during moderate- and severe-intensity exercise in hypoxic and normoxic conditions, following PL and BR supplementation……………………………………56

Chapter 6  Effects of short-term dietary nitrate supplementation on blood pressure, O₂ uptake kinetics, and muscle and cognitive function in older adults.

Table 6.1  Pulmonary gas exchange, ventilation, and heart rate during moderate-intensity exercise following PL and BR supplementation.64

Table 6.2  Muscle metabolic responses during low-intensity exercise following PL and BR supplementation……………………………………64
Table 6.3  
**Muscle metabolic responses during high-intensity exercise following PL and BR supplementation**

Table 6.4  
**Cognitive performance tests following PL and BR supplementation**

Table 6.5  
**$^1$H-MRS and ADC brain scan data following PL and BR supplementation**

Chapter 7  
**Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary $O_2$ uptake kinetics during moderate- and high-intensity cycle exercise.**

Table 7.1  
**Blood [lactate] and [glucose] and plasma [sodium] and [potassium] responses during moderate- and severe-intensity cycling, following BR and PL**

Table 7.2  
**Muscle [ATP], [PCr], [lactate], [HAD] and pH responses during moderate and severe-intensity cycling, following BR and PL. * = significantly different from ‘Rest’ (P < 0.05)**

Table 7.3  
**Oxygen uptake kinetics in response to moderate- and severe-intensity exercise, following PL and BR supplementation**

Table 7.4  
**Physiological and performance changes when participants considered as ‘responders’ and ‘non-responders’. * = significantly different between the two groups (P < 0.05). PL→BR, participants who consumed PL on visit 2 and BR on visit 3; BR→PL, participants who consumed BR on visit 2 and PL on visit 3**
Chapter 1

Introduction

Figure 1.1 Schematic illustration of the \( \dot{V}O_2 \) response to cycle exercise. At the onset of exercise, an oxygen deficit is incurred due to the \( \dot{V}O_2 \) response lagging behind the energy requirements of the task. The primary component of the \( \dot{V}O_2 \) response increases in an exponential fashion and attains a steady state within 120-180s.

Figure 1.2 The \( \dot{V}O_2 \) response to exercise above the CP in a healthy individual. Note that \( \dot{V}O_2 \) continues to increase beyond the primary component, leading to an end-exercise \( \dot{V}O_2 \) that is \( \sim 500 \) ml·min\(^{-1}\) higher than expected. This additional rise in \( \dot{V}O_2 \) is termed the ‘slow component’.

Chapter 2

Literature Review

Figure 2.1 A schematic diagram of the enterosalivary circulation of nitrate in the human body.

Figure 2.2 A schematic diagram outlining the pathways of NO generation and the roles it can have within the human body.

Figure 2.3 Illustration of the hyperbolic power-duration relationship.

Figure 2.4 A schematic diagram outlining the dependance of \( \dot{V}O_2 \) kinetics on muscle \( O_2 \) delivery in various populations.

Chapter 4

Effects of nitrate on the power-duration relationship for severe-intensity exercise

Figure 4.1 Group mean ± SD cycling times to exhaustion across four severe-intensity power outputs following both BR and PL.

Figure 4.2 Effects of BR on the power-duration relationship established from four severe-intensity prediction trials. The group mean ± SE power-duration profiles are shown in panel (A), and the group mean ± SE power-1/time relationships are shown in panel (B).
Figure 4.3  
Mean ± SD predicted time to complete given amounts of work following BR and PL as calculated using the CP and W’ estimates from the power-1/time model.

Chapter 5  
Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O₂ uptake dynamics during exercise in hypoxia and normoxia.

Figure 5.1  
Plasma [NO₂⁻] response during moderate- and severe-intensity exercise and recovery following PL and BR, in normoxia and hypoxia. Error bars indicate SE. H-BR was greater than H-PL at each time point and N-BR was greater than N-PL at each time point. A = P < 0.05 for N-BR compared to H-BR; b = P < 0.05 compared to moderate baseline; c = P < 0.05 compared to severe baseline. Where error bars are not visible, the size of the data point exceeds the error.

Figure 5.2  
Pulmonary O₂ uptake (\(\dot{V}O₂\)) responses during a step increment to a moderate-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles, with the PL responses being shown as open circles. The dotted vertical line denotes the abrupt ‘step’ transition from baseline to moderate-intensity cycling exercise. Error bars indicate the SE. A: Group mean response to moderate-intensity exercise in normoxia (~21% \(FIO₂\)). B: Group mean response to moderate-intensity exercise in hypoxia (~13.2 \(FIO₂\)); * = P < 0.05 compared to H-PL.

Figure 5.3  
Pulmonary O₂ uptake (\(\dot{V}O₂\)) responses and time-to-exhaustion during a step increment to a severe-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles, with the PL responses being shown as open circles. The dotted vertical line denotes the abrupt ‘step’ transition from baseline to moderate-intensity cycling exercise. Error bars indicate the SE. A: Group mean response to severe-intensity exercise in normoxia (~21% \(FIO₂\)). B: Group mean response to severe-intensity exercise in hypoxia (~13.2 \(FIO₂\)).
Chapter 6  **Effects of short-term dietary nitrate supplementation on blood pressure, O₂ uptake kinetics, and muscle and cognitive function in older adults.**

Figure 6.1  *Pulmonary oxygen uptake responses during a step increment to a moderate-intensity work rate in a representative subject following BR and PL supplementation.*

Figure 6.2  *Group mean muscle metabolic responses to low-intensity, leg-extension exercise following PL and BR supplementation. Changes in muscle [PCr] (panel A), [ADP] (panel B), [Pi] (panel C) from rest to steady state are represented.*

Figure 6.3  *Group mean intramuscular [PCr] response to 24-s high-intensity, leg-extension exercise and subsequent recovery, following PL and BR supplementation.*

Chapter 7  **Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O₂ uptake kinetics during moderate- and high-intensity cycle exercise.**

Figure 7.1  *Plasma [NO₃⁻] response during moderate- and severe-intensity exercise and recovery following BR (A & C) and PL (B & D). Error bars indicate the SE. BR was greater than PL at each time point. * P < 0.05 BR compared to PL; † P < 0.05 compared to baseline.*

Figure 7.2  *Plasma [NO₂⁻] response during moderate- and severe-intensity exercise and recovery following (A & C) and PL (B & D). Error bars indicate the SE. BR was greater than PL at each time point. *P < 0.05 BR compared to PL; † P < 0.05 compared to baseline.*

Figure 7.3  *Muscle [NO₃⁻] response during moderate- and severe-intensity exercise following BR (A & C) and PL (B & D). Error bars indicate*
the SE. BR was greater than PL at each time point. * P < 0.05 BR compared to PL. 

Figure 7.4 Pulmonary oxygen uptake (\(\dot{V}O_2\)) responses of A) the BR→PL group and B) the BR→PL group during a step increment to a moderate-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles, with the PL responses being shown as open circles. The dotted vertical line denotes the abrupt ‘step’ transition from baseline to moderate-intensity cycle exercise.

Figure 7.5 Pearson product-moment correlation coefficient between A) the change in muscle [NO\(_3\)] following BR (BR-PL; nmol/mg DW) and the change in exercise tolerance following BR (BR-PL; s); and B) the change in steady-state \(\dot{V}O_2\) following BR (BR-PL; ml/min) and the change in exercise tolerance following BR (BR-PL; s).
Symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ ]</td>
<td>concentration</td>
</tr>
<tr>
<td>Δ</td>
<td>difference</td>
</tr>
<tr>
<td>(^1)H-MRS</td>
<td>(^1)proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>6MWT</td>
<td>six-minute walk test</td>
</tr>
<tr>
<td>(^3^1)P-MRS</td>
<td>(^3^1)Phosphorous nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>A</td>
<td>exponential response amplitude</td>
</tr>
<tr>
<td>ADC</td>
<td>apparent diffusion coefficient</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMARES</td>
<td>advanced method for accurate, robust and efficient spectral fitting</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BH(_4)</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>BR</td>
<td>beetroot</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium</td>
</tr>
<tr>
<td>Ch</td>
<td>choline</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CO(_2)</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CON</td>
<td>control</td>
</tr>
<tr>
<td>CP</td>
<td>critical power</td>
</tr>
<tr>
<td>Cr</td>
<td>creatine</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GET</td>
<td>gas exchange threshold</td>
</tr>
<tr>
<td>H(^+)</td>
<td>hydrogen ion, proton</td>
</tr>
<tr>
<td>Hb</td>
<td>haemoglobin</td>
</tr>
<tr>
<td>HbO(_2)</td>
<td>oxygenated haemoglobin</td>
</tr>
<tr>
<td>Hb(_{tot})</td>
<td>total haemoglobin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HHb</td>
<td>deoxygenated haemoglobin</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>jMRUI</td>
<td>java-based magnetic resonance user interface</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium ion</td>
</tr>
<tr>
<td>kJ</td>
<td>kilojoules</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MR</td>
<td>magnetic resonance</td>
</tr>
<tr>
<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MRT</td>
<td>mean response time</td>
</tr>
<tr>
<td>mI</td>
<td>myo-inositol</td>
</tr>
<tr>
<td>NAA</td>
<td>N-acetyl aspartate</td>
</tr>
<tr>
<td>NaNO₃⁻</td>
<td>sodium nitrate</td>
</tr>
<tr>
<td>NIRS</td>
<td>near-infrared spectroscopy</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>nitrate</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide</td>
</tr>
<tr>
<td>P</td>
<td>power output</td>
</tr>
<tr>
<td>PCr</td>
<td>phosphocreatine</td>
</tr>
<tr>
<td>P₁</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PL</td>
<td>placebo</td>
</tr>
<tr>
<td>P/O</td>
<td>oxygen cost of ATP resynthesis</td>
</tr>
<tr>
<td>PRESS</td>
<td>point resolved spectroscopy</td>
</tr>
<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
</tr>
<tr>
<td>RMR</td>
<td>resting metabolic rate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SENSE</td>
<td>sensitivity encoding</td>
</tr>
<tr>
<td>τ</td>
<td>time constant</td>
</tr>
<tr>
<td>TD</td>
<td>exponential response time delay</td>
</tr>
<tr>
<td>T_{lim}</td>
<td>limit of tolerance</td>
</tr>
<tr>
<td>TT</td>
<td>time trial</td>
</tr>
<tr>
<td>VCl₃</td>
<td>vanadium chloride</td>
</tr>
<tr>
<td>( \dot{V} \text{CO}_2 )</td>
<td>pulmonary carbon dioxide output</td>
</tr>
<tr>
<td>( \dot{V}_E )</td>
<td>minute ventilation (expired)</td>
</tr>
<tr>
<td>( \dot{V} \text{O}_2 )</td>
<td>pulmonary oxygen uptake</td>
</tr>
<tr>
<td>( \dot{V} \text{O}_2\text{max} )</td>
<td>maximal oxygen uptake</td>
</tr>
<tr>
<td>( \dot{V} \text{O}_2\text{peak} )</td>
<td>peak oxygen uptake</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>W'</td>
<td>curvature constant of the power-duration relationship</td>
</tr>
</tbody>
</table>
Declaration

The material contained within this thesis is original work conducted and written by the author. The following publications and communications are a direct consequence of the work.

Refereed Journal Articles


Other publications


Works in review


Conference Activity

**Oral Presentation**: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O₂ uptake dynamics during exercise in hypoxia and normoxia. 19th annual Congress of the European College of Sports Science, VU University, Amsterdam, July 2014.


**Oral Presentation**: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O₂ uptake dynamics during exercise in hypoxia and normoxia. BASES Student Conference, University of Portsmouth, Portsmouth, April 2014.


**Oral Presentation**: ‘Effects of nitrate on the power-duration relationship for severe-intensity exercise’, BASES Student Conference, Cardiff Metropolitan University, Cardiff, March 2013.


*Awards & honours*

University of Exeter Strategic Development Funding -£4900 (2014)

Shortlisted for Exeter Impact Award- Outstanding impact in health and wellbeing (2013)


University of Exeter Researcher Development Travel Prize- £400 (2012).
Acknowledgements

The completion of this thesis would not have been possible without the input and support provided by a number of individuals for which I am extremely grateful.

Firstly, I would like to say a huge thank you to my fantastic PhD supervisors, Professor Andy Jones and Dr Anni Vanhatalo, I could not have asked for any more from a supervisory team. Your hard work, commitment and dedication to the role has been quite exceptional. The ‘open door’ policy you employ for discussion and your consistently prompt response to emails has made working with you a seamless task. Your productivity, professionalism and work ethic is second to none and in you both, I have an inspirational and influential, academic role model. The standard of your supervision and on-going support has been unquestionable and for that I am truly thankful.

I would also like to say thank you to Dr Stephen Bailey who really instigated my interest in a career in research. As a keen undergraduate, I was involved in each of Stephen’s PhD studies as either a participant or assistant and learned a lot from being immersed in the laboratory environment and from Stephen himself. His support and academic guidance has continued during my time as a PhD student, during designated meetings and over a few beers at the Raddy.

The research team that has been assembled here at Exeter, I believe, is somewhat unique to this institution. The close-knit team ethic provides a fantastic, supportive research environment, breeds healthy competition and promotes continued success. With that in mind, thanks must go to Dr Daryl Wilkerson, Dr Phil Skiba, Dr Weerapong Chidnok, Professor Peter Krstrup, Dr Magni Mohr, Dr Sarah Jackman, Jamie Blackwell, Lee Wylie, Matt Black, Ant Shephard, Sinead McDonagh and Chris Thompson for your assistance in aspects of my work and for contributing to the great team atmosphere that we have here at St Luke’s and long may it continue.

I must also acknowledge the input of a number of academics outside of our research team who have made important contributions to this thesis. Thanks to Dr. Jonathan Fulford for his help with data collection and analysis using magnetic resonance spectroscopy and cognitive function testing in Chapter 6; Professor Paul Winyard for his input to Chapter 6 and continued permission to use the NO analyser in his laboratory; Professor Nigel
Benjamin for his contributions to Chapter 6 and Professor Jens Bangsbo for his important role in Chapter 7.

Acknowledgement must also be made to the administrative, academic and support staff in Sport & Health Sciences. Thanks to David Childs, Len Maurer, Alison Hume, Julia Warner, Clare Fogarty and Rosa James-Watling who have made important contributions in ensuring that everything runs smoothly. Furthermore, to each subject who volunteered their time to participate in this research, to repeatedly exercise to exhaustion, to provide numerous blood and muscle biopsy samples and to consume copious amounts of beetroot juice, this research would not have been possible without your hard-work and commitment, so many thanks to them.

Finally, I would like to thank my family: Mum, Dunc, Mat, Gran and Sam. You have seen me through many years of education, providing love and support along the way.
Chapter 1: Introduction

History of nitric oxide physiology

The therapeutic effects of nitrate (NO$_3^-$), nitrite (NO$_2^-$) and nitric oxide (NO) were first realised during medieval times in ancient Chinese medicine. At the turn of the 20th century, a Daoist monk discovered hoards of medieval Buddhist manuscripts, paintings and documents in a grotto in the city of Dunhuang, West China. The documents, hidden for over 900 years, included medical recipes, one of which instructed patients to place potassium nitrate under the tongue and to swallow the saliva in order to treat symptoms of angina and digital ischemia. These specific instructions are particularly significant as they implicate the salivary-reducing bacteria in converting nitrate to nitrite (Bryan & Loscalzo, 2011). Furthermore, the longevity of Japanese (Sobko et al., 2009) and Mediterranean (Trichopoulou et al., 2000) populations is amongst the highest in the world. This is partly explained by the low occurrence of cardiovascular disease, which in turn can be attributed to the traditional diets typically consumed by these populations. One common feature of the traditional Japanese and Mediterranean diets is the high vegetable consumption, which would be expected to result in an increased ingestion of NO$_3^-$. Specific interest and scientific research into NO$_3^-$, NO$_2^-$ and NO began to soar following the discovery of the physiological role of NO in both health and disease states, along with the characterization of its metabolism into NO$_2^-$ and NO$_3^-$ in mammalian tissues. The discovery of the endothelium derived relaxing factor (EDRF) and NO pathway in the 1980’s represented a critical advance in the understanding of cell signalling and resulted in advancements in many clinical areas. This finding was considered so important that the Nobel Prize in Physiology or Medicine was awarded to its discoverers, Drs. Louis Ignarro, Robert Furchgott and Ferid Murad in 1998. More than a decade since the Nobel Prize was awarded and after over 100,000 published scientific papers, our understanding of the production, regulation and biological functions of NO and its derivatives is still incomplete. The importance of evolving this understanding is extremely important in developing therapeutic interventions in NO biology.

Hundreds of research papers are published in the field of nutrition and exercise every year. The findings of such studies can reveal physiological effects of particular foods, dietary supplements and substances. This knowledge can be utilised to aid athletic preparation, performance and/or recovery and can often be transferred into clinical populations to offset or prevent the negative effects of disease. A recent revelation in the nutrition and exercise
research sphere is that of dietary NO$_3^-$ supplementation, which has been shown to possess a number of ergogenic and therapeutic qualities, thought to occur due to increased NO bioavailability. These NO-mediated effects include smooth muscle relaxation causing subsequent vasodilation and lowered blood pressure (Webb et al., 2008), reductions in the oxygen cost of exercise (Larsen et al., 2007) and improved tolerance to exercise (Bailey et al., 2009). These outcomes provide important implications for a range of populations including the general public, for maintaining cardiovascular health and well-being; sports performers striving for excellence; and ageing or diseased individuals, looking to offset the negative impact of senescence or pathology. Therefore, interventions which may increase the bioavailability of NO have become a key focus of current research.

**Skeletal muscle bioenergetics**

In order to meet the energy requirements of contracting human skeletal muscle, the liberation of energy stored in the molecule adenosine tri-phosphate (ATP) is essential. Human skeletal muscle has only a limited store of ATP which can be depleted within a few seconds of the initiation of muscle contraction. To avert an abrupt and debilitating depletion in intramuscular ATP, the immediate and continued resynthesis of this molecule is imperative. During the first ~10s of intense exercise, the breakdown of stored muscle phosphocreatine (PCr) provides the necessary chemical energy in order to resynthesize adequate ATP. This process activates an additional anaerobic energy system known as anaerobic glycolysis, which metabolises glucose into lactate and 1 hydrogen ion (H$^+$). This process has a net yield of 2 ATP molecules per glucose molecule and is fuelled by the finite muscle glycogen reserves. As a result of ATP synthesis via this anaerobic energy pathway, metabolites associated with the process of muscle fatigue (inorganic phosphate (P$_i$) and H$^+$) accumulate (Allen et al., 2008). Although glycolysis releases anaerobic energy quickly, the yield of 2 ATP molecules is relatively small. In contrast, aerobic metabolic reactions provide for the greatest portion of energy transfer, particularly when exercise duration extends beyond ~2 minutes. The aerobic pathway utilises both carbohydrate and fat as substrates, which is important because the aerobic breakdown of a glucose molecule yields 38 ATP molecules (19 times as many as glycolysis), whilst fats are even more energy rich, although they require a longer period to be metabolised. In addition to this, the by-products of aerobic metabolism (H$_2$O and CO$_2$) are well regulated and therefore limited metabolic perturbation is associated with this energy pathway. The processes involved in the transfer of energy do not sequentially switch from PCr, glycolysis and oxidative phosphorylation; rather interplay between these pathways is evident.
Chapter 1: Introduction

**Oxygen uptake kinetics**

Once exercise has been initiated, muscle O$_2$ consumption must increase with rapid response kinetics in order to take advantage of the ‘efficient’ aerobic pathway. Oxygen uptake (VO$_2$) rises exponentially and does not reach ‘steady-state’ until 120-180 s following the onset of exercise performed below the gas exchange threshold (GET) (Jones & Poole, 2005). The steady-state in VO$_2$ represents the metabolic cost of a given bout of exercise, with a lower steady-state amplitude representing a lower energy cost of exercise. Prior to this steady-state being attained there is a discrepancy between the energy requirement and the amount of energy supplied by oxidative phosphorylation, which is termed the ‘oxygen deficit’. This exponential rise in VO$_2$ can limit the potential aerobic energy yield within the muscle. At the onset of constant work rate exercise, there is an early rapid increase which is initiated within the first breath. This initial increase in VO$_2$ (Phase I) is followed by a rapid exponential increase in VO$_2$ (Phase II) which has a time constant (time taken to achieve 63% of the change in VO$_2$) of 25-40 s in healthy individuals. This rapid exponential increase drives VO$_2$ toward the actual or initially anticipated steady-state within 180 s (Jones & Poole, 2005). Phase I (commonly referred to as the cardio-dynamic component) represents the O$_2$ exchange associated with the initial elevation of cardiac output and pulmonary blood flow, whereas Phase II (commonly termed the ‘primary component’) reflects the arrival at the lung of venous blood from the exercising muscles (Whipp and Wasserman, 1972; Linnarsson, 1974; Whipp et al., 1982). Importantly, a faster VO$_2$ response will elicit a smaller oxygen deficit, whereas extremely unfit or unhealthy individuals will have a slow response and will incur a high oxygen deficit and subsequently a greater degree of intracellular perturbation. Slow VO$_2$ kinetics result in a greater depletion of intramuscular [PCr], greater utilization of intramuscular glycogen stores and the accumulation of fatiguing metabolites, all of which may lead to reduced exercise tolerance.
Fig 1.1: Schematic illustration of the VO₂ response to cycle exercise. At the onset of exercise, an oxygen deficit is incurred due to the VO₂ response lagging behind the energy requirements of the task. The primary component of the VO₂ response increases in an exponential fashion and attains a steady state within 120-180s.

During exercise completed above the GET, a rise in the VO₂ response in addition to the primary component is evident. This additional superimposed elevation in VO₂ is termed the ‘slow component’ and can be stabilised during heavy-intensity exercise (below the critical power (CP)), but continues to drive the VO₂ to maximum during severe-intensity exercise (above CP). Importantly, the VO₂ slow component is associated with the depletion of muscle [PCr] and increased glycogen utilisation and metabolite accumulation within the exercising muscle (Poole et al., 1991; Rossiter et al., 2002; Krstrup et al., 2004).
Figure 1.2: The \( \dot{\text{VO}_2} \) response to exercise above the CP in a healthy individual. Note that \( \dot{\text{VO}_2} \) continues to increase beyond the primary component, leading to an end-exercise \( \dot{\text{VO}_2} \) that is \( \sim 500 \text{ ml/min} \) higher than expected. This additional rise in \( \dot{\text{VO}_2} \) is termed the ‘slow component’.

With this in mind, reducing the \( \dot{\text{VO}_2} \) steady-state, speeding the kinetic response and/or reducing the magnitude of the \( \dot{\text{VO}_2} \) slow component would be expected to reduce the extent of muscular [PCr] and [glycogen] degradation and curtail the accumulation of fatigue related metabolites. These alterations in \( \dot{\text{VO}_2} \) kinetics may improve severe-intensity exercise performance in young healthy individuals and may enhance tolerance to moderate and severe-intensity exercise in diseased and/or senescent populations.

**Cardiovascular health**

Hypertension is an important global public health issue due to its high prevalence and concomitant increase in risk of disease (Slama *et al.*, 2002; Calhoun *et al.*, 2002). Hypertension effects \( \sim 1 \) billion adults worldwide (Lloyd-Jones *et al.*, 2009) and is a predisposing risk factor for stroke, myocardial infarction, congestive heart failure, arterial aneurysm and renal failure (Hackman *et al.*, 2010; Pierdomenico *et al.*, 2009). Therefore, the prevention and management of hypertension is a major public health challenge, with a number of antihypertensive agents
being developed and tested in a variety of settings and populations. Existing literature collectively suggests that lowering arterial pressure can reduce cardiovascular morbidity and mortality (Lenfant et al., 2003). However, some of the treatments and medicines currently used can be expensive, result in unfavourable side effects and resistance to their therapeutic efficacy can be developed (Calhoun et al., 2008). Therefore, the identification of a relatively cheap, naturally occurring method of reducing blood pressure is important for the treatment and/or prevention of hypertension in the future.

The purpose of this thesis is to explore the use of dietary nitrate supplementation as a potential ergogenic intervention in modulating the $\dot{V}O_2$ kinetic and muscle metabolic response to exercise and to assess its therapeutic potential upon markers of cardiovascular health across healthy and senescent populations.
Nitric oxide (NO) is a soluble, gaseous signalling molecule known to play a critical role in a range of physiological functions within the human body and has a half-life in circulation, *in vivo*, of around 0.1s (Kelm *et al.*, 1990). From the regulation of blood flow, muscle contractility and mitochondrial respiration, to host defence, neurotransmission and the homeostasis of glucose and calcium (Bryan *et al.*, 2006; Dejam *et al.*, 2004; Stamler *et al.*, 2001), effective NO production is considered essential in order to maintain normal physiological functioning. Indeed, NO has emerged as one the most researched molecules in physiology and medicine in recent decades.

**NO production**

NO production via the NO synthase (NOS) enzymes is well established, with endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms of the enzymes having been described (Stamler *et al.*, 2001). These enzymes catalyze the complex five electron oxidation of L-arginine which yields NO and L-citrulline. This oxygen-dependent reaction requires a number of substrates and co-factors including oxygen, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄), haem and calmodulin (Alderton *et al.*, 2001). A reduced bioavailability of any of these co-factors can limit the production of NO via the NOS pathway (Crabtree *et al.*, 2009), which is associated with cardiovascular (Försterman, 2010) and metabolic disease (Wu *et al.*, 2009) as well as an attenuated tolerance to exercise (Lauer *et al.*, 2008). Furthermore, red blood cells have recently been identified to reversibly bind, transport and release NO within the cardiovascular system using an endothelial-type NOS, localized in the plasma membrane and cytoplasm of the red blood cell (Kleinbongard *et al.*, 2013).

An additional NO generating pathway has been identified (Benjamin *et al.*, 1994), in which NO is produced through the reduction of inorganic nitrate (NO₃⁻) to nitrite (NO₂⁻) and further to NO. This pathway offers a supplementary method to promote NO production when NO synthesis via the NOS pathway is impaired (Carlström *et al.*, 2010) and is a key focus of current research.
Chapter 2: Literature Review

Nitrate - nitrite - nitric oxide pathway

NO$_3^-$ and NO$_2^-$ are both generated endogenously in humans. The formation of NO$_3^-$ occurs via the reaction of NO$_2^-$ or NO with oxyhaemoglobin (Cooper, 1999), while NO$_2^-$ is generated through the reaction of NO with oxygen (Ignarro et al., 1993) or the oxidation of NO by ceruloplasmin (Shiva et al., 2006). To the general public, inorganic NO$_2^-$ and NO$_3^-$ are considered as undesired residues in the food chain, whilst biologists traditionally viewed them as inert oxidation end products of the metabolism of endogenous NO. However, a growing body of evidence indicates that NO$_3^-$ and NO$_2^-$ can be recycled in vivo to form bioactive NO under certain physiological conditions (Lundberg et al., 2004; Bryan 2006; van Faassen et al., 2009).

NO$_3^-$ is naturally ingested as part of a healthy diet, with 60-80% of daily NO$_3^-$ intake in a Western diet being made up of vegetables (Ysart et al., 1999). Of these, leafy green vegetables (lettuce, spinach, rocket) and beetroot have a particularly high NO$_3^-$ content (Bryan & Hord, 2010). Upon ingestion, the NO$_3^-$ is rapidly absorbed from the gut and passes into the systemic circulation within ~60 min (Lundberg et al., 2009), where it has a half-life of ~5h suspended in the plasma (McKnight et al., 1997). Up to 25% of this inorganic NO$_3^-$ is absorbed from the stomach into the circulation, where it is taken up by the salivary glands and concentrated in the saliva (Lundberg et al., 2008). Facultative anaerobic bacteria (Vionella species) in crypts of the dorsum of the tongue then reduce the NO$_3^-$ to NO$_2^-$ (Duncan et al., 1995). When swallowed into the acidic environment of the stomach, some of the NO$_2^-$ is further converted into nitric oxide (NO) (Benjamin et al., 1994), whilst the remainder is absorbed to increase circulating plasma NO$_2^-$ concentration [NO$_2^-$]. Dietary NO$_3^-$ supplementation, in the form of pharmacological sodium nitrate (NaNO$_3$) (Larsen et al., 2007, 2010, 2011), potassium nitrate (KNO$_3$) (Kapil et al., 2010) or natural NO$_3^-$ rich beetroot juice (Webb et al., 2008; Bailey et al., 2009, 2010; Vanhatalo et al., 2010a) is now considered a practical method of increasing circulating plasma [NO$_2^-$]. However, the expected increase in plasma [NO$_2^-$] following an oral NO$_3^-$ dose of this nature is attenuated via the use of antibacterial mouthwash (Govoni et al., 2008), highlighting the importance of the bacterial NO$_3^-$ reductases in the reduction of NO$_3^-$ to NO$_2^-$.
Finally, NO\(_2^-\) is reduced into bioactive NO. This reduction is actuated by a number of catalysts including deoxyhaemoglobin (Cosby \textit{et al}., 2003), deoxymyoglobin (Shiva \textit{et al}., 2007), xanthine oxidase (Zhang \textit{et al}., 1998), aldehyde oxidase (Li \textit{et al}., 2008), eNOS (Vanin \textit{et al}., 2007), and the mitochondrial electron transfer complexes (Kozlov \textit{et al}., 1999). This reduction reaction is enhanced in acidic (Modin \textit{et al}., 2001) and hypoxic (Castello \textit{et al}., 2006) environments, similar to those evident in skeletal muscle during exercise (Bailey \textit{et al}., 2010; Vanhatalo \textit{et al}., 2011). The existence of this NO\(_3^-\) - NO\(_2^-\) - NO pathway is important in the promotion of NO synthesis in conditions that may limit NO production via NOS, such as hypoxia and oxidative stress. Therefore, it is suggested that this pathway would be especially important in the generation of NO during exercise. The compensatory role of the NO\(_3^-\) - NO\(_2^-\) - NO pathway is supported by the findings that dietary NO\(_2^-\) (Bryan \textit{et al}., 2008) and NO\(_3^-\) (Carlström \textit{et al}., 2010) supplementation restores tissue and plasma [NO\(_2^-\)] and [NO\(_3^-\)] in eNOS knockout mice. In summary, the complementary nature of the NOS and NO\(_3^-\) - NO\(_2^-\) - NO pathways affirm that the synthesis of NO will occur during a broad range of cellular O\(_2\) tensions and redox states.
Chapter 2: Literature Review

Fig 2.2: A schematic diagram outlining the pathways of NO generation and the roles it can have within the human body. (From Bailey et al., 2012).

**Beetroot and typical NO\textsubscript{3} intake**

Red beetroot (*Beta vulgaris rubra*) is a member of the *chenopodiceae* family alongside Swiss chard and spinach. These plants, along with kale, lettuce, rocket and other leafy greens are known to contain high levels of NO\textsubscript{3}-. NO\textsubscript{3} is naturally ingested as part of a healthy diet, with 60-80% of daily NO\textsubscript{3} intake in a western diet being derived from vegetables (Ysart et al., 1999). The remaining contribution comes from processed meats, where it is added as a preservative, and in the water supply. The acceptable daily intake for NO\textsubscript{3} is 3.7mg/kg bw/day (EFSA, 2008), which equates to approximately 300 mg per day for an individual weighing between 80-85 kg. With the molecular mass 62 g/mol, the acceptable daily intake (ADI) of nitrate for an 80-85 kg individual is about ~5 mmol. However, estimates of average dietary NO\textsubscript{3} intake of adults in the US and Europe is 1-2 mmol/d. Vegetarians are likely to consume more NO\textsubscript{3} and it has recently been highlighted that individuals who comply with the Dietary Approaches to Stop Hypertension (DASH) diet (Appel et al., 1997) will consume ~ 20 mmol/d, nearly five times the ADI.

In addition to high NO\textsubscript{3} concentrations, beetroot contains potassium, magnesium and iron as well as vitamins A, B6 and C, and folic acid. Furthermore beetroot contains polyphenols, including phenolic acids, flavonoids, betaine and a number of antioxidants including betacyanin, with some of these compounds being potentially metabolically active. For example, the amino acid betaine has been used in the treatment of cardiovascular disease (Borsook et al., 1951; Van Zandt et al., 1951), and betaine supplementation has been reported.
to elicit improvements in muscular endurance, strength, and power (Hoffman *et al.*, 2009; Maresh *et al.*, 2008). In addition, some of the polyphenols found in beetroot juice, including quercetin and resveratrol, have been linked with mitochondrial biogenesis and an associated increase in aerobic capacity (Davis *et al.*, 2009; Lagouge *et al.*, 2006; Cureton *et al.*, 2009, Ganio *et al.*, 2010). The high antioxidant content of beetroot may also provide protection against exercise-induced oxidative stress (Kanner *et al.*, 2001). Whilst beetroot juice supplementation has the potential to affect exercise efficiency and performance via numerous pathways, research has established that the cardiovascular and physiological changes observed following beetroot juice supplementation can be ascribed exclusively to its high NO$_3^-$ content, by using a NO$_3^-$ depleted beetroot juice placebo (Lansley *et al.*, 2011b).

*Plasma nitrate and nitrite concentrations*

The reported levels of NO$_3^-$ and NO$_2^-$ in human plasma will invariably differ between individuals based on age, health and fitness status and nutritional intake. Reported values may also differ between measurement techniques. However, the typical plasma NO$_3^-$ concentration, at rest, in a human subject would be expected to be around 30 µmol (Jungersten *et al.*, 1996) with NO$_2^-$ concentration being around 300 nmol (Kleinbongard *et al.*, 2003). Interestingly, baseline plasma [NO$_2^-$] and [NO$_3^-$] and/or the change in the concentrations of these metabolites during exercise is thought to be associated with exercise performance (Poveda *et al.*, 1997; Dreissigacker *et al.*, 2010; Totzeck *et al.*, 2012). Previous research has consistently reported that both acute and chronic dietary nitrate supplementation results in elevated circulating plasma [NO$_2^-$] (Bailey *et al.*, 2009; Vanhatalo *et al.*, 2010a) and plasma [NO$_3^-$] (Larsen *et al.*, 2010; Kapil *et al.*, 2010; Wylie *et al.*, 2013a). It was recently reported that during high-intensity, intermittent running exercise, plasma [NO$_2^-$] was significantly ‘depleted’ following exhaustive exercise and showed a tendency to ‘replenish’ following 15-min of passive recovery (Wylie *et al.*, 2013b). Conversely, plasma [NO$_3^-$] increased during exercise and appeared to revert back to resting values during recovery. Previous research has reported increases (Allen *et al.*, 2010; Rassaf *et al.*, 2007) but more commonly, decreases (Bescós *et al.*, 2011; Dreissigacker *et al.*, 2010; Larsen *et al.*, 2010; Gladwin *et al.*, 2000) in [NO$_2^-$] as a result of exercise. It is currently not known to what extent the ‘depletion’ of plasma [NO$_2^-$] and [NO$_3^-$] is influenced by environmental conditions (hypoxia) and/or exercise intensity.
Chapter 2: Literature Review

Toxicity
Concerns related to adverse effects of inorganic NO$_2^-$ and NO$_3^-$ have previously been proposed in the literature including the development of methaemoglobinemia (Comly et al., 1945), increased nitration of proteins (Beckman et al., 2002) and potential carcinogenic effects (Newberne et al., 1976). The NO$_3^-$ anion itself is considered relatively inert, with any toxicity being related to its bioconversion to NO$_2^-$, which is thought to be considerably more reactive.

Methaemoglobinemia
Haemoglobin contains four heme groups with iron in the reduced form (Fe$^{2+}$). Methaemoglobin is produced when haemoglobin undergoes oxidation and an electron is removed from one of the iron atoms of the heme groups, converting the ferrous (Fe$^{2+}$) iron to the ferric (Fe$^{3+}$) state (Stryer, 1988). This renders the haemoglobin molecule unable to bind to oxygen and results in a left shift of the oxygen-haemoglobin dissociation curve (Goldfrank et al., 1978). This can result in methaemoglobinemia which can cause cellular hypoxia. Concern about NO$_3^-$ and methaemoglobinemia stemmed from early research by Comly et al. (1945) who reported cases of infant methaemoglobineamia from well-water with high concentrations of NO$_3^-$. This remains the origin of the regulation of NO$_3^-$ content of drinking water in the US and Europe although Avery et al. (1999) argues that NO$_3^-$, without bacterial contamination is unlikely to cause methaemoglobinemia.

Nitration of proteins
A potential adverse effect of prolonged dietary NO$_3^-$ supplementation might be the generation of peroxynitrite and other reactive nitrogen species capable of nitration reactions which can alter protein structure and function (Beckman et al., 2002). 3-Nitrotyrosine is commonly used as a marker of nitration reactions and it was demonstrated that no differences in nitrotyrosine staining was evident between 3-days of sodium NO$_3^-$ supplementation and placebo groups (Larsen et al., 2011). The ingestion of NO$_3^-$ accompanied with antioxidants, polyphenols and vitamins found in beetroot juice may also help to offset any possibility of detrimental nitration reactions occurring.

Carcinogenic properties
The theoretical transformation of NO$_3^-$ to N-nitrosamines by dinitrogen trioxide with secondary amines was proposed by Tannenbaum et al. (1976). Subsequently it was shown that N-nitrosamines could cause hepatic tumors in laboratory animals. One study directly linked
dietary NO$_2^-$ with lymphoma in rats (Newberne et al., 1976), whilst other studies also suggested links between NO$_3^-$ intake and cancer (Magee et al., 1956). It is important to emphasize that the Newberne study utilized sodium NO$_2^-$, as opposed to NO$_3^-$, and the relative dose administered was in excess of anything that humans have been exposed to in supplementation studies. Therefore, the Joint FAO/WHO Expert Committee on Food Additives concluded that the reviewed epidemiological studies showed no consistently increased risk for cancer with increasing consumption of NO$_3^-$ and that the data do not provide evidence that NO$_3^-$ is carcinogenic to humans (Speijers et al., 2003).

**Summary**

Effective NO production is considered essential in order to maintain normal physiological functioning. In addition to the endogenous NOS production of NO, another NO generating pathway has been identified (Benjamin et al., 1994). This pathway produces NO via the reduction of inorganic NO$_3^-$ to NO$_2^-$ and further to NO, offering a complementary method of promoting NO production when NO synthesis via the NOS pathway is impaired (Carlström et al., 2010). Increasing dietary NO$_3^-$ intake can help to promote NO production via this NO$_3^-$ - NO$_2^-$ - NO pathway and can be achieved by consuming vegetables rich in NO$_3^-$, including leafy green vegetables and beetroot. If consumed in extreme doses, NO$_3^-$ can potentially have detrimental side effects, although this risk is reduced if ingested from vegetable sources. Interventions designed to increase NO$_3^-$ ingestion will help to maintain normal physiological function and may help to offset metabolic and cardiovascular disease.

**Dietary NO$_3^-$ supplementation**

Typically, increasing dietary NO$_3^-$ intake will increase circulating NO$_2^-$ and elevate the bioavailability of NO. This elevation in NO can have a number of physiological effects within the human body. These effects and the proposed mechanisms behind them are outlined in the following section.

**Blood pressure**

The beneficial effects of a vegetable-rich diet upon cardiovascular health (Gilchrist et al., 2010) and longevity (Visioli et al., 2005) have been well described. These positive effects have been attributed, in part, to inorganic NO$_3^-$ and its reduction to NO. There is now substantial
evidence that dietary NO$_3^-$ supplementation, either in the form of NaNO$_3$ or NO$_3^-$-rich beetroot juice, can significantly reduce resting blood pressure in young healthy adults (Bailey et al., 2010; Larsen et al., 2006; Vanhatalo et al., 2010a; Webb et al., 2008). Typically a reduction in systolic blood pressure in the region of ~ 5 mmHg is evident following supplementation. Increased NO bioavailability stimulates smooth muscle relaxation via the synthesis of cyclic guanosine monophosphate (cGMP) (Murad, 1986). It is this NO-mediated smooth muscle relaxation that is considered to be responsible for reductions in BP following NO$_3^-$ supplementation (Larsen et al., 2006, Webb et al., 2008). Increased dietary NO$_3^-$ intake may therefore provide a practical therapeutic and/or prophylactic intervention for reducing the risk of hypertension.

**Oxygen uptake**

*Moderate-intensity exercise*

In 2007, Larsen and colleagues from the Karolinska Institutet, Sweden, reported that the oxygen demand of submaximal cycle exercise was reduced following 3 days of dietary supplementation with NaNO$_3$ (Larsen et al., 2007). This finding was surprising considering that the oxygen cost of moderate-intensity exercise is considered to be independent of factors such as age, health status and physical fitness (Jones & Poole, 2005). Furthermore, this parameter is known to be unaffected by prior exercise (Burnley et al., 2000), erythropoietin administration (Wilkerson et al., 2005), hyperoxia (Wilkerson et al., 2006), exercise training (Bailey et al., 2009a) and intravenous antioxidant infusion (Bailey et al., 2011). Therefore, the finding that a short-term dietary intervention improved the efficiency of exercise had considerable impact and novelty in exercise physiology research.

In 2009, Bailey and colleagues (Bailey et al., 2009) also reported a reduction in the oxygen cost of moderate-intensity cycle exercise of ~ 5%. However, this study utilized a natural NO$_3^-$-rich beetroot juice as the dietary supplement. It has been reported that similar reductions in moderate-intensity exercise are elicited following both acute and chronic supplementation. Ingestion of 5.2 mmol of NO$_3^-$, 2.5 h prior to exercise elicited a reduction in O$_2$ cost of exercise, with similar reductions seen following 5 and 15 days of continued supplementation (Vanhatalo et al., 2010). This study showed that longer term NO$_3^-$ supplementation did not elicit any greater improvements in exercise efficiency, but also, that tolerance to the intervention did not develop. In addition to consistent observations in cycle exercise (Larsen et al., 2007, 2010, 2011; Vanhatalo et al. 2010; Bailey et al., 2009), the NO$_3^-$ mediated reduction
Chapter 2: Literature Review

in sub-maximal $\dot{V}O_2$ has also been reported in walking, running (Lansley et al., 2011), flat water kayaking (Muggeridge et al., 2012) and two-legged knee-extension exercise (Bailey et al., 2010).

The mechanistic bases underpinning these effects of NO$_3^-$ supplementation are yet to be fully understood although both reduced ATP cost of muscle power output (Bailey et al., 2010) and increased mitochondrial efficiency (P/O ratio) (Larsen et al., 2011) have been reported. Using $^{31}$P-MRS, Bailey et al., (2010) investigated the first of these mechanisms. Their study revealed that estimated ATP turnover rates from PCr hydrolysis and oxidative phosphorylation were reduced as a result of NO$_3^-$ supplementation and subsequently resulted in a significant reduction in estimated total ATP turnover rate during low- and high-intensity knee extensor exercise. In addition to this, the accumulation of ADP and P$_i$ and the magnitude of PCr depletion were blunted following NO$_3^-$ supplementation, indicating that NO$_3^-$ supplementation improves the coupling of ATP hydrolysis and muscle force production. According to existing respiratory control models (Bose et al., 2003; Brown., 1992), the observed changes in [ADP], [P$_i$] and [PCr] would reduce the stimuli for increasing oxidative phosphorylation and could explain the lower $\dot{V}O_2$ observed following NO$_3^-$ supplementation.

The reduction in $\dot{V}O_2$ evident during low-intensity exercise following NO$_3^-$ supplementation could be explained by the reduced ATP cost of muscle force production outlined by Bailey et al., (2010). However, an increase in mitochondrial P/O ratio could also play a crucial role in the reduction of $\dot{V}O_2$ and this was investigated by Larsen et al. (2011). In this study the investigators isolated mitochondria harvested from vastus lateralis muscle of humans supplemented with NaNO$_3^-$ and added them to a reaction medium containing pyruvate and maltate in order to monitor mitochondrial respiration. A submaximal concentration of ADP was infused at a rate selected to mimic the metabolic rate in vivo (Kuznetsov et al., 1996) with results indicating that the mitochondrial P/O ratio (amount of ADP infused divided by O$_2$ consumed) was increased as a result of NO$_3^-$ supplementation. In addition to this, the respiratory control ratio (ratio between state 3 and state 4 respiration) and the maximal rate of ATP production through oxidative phosphorylation was increased following NO$_3^-$ supplementation. Furthermore state 2 respiration (indicative of proton leakage through the inner mitochondrial membrane) and uncoupled, state 4 respiration were reduced as a result of supplementation. These data suggest that NO$_3^-$ supplementation can reduce proton leakage and uncoupled respiration, subsequently improving mitochondrial P/O ratio. Interestingly, this
improved P/O ratio was significantly correlated to the reduction in whole body \( \dot{V}O_2 \) during exercise (Larsen et al., 2011).

It is currently not known to what extent this improved mitochondrial efficiency might influence skeletal muscle energy metabolism at rest. If the resting metabolic rate (RMR) is significantly reduced following NO\(_3^-\) supplementation, this could have implications for daily energy expenditure and weight management. The influence of NO\(_3^-\) supplementation upon RMR is currently unknown.

**Severe-intensity exercise**

The \( \dot{V}O_2 \) slow component is associated with the depletion of muscle [PCr], increased glycogen utilisation and the accumulation of fatigue related metabolites. Therefore, a reduction in the amplitude of the slow component is likely to have beneficial effects upon exercise tolerance and performance. NO\(_3^-\) supplementation has been shown to reduce the amplitude of the \( \dot{V}O_2 \) slow component in response to constant-work-rate, severe-intensity cycle and two-legged knee-extensor exercise (Bailey et al., 2009, 2010), while reductions in end-exercise \( \dot{V}O_2 \) during running have also been reported (Lansley et al., 2011).

The purpose of these studies was to investigate the effect of NO\(_3^-\) on \( \dot{V}O_2 \) kinetics and to assess whether alterations in this response could influence exercise tolerance. In order to compare the interaction between \( \dot{V}O_2 \) kinetics and exercise tolerance, it was imperative to assess these two parameters in the same test. The conventional approach to assess \( \dot{V}O_2 \) kinetics requires the subject to complete a ‘step’ exercise, where the work rate is abruptly increased from a low baseline to a higher target work rate. These protocols enhance the validity of the investigation of \( \dot{V}O_2 \) kinetics as a determinant of exercise tolerance. In addition to the alterations in \( \dot{V}O_2 \), improved exercise tolerance during severe-intensity constant-work-rate cycling (16%; Bailey et al., 2009), running (~15%; Lansley et al., 2011) and two-legged knee-extension (25%; Bailey et al., 2010) exercise has been reported following NO\(_3^-\) supplementation. Furthermore, improvements to incremental exercise tolerance have been reported during single-legged knee extension (5%; Lansley et al., 2011) with a trend for improvement (7%) reported during combined incremental arm and leg exercise (Larsen et al., 2010). Improved incremental exercise tolerance has also been reported during cycle exercise in hypoxia (5%; Masschelein et al., 2012). Tolerance to incremental exercise was also improved in patients with peripheral arterial disease as a result of NO\(_3^-\) supplementation (Kenjale et al., 2011). However,
incremental exercise tolerance is not always improved following NO3 supplementation (Bescos et al., 2011). During incremental exercise protocols, NO3 supplementation has been reported to both increase (Vanhatalo et al., 2010) and decrease (Larsen et al., 2010; Bescos et al., 2011) the VO2max. Explanations of the increase in VO2max include NO-mediated changes in local perfusion in skeletal muscle (Thomas et al., 2001), possible effects on cardiac output (Jones et al., 2004) and increased mitochondrial mass (Nisoli et al., 2004). Proposed mechanisms behind a decreased VO2max include reductions in the ATP cost of muscle force production (Bailey et al., 2010) and improved mitochondrial efficiency Larsen et al., 2011), although these alterations have only been identified during low-intensity exercise.

Power-duration relationship

Although the increases in exercise tolerance during single constant power output exercise bouts indicates a physiological benefit of NO3 supplementation (Bailey et al., 2009; Lansley et al., 2011), it has been proposed that the magnitude of the changes elicited after an intervention can be difficult to interpret because of the shape of the power-duration relationship (Whipp & Ward, 2009). While accepting that improved exercise tolerance to any particular constant work rate is reflective of a physiological benefit of an intervention, it does not act as a sufficient quantitative measure of the actual improvement in function as it provides data from just a single point of that relationship. Ideally, characterisation of the pre- and post-intervention power-duration relationship is necessary (Whipp & Ward, 2009).

It is a familiar occurrence that exercising at a relatively fast yet comfortable pace can be maintained for an appreciable amount of time without feeling too tired. However, if the speed is even slightly increased, this can result in significant increases in perceived effort and can substantially reduce the tolerable duration of exercise. These experiences have genuine mathematical and physiological bases, which are defined by the critical power (CP) concept. The CP and W’ characterize the hyperbolic power-duration relationship that is evident during high-intensity exercise (Fukuba et al., 2003; Monod & Scherrer 1965; Poole et al., 1988). The CP is defined as the power-asymptote of the relationship and demarcates the boundary between ‘heavy’ intensity (work rates at which physiological steady-state is attained) and ‘severe’ intensity exercise (work rates at which physiological steady-state is not attained) (Jones et al., 2008; Poole et al., 1988). Thus, the CP theoretically represents the highest work rate that can be maintained via predominantly aerobic metabolism, where pulmonary oxygen uptake (VO2), blood lactate and concentrations of intramuscular metabolites such as phosphocreatine ([PCr]),
[H+] and inorganic phosphate ([P_i]) can be stabilized (Jones et al., 2008; Poole et al., 1988). The W' represents the curvature constant of the relationship and can be considered as the finite work capacity available above the CP before the limit of tolerance is reached (Fukuba et al., 2003; Vanhatalo et al., 2007). This limited energy reserve is thought to be largely “anaerobic”, utilising energy from anaerobic glycolysis and intramuscular high-energy phosphates, with an additional yet modest contribution from myoglobin and haemoglobin bound oxygen stores (Miura et al., 1999; Monod & Scherrer 1965; Moritani et al., 1981). The two-parameter CP model essentially defines a bioenergetic supply-and-demand system comprised of two components. In this sense, the concept lends itself to mathematical modelling and can be represented in a number of forms.

Estimates of CP and W’ from prediction trials can be calculated using three different models, using the following equations:

The hyperbolic power-duration relationship:

\[ T_{lim} = \frac{W'}{(P-CP)} \]  
\[ \text{(Eqn. 1)} \]

where P is a given severe-intensity power output (Hill, 1993; Jones et al., 2010; Vanhatalo et al., 2011). The linear transformations of this relationship are the power-1/time equation:

\[ P = \frac{W' \cdot 1/T_{lim} + CP}{1/T_{lim} + CP} \]  
\[ \text{(Eqn. 2)} \]

and the work-time equation, where P is replaced with work done (W) per unit time:

\[ W = CP \cdot T_{lim} + W' \]  
\[ \text{(Eqn. 3)} \]

It is evident that when the CP and W’ are known, performance time within the severe domain (indicated by T_{lim}) can be accurately predicted by rearranging Eqn. 2 (Hill, 1993; Jones et al., 2010; Vanhatalo et al., 2011). As such, estimates of CP and W’ can be used to predict the time taken to complete total work done (W) targets using the following equation:

\[ T_{lim} = \frac{(W-W')}{CP} \]  
\[ \text{(Eqn. 4)} \]

The CP and W’ are important determinants of sport and exercise performance (Jones et al., 2010; Vanhatalo et al., 2011). It is important to stress that exercise performance within the severe domain is a function of both the CP and the W’, which act in concert to determine the shortest possible time required to complete a given target total work done. The early work of A.V. Hill in the 1920’s attempted to understand the physiological determinants of physical
performance, with the formulation of velocity-time curves based on athletic world records (Hill, 1925). Fundamental to his 1922 Nobel prize accolade was his demonstration that both aerobic and anaerobic energy sources were recruited and important in supporting high-intensity muscle contraction. Not surprisingly, our current understanding of the power-duration relationship revolves around the coordinated function of these two energy sources.

Figure 2.3: Illustration of the hyperbolic power-duration relationship. When time-to exhaustion, during exercise at four different intensities above the critical power (severe-intensity), is plotted against the power output, time to exhaustion increases hyperbolically. The filled circles represent the time to exhaustion at a given power output. The solid line hyperbola characterises the power-duration relationship while the dashed line represents the critical power. The \( W' \) is represented by the shaded rectangles, which depicts the rate of \( W' \) utilisation differing dependent upon the magnitude of the power output. The magnitude of the \( W' \) is identical for all four power outputs and exhaustion occurs when the \( W' \) has been fully utilised.

Evidently, the synergistic relationship between these two parameters dictates exercise tolerance during severe-intensity exercise (Jones et al., 2008; Vanhatalo et al., 2011). In an attempt to modulate these two indices, various interventions have been employed. Endurance training (Jenkins & Quigley, 1992), high-intensity interval training (Vanhatalo et al., 2008, Gaesser et al., 1988, Poole et al., 1990) and hyperoxia (Vanhatalo et al., 2010) have been shown to elicit improvements in CP. Typically, interventions that enhance CP result in a trend toward a decrease in \( W' \) (Vanhatalo et al., 2008; Jenkins & Quigley, 1992). However, the \( W' \) can be improved by utilising the correct ‘priming’ exercise and pacing strategy via alterations to \( \dot{VO}_2 \)
kinetics and VO$_{2\text{max}}$ (Bailey et al., 2009; Jones et al., 2003; Jones et al., 2008). A recent study investigated the effect of sodium bicarbonate supplementation upon CP and W' derived from a 3-min all out test, but showed no changes to either parameter (Vanhatalo et al., 2010). The use of creatine monohydrate supplementation has produced mixed results with regard to improvements in W'. Creatine supplementation is known to increase intramuscular PCR concentrations and has been shown to result in a significant increase in the conventionally estimated W' parameter (Miura et al., 1999; Smith et al., 1998), although no change was reported in a subsequent study (Eckerson et al., 2005). Collectively these findings suggest that both CP and W' can be altered via a number of training and environmental interventions, although these parameters appear to be less sensitive to nutritional supplementation.

As exercise performance in the severe domain is a function of CP and W', the reported improvements in exercise tolerance and performance following NO$_3^-$ supplementation may be as a result of a beneficial shift in the power-duration relationship. A rightward shift in the power-duration relationship as a result of NO$_3^-$ supplementation would enable an individual to exercise at a given power output for a longer duration or to exercise at a higher power-output for the same duration. The increased power output to VO$_2$ ratio evidenced following NO$_3^-$ supplementation (Bailey et al., 2009; Cermak et al., 2012; Lansley et al., 2011; Larsen et al., 2007) suggests that NO$_3^-$ might increase CP, whilst the recently reported effects of NO$_3^-$ supplementation upon blood flow and contractile function of type II fibres (Ferguson et al., 2013; Hernandez et al., 2012), indicate potential for improvements in W'.

As discussed earlier, it is important to characterise the power-duration relationship before and after an intervention in order to gain a true appreciation of it ergogenic effects. It is therefore important to consider 3 or more constant power output exercise bouts in BR and PL conditions in order to accurately calculate the power-duration relationship, which will allow us to determine, with more confidence, the beneficial effects of NO$_3^-$ supplementation upon exercise tolerance and performance.

**Exercise performance**

The potential ergogenic effects of NO$_3^-$ supplementation are of particular interest to athletes and coaches. While ‘step’ and incremental exercise protocols are useful for characterizing parameters of the VO$_2$ response, they do limit the generalizability of findings to ‘true’ endurance sports performance where success is not determined by the ability to sustain a given power output for the longest duration. The magnitude of improvements reported in constant-
work-rate exercise (15%-25%) is considerably larger than improvement in ‘time-trial’ type exercise performance. It is estimated that a 20% improvement in time to exhaustion would be expected to correspond to an improvement in performance of 1-2% during a time-trial protocol (Hopkins et al., 1999). Therefore, it was important to assess whether NO₃⁻ supplementation would improve performance in a time-trial performance test, which closely replicates sports performance with the aim of covering a set distance in the fastest possible time. It was subsequently shown that NO₃⁻ supplementation improves time-trial performance during cycling over 16, (2.9%; Lansley et al., 2011), 10 (1.2%; Cermak et al., 2012) and 4 km (2.8%; Lansley et al., 2011), rowing ergometer repetitions (1%; Bond et al., 2012) and 5km running (3.2%; P=0.06; Murphy et al., 2012). However, some studies have reported no beneficial effect of NO₃⁻ on time-trial exercise during both cycle (Bescos et al., 2012; Cermak et al., 2012) and running (Peacock et al., 2012) trials. It should be noted that recent studies indicate that nitrate supplementation may be less effective as an ergogenic aid in highly-trained endurance athletes, at least when nitrate is ingested acutely and/or longer duration, lower-intensity endurance performance is assessed (Bescos et al., 2012, Cermak et al., 2012, Wilkerson et al., 2012). Compared to less well-trained subjects, endurance athletes have higher baseline plasma [NO₂⁻], greater training-related NOS activity, a higher proportion of type I fibres, and greater mitochondrial and capillary density, all of which may reduce the potential benefits of nitrate supplementation (Wilkerson et al., 2012).

Additional proposed mechanisms for NO₃⁻-mediated alterations in the VO₂ response and improved exercise performance include that of changes to intracellular Ca²⁺ handling. In 2012, Hernandez and colleagues reported that fast-twitch skeletal muscle harvested from mice supplemented with NO₃⁻ in drinking water displayed increased tetanic [Ca²⁺], which was coupled with an increased contractile force at low stimulation frequencies. These changes were accompanied by altered protein expression, with both calsequestrin 1 and dihydropyridine receptor (key proteins involved in sarcoplasmic reticulum expression Ca²⁺ handling) being increased. These findings show that fast twitch muscles of NO₃⁻ supplemented mice can be activated at a lower frequency to achieve the same force output, which would subsequently reduce the effort for a given task. This also suggests that for a given force output, a reduced number of motor units would need to be recruited and is consistent with existing mechanistic proposals (Bailey et al., 2010). Furthermore, it has been shown that in the healthy rat model, NO₃⁻ supplementation with beetroot juice for 5 days can increase total hind limb muscle blood flow and vascular conductance (Ferguson et al., 2013). Interestingly, the increases in blood
flow and vascular conductance were targeted in the muscles and muscle parts comprised of principally type II fibers. This apparent preferential muscle O\textsubscript{2} delivery to Type II fibers offers important information regarding the NO\textsuperscript{3−} -mediated vascular and metabolic control that has previously been reported in humans during exercise (Larsen \textit{et al.}, 2007; Bailey \textit{et al.}, 2009, Bailey \textit{et al.}, 2010). In order to add to the existing proposed mechanisms behind the beneficial effects of NO\textsuperscript{3−} supplementation, direct assessment of human skeletal muscle metabolism during exercise must be investigated.

\textbf{Muscle metabolism}

Following the onset of exercise, an immediate increase in ATP turnover and an exponential rise in oxygen (O\textsubscript{2}) consumption are evident within the contracting muscle cells. This disparity in the rates of muscle ATP utilization and ATP supply via oxidative phosphorylation obligates a compensatory energy contribution from substrate-level phosphorylation (Poole \textit{et al.}, 2008). As previously discussed, pulmonary VO\textsubscript{2}, which provides a close representation of muscle VO\textsubscript{2} (Grassi \textit{et al.}, 1996; Krstrup \textit{et al.}, 2009), attains a ‘steady-state’ within 120-180 s following the onset of moderate-intensity exercise (below the gas exchange threshold; GET) (Whipp \textit{et al.}, 1982). However, during heavy (above GET but below critical power) and severe-intensity (above critical power) exercise, an additional phase of the VO\textsubscript{2} response, the VO\textsubscript{2} ‘slow component’, is evident which delays and/or prevents the attainment of a steady state. The development of the VO\textsubscript{2} slow component is closely related to accelerated muscle PCr (Rossiter \textit{et al.}, 2002) and glycogen utilization (Krupstrup \textit{et al.}, 2004), and to the accumulation of fatigue associated metabolites (H\textsuperscript{+}, P\textsubscript{i}, ADP). Interventions that alter the VO\textsubscript{2} response during exercise and modulate the rate at which the body’s energy stores are depleted and fatiguing metabolites are accumulated are therefore likely to have important implications for exercise tolerance (Jones \textit{et al.}, 2009).

The effects of NO\textsuperscript{3−} supplementation upon the VO\textsubscript{2} response to exercise are well documented. While non-invasive measures (\textsuperscript{31}P-MRS) of muscle metabolism during exercise, following NO\textsuperscript{3−} supplementation, have been recorded (Bailey \textit{et al.}, 2010), the effects of NO\textsuperscript{3−} supplementation upon muscle metabolic responses, measured via skeletal muscle biopsy technique, have yet to be established in humans. This would allow a direct measure of energy stores and fatigue associated metabolites before, during and after exercise in human skeletal muscle. This would provide insight into how NO\textsuperscript{3−} supplementation can affect the muscle metabolic response to exercise in humans.
It is also currently unknown whether NO\textsuperscript{3−} supplementation can increase skeletal muscle [NO\textsubscript{3−}] in human subjects. Stable metabolites of NO (NOx) have previously been measured at rest in the muscle interstitium in young human adults with values of ~ 120 μM being reported (Nyberg et al., 2012), while skeletal muscle [NO\textsubscript{3−}] of sedentary, resting rats have been reported as ~46 nM g\textsuperscript{−1} (Perez et al., 2002). Assessing the effects of NO\textsubscript{3−} supplementation upon skeletal muscle [NO\textsubscript{3−}] in humans is important. This may elucidate whether skeletal muscle [NO\textsubscript{3−}] is influenced by NO\textsubscript{3−} supplementation and whether changes in this parameter may influence muscle metabolic and pulmonary oxygen uptake responses to exercise and improve exercise tolerance.

**Hypoxia**

As previously discussed, the reduction of NO\textsubscript{2−} to bioactive NO in the NO\textsubscript{3−} - NO\textsubscript{2−} - NO pathway is enhanced in acidic (Modin et al., 2001) and hypoxic (Castello et al., 2006) environments, similar to those evident in skeletal muscle during exercise (Bailey et al., 2010; Vanhatalo et al., 2011). The existence of the NO\textsubscript{3−} - NO\textsubscript{2−} - NO pathway is important in the promotion of NO synthesis in conditions that may limit NO production via NOS, such as hypoxia. Therefore, it is suggested that this pathway would be especially important in the generation of NO during exercise in hypoxia. Hypoxia is defined as a decrease in the oxygen supply to a level insufficient to maintain cellular function (Gilany et al., 2010) or an imbalance of O\textsubscript{2} delivery versus O\textsubscript{2} demand, and is one of the most frequently encountered conditions or stresses in disease (Brahimi-Hom et al., 2007). Hypoxia leads to a global down-regulation of protein synthesis (Koritzinsky et al., 2006) and specifically regulates the expression of many genes with various important roles in mammalian cells (Shih et al., 1998). The fundamental importance of O\textsubscript{2} for all aerobic organisms including mammals has led to the evolution of a complex cellular response to hypoxia. At the heart of this response is the hypoxia inducible factor (HIF) (Wang et al., 1995), which is known to be modified by NO (Hagen et al., 2003).

A reduced fraction of O\textsubscript{2} in inspired air results in a reduction in arterial oxygen concentration and a decrease in intracellular partial pressure of O\textsubscript{2} (PO\textsubscript{2}) (Richardson et al., 1995). In order to restore sufficient O\textsubscript{2} supply, local blood flow is increased via hypoxia induced vasodilation (Heinonen et al., 2010). This compensatory increase in blood flow is thought to be mediated in part by NO (Casey et al., 2010) as well as adenosine (Berne et al., 1963), ATP-sensitive potassium channels (Daut et al., 1990) and prostaglandins (Crecelius et al., 2011). Compared to normoxia, submaximal constant-work-rate exercise in hypoxia is associated with the same VVo\textsubscript{2} but greater muscle metabolic perturbation (Wilkins et al., 2006). A reduction in
intracellular $\text{PO}_2$ commands an increased concentration of mitochondrial respiration regulators, in order to maintain the required rate of oxidative ATP turnover. Specifically, concentrations of ADP, $\text{P}_i$ and NADH are increased via elevated rates of PCr hydrolysis and glycolysis (Hogan et al., 1999). Subsequently, at work rates below 50% of maximum, hypoxia accelerates the depletion of PCr and glycogen and speeds the accumulation of fatigue-related metabolites (ADP, $\text{P}_i$, $\text{H}^+$). This hypoxia induced skeletal muscle metabolic perturbation (Linnarsson et al., 1974) contributes to impaired exercise tolerance (Allen et al., 2008) and reduced functional capacity in many disease conditions (Ellis et al., 2010; Kenjale et al., 2011) and at altitude (Amann & Calbet, 2008).

NO plays a key role in the physiological response and adaptation to hypoxia and is implicated as a major mediator in a number of pathways for hypoxic vasodilatation including regulation of muscle perfusion and matching of energy supply and demand (Casey et al., 2010). $\text{NO}_2^-$ may also promote hypoxic vasodilatation in an NO-independent manner (Dalsgaard et al., 2007). NO is also known to redistribute intracellular oxygen in hypoxia by preventing the stabilization of HIF1α (Hagen et al., 2003) and to modulate oxygen delivery to the tissue (Thomas et al., 2001). This outlines an additional physiological response of NO in enhancing tissue cellular respiration, in addition to vasodilation and may prove important when exercising in hypoxic environments. As mentioned before, production of NO via NOS in hypoxia can be compromised but the reduction of $\text{NO}_2^-$ to NO in the NO$_3^-$ - NO$_2^-$ - NO pathway is enhanced in hypoxic conditions (Castello et al., 2006). Increasing the bioavailability of NO during exercise in hypoxia may facilitate the physiological response to hypoxia and contribute to improving tolerance to such exercise, in young healthy individuals (Vanhatalo et al., 2011). It may also be reasonable to hypothesize that NO$_3^-$ supplementation can have greater beneficial physiological effects in hypoxic compared to normoxia. Furthermore, it is currently unknown to what extent NO$_3^-$ supplementation, FIO$_2$ and/or exercise intensity can affect the metabolism of NO. Characterizing the kinetic profile of [NO$_3^-$] and [NO$_2^-$] during exercise bouts of different intensities, with a different FIO$_2$ and following NO$_3^-$ supplementation will allow important insight to the plasticity of NO metabolism by manipulation of exercise intensity, FIO$_2$ and NO bioavailability.

Hypoxia plays an integral role in the reduced functional capacity evidenced in many clinical conditions (Ellis et al., 2010; Allen et al., 2012). Understanding the potential beneficial effects of NO$_3^-$ supplementation upon NO metabolism in hypoxic environments may have important implications for diseased and/or ageing populations. Specifically, NO$_3^-$ supplementation may
help to improve exercise tolerance in hypoxia, in a young healthy population, which could translate into improved functional capacity and life quality in diseased and/or aged populations.

**Senescence**

Given our ever increasing life expectancy and the subsequent, continued growth in the older population, reduced functional and cognitive capacities are not only likely to have a detrimental effect upon the quality of life of ageing individuals but pose an increasing financial burden on healthcare systems around the world. The prevention or attenuation of these age-related conditions needs to be addressed.

The ageing process is associated with a number of metabolic, cardiovascular (Cheitlin, 2003) and cognitive (Glisky, 2007) alterations even within healthy, older populations. With age, a progressive decline in the oxidative and operative scope of our vital physiological systems ensues. The muscular and cardiovascular systems of an ageing individual encounter functional and structural alterations that may hinder muscle O$_2$ delivery, the matching of that O$_2$ delivery to $\dot{V}O_2$ requirements, and mitochondrial oxidative function (Barstow & Scheuermann, 2005). Existing literature illustrates an age-related diminished ability to increase cardiac output during exercise (Lakatta, 1999) such that muscle blood flow is impaired (Wahren et al., 1974). Capillary density, capillary-to-fibre ratio, mitochondrial volume density and oxidative function have also been shown to diminish with age (Coggan et al., 1993 and Conley et al., 2000). Furthermore, decrements in $\dot{V}O_{2\text{max}}$ apparent in ageing individuals could be attributed to impaired O$_2$ extraction at the muscle (McGuire et al., 2001). Ageing is also associated with microcirculatory changes including a redistribution of blood flow amongst a variety of organs and muscles (Musch et al, 2004) and altered capillary haemodynamics (Russell et al, 2003). Such changes may contribute to the impaired O$_2$ diffusing capacity and decreased O$_2$ extraction documented in older individuals.

Senescent individuals also evidence a profound slowing of their $\dot{V}O_2$ kinetics in response to moderate-intensity exercise (Scheuermann et al., 2002). $\dot{V}O_2$ kinetics are known to be influenced by muscle O$_2$ delivery or arterial O$_2$ content in some circumstances as represented in Fig 3.2. The diagram demonstrates that there is a ‘tipping point’ in the relationship between the speed of $\dot{V}O_2$ kinetics and muscle O$_2$ delivery. To the left of this tipping point, the kinetics is O$_2$ delivery dependent, whereas to the right of the tipping point, the kinetics is determined by O$_2$ utilisation. Therefore, specific human populations and/or experimental conditions may occupy distinct and predictable positions on the continuum (Burnley, 2008). The kinetics of well-trained young individuals performing upright cycle exercise is not O$_2$ delivery dependent.
even at work rates that elicit VO$_{2\text{max}}$ (Poole et al., 2008) and in support of this, administering hyperoxic inspirate does not speed the kinetics (Wilkerson et al., 2006). However, due to age-related changes in muscle blood flow (chronic cardiovascular, respiratory, and/or muscular pathologies), older individuals may reside on the left-hand side of this schematic diagram. Therefore therapeutic interventions aimed at improving muscle O$_2$ delivery may enhance exercise tolerance by speeding VO$_2$ kinetics. In support of this notion, a bout of heavy priming exercise speeded VO$_2$ kinetics at the onset of a subsequent moderate-intensity exercise bout in older but not younger individuals (Scheuermann et al., 2002). Furthermore, the speeding of VO$_2$ kinetics and those of heart rate has been significantly correlated in older individuals (Babcock et al., 1994). This suggests that improved O$_2$ delivery may, in part, be responsible for the speed of VO$_2$ kinetics in older people. However, conflicting research suggests that the slow VO$_2$ kinetics following the onset of moderate-intensity exercise, evident in older populations, are modulated by structural (mitochondrial, microvascular) and/or functional alterations within the exercising musculature (Bell et al., 1999, 2001). Despite the mixed findings, an intervention which could increase muscle O$_2$ delivery may be expected to speed VO$_2$ kinetics in an older population.

![Fig 2.4: A schematic diagram outlining the the dependence of VO$_2$ kinetics on muscle O$_2$ delivery in various populations (From Poole & Jones, 2005).](image)

Another notable change associated with senescence is the progression of vascular endothelial dysfunction which is often evidenced by reduced endothelium-dependant dilatation (Lakatta & Levy, 2003). Endothelial dysfunction is characterised, in part, by excessive superoxide
production (Darley-Usmar et al., 1995) which decreases the bioavailability of NO. The increased superoxide reacts with NO to form peroxynitrite and oxidises BH₄, an essential co-factor in NO production by eNOS (Seals et al., 2011). Alternative mechanisms underpinning this age-dependant reduction in endothelial function and NO bioavailability include a reduced availability of L-arginine (Morris, 2000), reduced eNOS activity and reduced circulating nitrite concentrations (Sindler et al., 2011). Collectively, these changes can contribute to the activities of everyday life requiring ageing individuals to work within the upper end of their exercise capacity, resulting in heightened metabolic stress, although lifelong physical activity appears to offset the reduction in NO bioavailability (Nyberg et al., 2012). An intervention which has the potential to improve muscle O₂ delivery, speed VO₂ kinetics, improve mitochondrial efficiency, that possesses antioxidant properties and which may help to increase the bioavailability of NO, such as dietary NO₃⁻ supplementation, may provide therapeutic effects for an older population.

Increased NO bioavailability may also provide a means of enhancing brain blood flow and improving cognitive function in older age. Aging causes alterations in brain size, vasculature and cognition. In addition to the brain shrinking with age (Svennerholm, 1997), the capacity of the brain to produce ATP via oxidative phosphorylation also decreases (Boveris et al., 2008) and in combination with age-associated chronic ischemia of white matter (Presley et al., 2011), cognitive decline ensues. Furthermore, age-related mitochondrial dysfunction has been associated with the neuronal loss seen in a number of neurodegenerative diseases (Chagnon et al., 1995). A recent study demonstrated that NO₂⁻ infusion led to increases in cerebral blood flow in rats measured using laser Doppler flowmetry (Rifkind et al., 2007). Subsequent research demonstrated that a high-NO₃⁻ diet elevated plasma NO₂⁻ and increased cerebral blood flow in older adult humans in critical brain areas known to be involved in executive functioning, using perfusion MRI techniques (Presley et al., 2011). Furthermore, recent research has identified that NO plays an important role in neurotransmission and the coupling of neural activity to local cerebral blood flow (Piknova et al., 2011). Therefore dietary NO₃⁻ may have the potential to modify cerebrovascular physiology, enhance cognitive function and may offset the influence of aging on cognitive decline and dementia (Holland et al., 2008).

**Summary**
Dietary NO₃⁻ supplementation can have a number of physiological effects within the human body via increasing the bioavailability of NO. These effects include reducing resting blood
pressure (Bailey et al., 2010; Larsen et al., 2006; Vanhatalo et al., 2010a; Webb et al., 2008), modulating the VO₂ response to moderate-intensity (Larsen et al., 2007, 2010, 2011; Bailey et al., 2009; Vanhatalo et al. 2010; Lansley et al., 2011; Muggeridge et al., 2012) and severe-intensity exercise (Bailey et al., 2009, 2010; Lansley et al., 2011), as well as improving exercise tolerance (Bailey et al., 2009, 2010; Lansley et al., 2011; Kenjale et al., 2011) and perhaps athletic performance (Lansley et al., 2011, Cermak et al., 2012; Bond et al., 2012). NO₃⁻ supplementation can also increase cerebral blood flow in older human individuals (Presley et al., 2011). NO-mediated smooth muscle relaxation via the synthesis of cyclic guanosine monophosphate (cGMP) (Murad, 1986) is considered responsible for reductions in BP following NO₃⁻ supplementation. A reduced ATP cost of muscle power output (Bailey et al., 2010), increased mitochondrial efficiency (Larsen et al., 2011), improved intracellular Ca²⁺ handling in increased force production (Hernandez et al., 2012) and/or elevated muscle oxygen delivery during exercise preferentially to type II muscle fibres (Ferguson et al., 2013) are proposed explanations of changes to the VO₂ response and improved exercise capacity.

Despite the publication of many dietary NO₃⁻ supplementation research papers in recent years, a number of outstanding questions remain to be answered regarding the ergogenic and therapeutic capabilities of dietary NO₃⁻ supplementation. Among others, these include investigating the effects of NO₃⁻ supplementation upon the power-duration relationship; NO metabolism in differing environmental conditions; and effectiveness of NO₃⁻ supplementation in an older population. Furthermore, a deeper understanding of the effects that NO₃⁻ can have upon muscle metabolic responses to exercise is required and the mechanistic bases behind the reported effects of supplementation.

Aims

This thesis aims to add to the existing understanding of dietary nitrate supplementation as a potential ergogenic and therapeutic aid. The following research questions will be addressed:

1) Does dietary NO₃⁻ supplementation modulate the power-duration relationship for severe-intensity exercise in young, healthy, recreationally active males?
   - Does dietary NO₃⁻ improve exercise tolerance at intensities spanning the severe domain?
   - Does dietary NO₃⁻ increase critical power and/or W'?
   - Does dietary NO₃⁻ decrease resting metabolic rate?
Chapter 2: Literature Review

2) How does dietary NO₃⁻ supplementation affect NO metabolism and does it have beneficial effects on exercise tolerance in hypoxic conditions?
   - What is the kinetic response of plasma [NO₃⁻] and [NO₂⁻] during moderate- and severe-intensity exercise and is this different between normoxia and hypoxia?
   - Are the negative effects of hypoxia on exercise tolerance off-set as a result of dietary nitrate supplementation?

3) Are the beneficial effects of dietary NO₃⁻ supplementation elicited in young, healthy participants also evident in a healthy, older population?
   - Can NO₃⁻ supplementation reduce resting blood pressure in older individuals?
   - Does dietary NO₃⁻ modulate intramuscular metabolite responses in response to low- and high-intensity exercise?
   - Can dietary NO₃⁻ improve functional capacity in older adults?
   - Does dietary NO₃⁻ alter cerebral blood flow or the concentrations of important cerebral metabolites?
   - Can dietary NO₃⁻ be beneficial to cerebral health and cognitive function in an older population?

4) How does dietary NO₃⁻ supplementation influence skeletal muscle [NO₃⁻], pulmonary VO₂ and muscle metabolic responses to constant-work-rate moderate- and severe-intensity exercise.
   - Are NO₃⁻ levels increased within skeletal muscle tissue as a result of dietary NO₃⁻ supplementation?
   - Is the muscle metabolic response to exercise altered as a result of dietary NO₃⁻ supplementation?
   - Do changes in the muscle metabolic response to exercise elucidate the mechanistic bases of previously reported improvements in exercise efficiency and exercise tolerance?

**Hypotheses**
This thesis will address the following hypotheses:

1) NO₃⁻ supplementation will 1) improve exercise tolerance across a range of severe-intensity exercise bouts by increasing the CP and/or W' and 2) NO₃⁻ supplementation will reduce the resting metabolic rate.
Chapter 2: Literature Review

2) A reduced fraction of inspired O₂ (FIO₂) will accentuate the depletion of NO₂⁻ during exercise compared to normoxia. NO₃⁻ supplementation will improve tolerance to severe-intensity, constant-work-rate cycle exercise in hypoxia, and this improvement will be greater than the effect of NO₃⁻ on exercise tolerance in normoxia.

3) Dietary supplementation with NO₃⁻ -rich beetroot juice will reduce resting blood pressure, speed the VO₂ kinetics and lower the O₂ cost of treadmill walking, and improve functional capacity and cognitive function in healthy older adults.

4) Muscle [NO₃⁻] will be elevated as a result of NO₃⁻ supplementation. The magnitude of muscle PCr degradation and fatiguing metabolite accumulation will be attenuated and exercise tolerance will be improved as a result of NO₃⁻ supplementation.
Chapter 3: General Methods

Ethical approval and informed consent
The four experimental chapters (Chapters 4-7) included in this thesis required 268 exercise tests. All tests were conducted in an air conditioned exercise physiology laboratory or MRI suite at sea level with an ambient temperature of 18-22°C. Each study included in this thesis was approved by the Sport and Health Sciences (SHS, University of Exeter) Ethics Committee prior to the commencement of data collection. Prior to agreeing to take part in these studies, subjects were provided with a Participant Information Sheet which outlined a detailed description of the experimental protocol and exactly what they would be required to do. The potential risks and benefits of their participation were clearly explained in the information sheet and subjects were informed that, while their anonymity would be preserved and their data stored safely, the group data may be published in academic journals or presented at national/international conferences. Participants were told that they were free to withdraw from the study at any point with no disadvantage to themselves. Any additional questions or concerns that the subjects had were answered before subjects provided written, informed consent to participate.

Health and safety
All testing procedures followed the health and safety guidelines of SHS. Due care and attention was paid to ensure that the laboratory was clean, safe and suitable for the exercise testing of human subjects. Work surfaces, trolleys, ergometers and floors were thoroughly cleaned using dilute Virkon disinfectant. All respiratory equipment was disinfected by submerging in Virkon solution for a minimum of 15 min, then rinsed and dried prior to use. Experimenters involved in the collection of blood wore disposable latex gloves during sampling. All sharps and biohazard materials were disposed of immediately after use for later incineration in accordance with institutional risk assessments.

Subjects
Subjects for studies in Chapters 4, 5 and 7 were recruited from the University and local community and were 23 ± 4 years of age. These subjects were non-smokers who were free from disease and were not using dietary supplements at the time of data collection. The subjects were recreationally active, participating in regular structured and/or competitive sport, although were not elite-level athletes. Subjects for the experiment in Chapter 6 were
recruited from the local area and from the Exeter Clinical Research Facility, Peninsula Research Bank. These individuals were screened prior to recruitment to ensure suitability for participation. The subjects were 64 ± 3 years of age and were ostensibly healthy, free from any disease or condition that may limit walking or knee-extension exercise. In all studies, subjects were instructed to attend the laboratory at least 3 h postprandial in a rested, fully-hydrated state, having completed no strenuous exercise within the previous 24 h. Subjects were also instructed to avoid alcohol and caffeine 24 and 6 hours, preceding each exercise test, respectively. It was ensured that each subject underwent testing at the same time of day (± 2 hr). Subjects’ mass, stature and age were recorded prior to the initiation of testing in order to provide descriptive data of the subject group.

Supplementation
Nitrate supplementation was administered in the form of beetroot juice, which was supplied by Beet it, James White Drinks, Ipswich, UK. In each experimental chapter participants were instructed to ingest either nitrate-rich beetroot juice or nitrate-depleted beetroot juice, in a double-blind randomized, crossover study design. The placebo beverage was created by passage of the juice, before pasteurisation, through a column containing Purolite A520E ion exchange resin, which selectively removes NO$_3^-$ ions (Lansley et al. 2011). The placebo was similar to the BR in appearance, taste and smell.

In Chapter 4, the supplements were either nitrate-rich BR (2 x 250 ml/day of organic beetroot juice providing a total of 8.2 mmol nitrate per day) or nitrate-depleted PL (2 x 250 ml/day of organic beetroot juice providing a total of 0.006 mmol nitrate per day). In Chapters 5, 6 and 7, the supplements were either concentrated nitrate-rich BR (2 x 70 ml/day of concentrated organic beetroot juice providing 9.6 mmol nitrate per day) or nitrate-depleted PL (2 x 70 ml/day of concentrated organic beetroot juice providing a total of 0.006 mmol nitrate per day). Participants were instructed to consume the beverages in the morning and afternoon of days 1 and 2 of supplementation, then in the morning and 2.5 h before the exercise on testing days.

A washout period of at least 72 h separated each supplementation period. Subjects were instructed to follow their normal dietary habits throughout the testing period and to replicate their diet between conditions during the supplementation periods. Subjects were told that supplementation may cause beeturia (red urine) and red stools temporarily but that
this side effect was harmless. The supplementation was well tolerated by all participants with no adverse effects reported.

**Blood pressure**

Prior to every exercise test in chapters 4-7, blood pressure of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro 100v2, GE Medical Systems, Tampa, USA). Subjects were seated in a rested state for 10 min before four measurements were taken. The mean of the final three measurements were recorded. The automated sphygmomanometer meets or exceeds American National Standards Institute/Association for the Advancement of Medical Instrumentation standards (Dinamap Pro).

The reliability of systolic blood pressure measurement was determined by repeat assessment performed over five days. A subject was asked to remain seated in a rested position for 10 min before four blood pressure measurements were taken, on 5 separate days. The coefficients of variation for systolic blood pressure were 1.0-1.1% (intra-test variation, using the four measurements on one day) and 0.7% (inter-test variation, using values obtained on the 5 tests performed on separate days) at an overall systolic BP of 121 mmHg. Thus the absolute error associated with the experimenter and equipment used for the measurement of systolic BP within this thesis was less than 1 mmHg.

**Heart rate**

With exception to the exercise tests conducted within the bore of the magnetic resonance scanner, heart rate (HR) was measured during all exercise tests. During Chapters 4, 6 and 7 two second average values for HR were recorded using short-range radio telemetry (model S610, Polar Electro, Oy, Kempele, Finland).

**Measurement of lactate, glucose, potassium and sodium concentrations**

In Chapter 4, small fingertip blood samples were collected to determine whole blood lactate concentration ([lactate]) during the ‘unloaded’ baseline and immediately following exhaustion. Prior to obtaining the sample, the tip of the finger was cleaned thoroughly with alcohol and a disposable safety lancet (Safety-Lanzette, Sarstedt) was used to puncture the skin. The first drops of blood were wiped away and ~20-25µL of free-flowing blood was collected into a heparinized microvette (Microvette CB 300, Sarstedt) and analysed using
an automated blood lactate and glucose analyser (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH, USA). The analyser was calibrated hourly or every ten samples and daily maintenance was undertaken in accordance with the manufacturer’s recommendations.

In Chapters 5 and 7, a 20 gauge cannula (Insyte-W™ Becton-Dickinson, Madrid, Spain) was inserted into the subject’s antecubital vein to enable frequent blood sampling before, during and after the exercise protocol. The cannula was kept patent with an infusion of 0.9% saline at 10 ml·h⁻¹ using a syringe driver (Terumo NV, Leuven, Belgium). Blood samples were drawn into 5-ml lithium-heparin tubes (Vacutainer, Becton-Dickinson, New Jersey, USA). 200 μl of blood was immediately haemolysed in 200μl of cold Triton X-100 buffer solution (Triton X-100, Amresco, Salon, OH) and analysed to determine blood [lactate] and [glucose] (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining whole blood was then centrifuged at 4000 rpm for 8 min (Hettich EBA 20, Germany) before plasma was extracted and analysed for [K⁺] and [Na⁺] using an ion-selective analyser (9180 Electrolyte Analyzer, F. Hoffman-La Roche, Basel, Switzerland). The analyser is automatically calibrated, hourly.

**Measurement of nitrate and nitrite concentrations**

In Chapters 5 and 7, separate blood samples for the determination of plasma [NO₂⁻] and [NO₃⁻] were collected via the cannula into lithium-heparin tubes. In Chapters 4 and 6, venous blood samples were obtained from the antecubital fossa using venepuncture, with all samples being immediately centrifuged at 4000 rpm and 4 °C for 8 min. Plasma was extracted and immediately frozen at -80 °C for later analysis of [NO₂⁻] and [NO₃⁻] via chemiluminescence.

Prior to, and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO₂⁻. Plasma NO₂⁻ and NO₃⁻ were analysed by gas phase chemiluminescence analysis. This initially required NO₂⁻ and NO₃⁻ to be reduced to NO gas. For reduction of NO₂⁻, undiluted plasma was injected into a glass purge vessel containing 5 ml glacial acetic acid and 1 ml NaI solution. For NO₃⁻ reduction, plasma samples were deproteinised in an aqueous solution of zinc sulphate (10% w/v) and 1 M sodium hydroxide, prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). Quantification of NO was enabled by the detection of light
emitted during the production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentrations of NO₂⁻ and NO₃⁻ were determined by plotting signal area (mV) against a calibration plot of 25 nM to 1 µM sodium nitrite and 100 nM to 10 µM sodium nitrate, respectively. The coefficients of variation for duplicate samples of nitrate and nitrite using these techniques were 1.9% and 8.5%, respectively.

**Pulmonary gas exchange**

Pulmonary gas exchange and ventilation were measured breath-by-breath in all laboratory tests excluding those tests conducted within the bore of the magnetic resonance scanner. All pulmonary gas exchange analysis was performed using a metabolic cart system that was made up of a bidirectional TripleV digital transducer and differential paramagnetic (O₂) and infrared absorption (CO₂) (Jaeger Oxycon Pro, Hoechberg, Germany). The gas analyser was calibrated before each test with gases of known concentration and the volume sensor was calibrated using a 3-liter syringe (Hans Rudolph, Kansas City, MO). During all tests subjects wore a nose clip and breathed through a low-dead-space, low-resistance mouthpiece that was connected securely to the transducer. A capillary line continuously sampled \( \dot{V}_O_2 \), \( \dot{V}_C_O_2 \) and minute ventilation (\( \dot{V}_E \)) and displayed these variables breath-by-breath. Upon completion of each test, raw breath-by-breath gas exchange and ventilation data were exported for analysis.

The reliability of pulmonary gas exchange measurement was determined by repeated bouts of moderate-intensity exercise performed over several days. A subject performed square-wave exercise from a 3 min baseline pedaling period at 20 W to a power output of 100 W. The coefficients of variation for steady state \( \dot{V}_O_2 \) were 1.3-1.8% (intra-test variation, using six 30 s bins from 3 min to 6 min of exercise) and 2% (inter-test variation, using 5 tests performed on separate days) at an absolute \( \dot{V}_O_2 \) of 2090 ml·min⁻¹. Thus, the typical error associated with the measurement of steady-state \( \dot{V}_O_2 \) on separate days was 40-50 ml·min⁻¹, or 1.9-2.3%.

**Normalisation of exercise intensity**

In Chapters 4, 5 and 7 a preliminary ramp incremental exercise test to exhaustion was completed. These tests consisted of a three minute unloaded baseline period, followed by a
continuous linear (ramp) increase in work rate of 30 W min\(^{-1}\) until the subject was unable to continue. The test was terminated when the subject dropped 10 rpm below the test cadence (80 rpm). The height and configuration of both handlebar and saddle were recorded following the ramp test so that the same settings could be reproduced on all subsequent tests. Pulmonary gas exchange was continuously measured throughout these incremental tests in order to determine the gas exchange threshold (GET), \(\dot{V}O_2\text{peak}\) and to calculate appropriate work rates for subsequent constant work rate exercise tests. In chapter 6, the incremental test was not continued until exhaustion and the data were used to establish the GET only.

The breath-by-breath pulmonary gas-exchange data were averaged over consecutive 10-s periods. The GET was determined by identifying the first disproportionate increase in CO\(_2\) production (\(\dot{V}CO_2\)) from visual inspection of individual plots of \(\dot{V}CO_2\) vs. \(\dot{V}O_2\) and an increase in ventilatory equivalent of \(O_2\) (\(\dot{V}E/\dot{V}O_2\)) with no increase in ventilatory equivalent of \(CO_2\) (\(\dot{V}E/\dot{V}CO_2\)). The \(\dot{V}O_2\text{peak}\) was defined as the highest 30-s rolling average value.

In Chapters 4-7, constant work rate exercise tests were employed in order to assess pulmonary \(\dot{V}O_2\) kinetics in response to exercise. These tests involved an abrupt transition from a lower to a higher work rate. All constant work rate trials were performed at power outputs which were predetermined based on the results of the preliminary ramp incremental tests. When prescribing work rates based upon pulmonary gas exchange data from incremental exercise tests it is important to consider the \(\dot{V}O_2\) mean response time and correct for this accordingly. The \(\dot{V}O_2\) mean response time is assumed to approximate two-thirds of the ramp rate during incremental exercise (Whipp \textit{et al.}, 1981). Therefore, the work rates associated with the GET and \(\dot{V}O_2\text{peak}\) used to normalise exercise intensity in chapters 4-7 reflect work rates that are 20 W less than the work rates coinciding with the appearance of GET and \(\dot{V}O_2\text{peak}\) during the incremental ramp tests.

The moderate-intensity work rates utilised in Chapters 5, 6 and 7 were calculated as 80\% of the GET. In Chapters 5 and 7, severe-intensity exercise was calculated as 75\% \(\Delta\) (a power output representing the power output at GET plus 75\% of the difference between the power outputs at \(\dot{V}O_2\text{peak}\) and GET). In Chapter 4, four different severe-intensity work rates were imposed and these were 60\%\(\Delta\), 70\%\(\Delta\), 80\%\(\Delta\) and 100\% \(\dot{V}O_2\text{peak}\).
Chapter 3: General Methods

Mathematical modelling of $\dot{V}O_2$ data

Typically, raw, breath-by-breath pulmonary $\dot{V}O_2$ data displays a considerable inherent ‘noise’ in its signal, which can obscure the underlying response characteristics. Despite this ‘noise’, the dynamics of pulmonary $\dot{V}O_2$ are considered closely matched to those of the contracting muscle (within ~ 10%) and can be considered valid and as long as a number of data editing procedures are adhered to prior to the modelling of the response (Barstow et al., 1990; Poole et al., 1991; Grassi et al., 1996; Krstrup et al., 2009).

Each individual transition was initially inspected prior to exclude data that were thought to inappropriately reflect the underlying physiological response (errant breaths caused by coughing, swallowing etc.). During this process, great care was taken to only remove definitive outliers using a criterion that those data points lying more than four standard deviations from the five-breath rolling average were removed. Following this ‘filtering’ process, data were linearly interpolated using a dedicated algorithm to provide second-by-second values. This is necessary because signal-to-noise ratio can also be enhanced by time-aligning and averaging repeat transitions of identical trials (Lamarra et al., 1987). It has also been shown that breath-to-breath ‘noise’ in the $\dot{V}O_2$ signal is influenced by the response amplitude (Lamarra et al., 1987). Therefore, in experimental chapters 5-7, repeat-transitions to moderate-intensity exercise were conducted in order to ensemble average the data in an attempt reduce the negative impact of noise on the confidence in the modelled parameter estimates. In these chapters a moderate exercise transition often preceded a subsequent moderate or severe exercise transition following passive recovery. This was done to reduce the total number of laboratory visits. Importantly, the performance of moderate exercise prior to a subsequent moderate or severe exercise bout does not influence the $\dot{V}O_2$ response during the subsequent bout (Burnley et al., 2000).

The on-kinetics of the $\dot{V}O_2$ response to exercise was defined using parameters derived from the fitting of an exponential curve. Once filtering, interpolation and where necessary, averaging of the breath-by-breath data was complete, the second-by-second files were transferred into a purpose-written modelling program that described the $\dot{V}O_2$ response using a nonlinear least-square regression algorithm. The program uses an iterative process that minimises the sum of the squared error between the fitted function and the observed data. Prior to fitting the exponential curve, the first 20 s of data after the onset of exercise were
deleted to ensure that the cardio-dynamic phase of the \( \dot{V}O_2 \) response (phase I) did not influence the phase II fit. For moderate-intensity exercise (Chapters 5 and 7), a single-exponential model was used to characterise the \( \dot{V}O_2 \) response, as described in following equation:

\[
\dot{V}O_2(t) = \dot{V}O_{2\text{baseline}} + A_p [1 - e^{-(t-TD_p/\tau_p)}]
\]

(Eqn. 5)

where \( \dot{V}O_2(t) \) represents the absolute \( \dot{V}O_2 \) at a given time \( t \); \( \dot{V}O_{2\text{baseline}} \) represents the mean \( \dot{V}O_2 \) in the baseline period; \( A_p, TD_p \), and \( \tau_p \) represent the amplitude, time delay and time constant, respectively, describing the phase II increase in \( \dot{V}O_2 \) above baseline.

In Chapters 5 and 7 the fitting strategy was subsequently used to identify the onset of any ‘slow component’ in the \( \dot{V}O_2 \) response to severe-intensity exercise as previously described (Rossiter et al., 2001). The fitting window was lengthened iteratively until the exponential model-fit demonstrated a discernible departure from the measured response profile. Identification, via visual inspection, of the flat residual plot profile (signifying a good fit to measured data) systematically differing from zero, gave indication of the delayed slow component onset. The magnitude of the slow component for \( \dot{V}O_2 \) was measured from the phase II steady state amplitude and the amplitude of the final value, averaged over the last 30 s of exercise.

For severe-intensity exercise in Chapter 4 a bi-exponential model was used to characterise the \( \dot{V}O_2 \) response which is described in the following equation:

\[
\dot{V}O_2(t) = \dot{V}O_{2\text{baseline}} + A_p [1 - e^{-(t-TD_p/\tau_p)}] + A_s [1 - e^{-(t-TD_s/\tau_s)}]
\]

(Eqn. 6)

Where \( \dot{V}O_2(t) \) represents the absolute \( \dot{V}O_2 \) at a given time \( t \); \( \dot{V}O_{2\text{baseline}} \) represents the mean \( \dot{V}O_2 \) during the final 90 s of the baseline period; \( A_p, TD_p \), and \( \tau_p \) represent the amplitude, time delay, and time constant, respectively, describing the phase II increase in \( \dot{V}O_2 \) above baseline; and \( A_s, TD_s \), and \( \tau_s \) represent the amplitude of, time delay before the onset of, and the time constant describing the development of the \( \dot{V}O_2 \) slow component, respectively. Furthermore, the \( \dot{V}O_2 \) mean response time (MRT) was determined in Chapter 6 by fitting a single exponential curve without time delay to all data from \( t = 0 \). This parameter provides information on the overall \( \dot{V}O_2 \) kinetics with no distinction made for various phases of the
response. This can be useful for estimating the O$_2$ deficit during exercise which was of particular interest in Chapter 6.

Statistical methods
Statistical analyses in all experimental chapters were conducted using the Statistical Package for Social Sciences (SPSS v.19). Specific statistical analysis conducted is outlined in each individual experimental chapter. Prior to any statistical analyses the data was appropriately screened for normality using recognized procedures. Statistical significance was accepted at $P < 0.05$ with all data being presented as means ± SD unless otherwise stipulated.
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

Effects of Nitrate on the Power–Duration Relationship for Severe-Intensity Exercise

JAMES KELLY, ANNI VANHATALO, DARYL P. WILKERTON, LEE J. WYLIE, and ANDREW M. JONES

Sport and Health Sciences, College of Life and Environmental Sciences, St. Luke’s Campus, University of Exeter, Heavitree Road, Exeter, UNITED KINGDOM

ABSTRACT

KELLY, J., A. VANHATALO, D. P. WILKERTON, L. J. WYLIE, and A. M. JONES. Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise. Med. Sci. Sports Exerc., Vol. 45, No. 9, pp. 1798–1806, 2013. Purpose: The power asymptote (critical power [CP]) and curvature constant ($W'$) of the power–duration relationship dictate the tolerance to severe-intensity exercise. We tested the hypothesis that dietary nitrate supplementation would increase the CP and/or the $W'$ during cycling exercise. Methods: In a double-blind, randomized, crossover study, nine recreationally active male subjects supplemented their diet with either nitrate-rich concentrated beetroot juice (BR; 2 × 250 mL·d$^{-1}$, ~8.2 mmol·d$^{-1}$ nitrate) or a nitrate-depleted BR placebo (PL; 2 × 250 mL·d$^{-1}$, ~0.06 mmol·d$^{-1}$ nitrate). In each condition, the subjects completed four separate severe-intensity exercise bouts to exhaustion at 60% of the difference between the gas exchange threshold and the peak power attained during incremental exercise (60% $\Delta$, 70% $\Delta$, 80% $\Delta$, and 100% peak power), and the results were used to establish CP and $W'$. Results: Nitrate supplementation improved exercise tolerance during exercise at 60% $\Delta$ (BR, 696 ± 120 vs PL, 593 ± 68 s; $P < 0.05$), 70% $\Delta$ (BR, 452 ± 106 vs PL, 390 ± 86 s; $P < 0.05$), and 80% $\Delta$ (BR, 294 ± 50 vs PL, 263 ± 50 s; $P < 0.05$) but not 100% peak power (BR, 182 ± 37 vs PL, 166 ± 26 s; $P = 0.10$). Neither CP (BR, 221 ± 27 vs PL, 218 ± 26 W) nor $W'$ (BR, 19.3 ± 4.6 vs PL, 17.8 ± 3 kJ) were significantly altered by BR. Conclusion: Dietary nitrate supplementation improved endurance during severe-intensity exercise in recreationally active subjects without significantly increasing either the CP or the $W'$. Key Words: MAXIMAL EXERCISE, ENDURANCE, BEETROOT JUICE, CRITICAL POWER.

The critical power (CP) and the $W'$ are the two parameters that characterize the hyperbolic power–duration relationship, which is evident during high-intensity exercise (30,33). The CP is the power asymptote of the relationship and demarcates the boundary between high-intensity exercise, within which a physiological steady state is attained, and severe-intensity exercise, which by definition does not permit steady-state behavior (20). Thus, the CP theoretically represents the highest power output that can be maintained via predominantly aerobic metabolism, where pulmonary oxygen uptake (VO$_2$), blood lactate and concentrations of intramuscular metabolites such as phosphocreatine ([PCr]), [H$^+$], and inorganic phosphate ([P$_i$]) can be stabilized (21,33). The $W'$ represents the curvature constant of the relationship and can be considered as the finite work capacity available above the CP before the limit of tolerance ($T_{lim}$) is reached (30,33). The physiological determinants of the $W'$ are debated (12,29,38).

The hyperbolic power–duration relationship is given as follows:

$$ T_{lim} = W'/(P - CP) $$

[1]

where P is a given severe-intensity power output (17,20,40). The linear transformations of this relationship are the power–1/time equation:

$$ P = W'(1/T_{lim}) + CP $$

[2]

and the work–time equation, where P is replaced with work done (W) per unit time:

$$ W = CP T_{lim} + W' $$

[3]

It is evident that when the CP and $W'$ are known, performance time for a given amount of work within the severe domain (indicated by $T_{lim}$) can be accurately predicted by rearranging equation 3 (17,20,40):

$$ T_{lim} = (W - W')/CP $$

[4]

The CP and the $W'$ are important determinants of sport and exercise performance (20,40). Importantly, equations 1–4 indicate that performance in the severe domain is a function of both the CP and the $W'$, which act in concert to determine the shortest possible time required to complete a given target total work done.

There is a growing body of evidence to suggest that supplementing an individual’s diet with inorganic nitrate (NO$_3^-$) can have beneficial effects on cardiovascular health.
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

and exercise performance. The NO$_3^-$ anion itself is inert, and its in vivo conversion to bioactive nitrite (NO$_2^-$) and nitric oxide (NO) is likely responsible for the biological effects observed. Upon ingestion, up to 25% of the inorganic NO$_3^-$ enters the enterosalivary circulation and is concentrated in the saliva (28). Facultative anaerobic bacteria in the oral cavity then reduce the NO$_3^-$ to NO$_2^-$ (10). When swallowed into the acidic environment of the stomach, some of the NO$_2^-$ is further converted into NO, whereas the remainder is absorbed to increase circulating plasma NO$_3^-$ concentration [NO$_2^-$]. This NO$_2^-$ may be reduced further to NO, particularly in tissues that may be relatively hypoxic, such as contracting skeletal muscle (34). NO is a physiological signaling molecule with various functions in the body, including the regulation of vascular tone, blood flow, muscle contractility, and mitochondrial respiration (8,15,35).

It is now widely accepted that dietary nitrate supplementation via nitrate-rich beets, nitrate-rich beetroot juice (BR) can significantly reduce resting blood pressure (BP) in young, normotensive adults (24,41). Moreover, dietary nitrate supplementation has been shown to reduce the O$_2$ cost of moderate-intensity exercise (1,25,36). This improved muscle efficiency may potentially result from NO-mediated enhanced mitochondrial efficiency (26) and/or a reduced ATP cost of muscle force production (2). It is not known to what extent improved mitochondrial efficiency after nitrate supplementation (26) might influence skeletal muscle energy metabolism at rest. If the resting metabolic rate (RMR) is significantly reduced after nitrate intake, this could have implications for daily energy expenditure and weight management. The influence of nitrate supplementation on RMR is yet to be examined.

In recreationally active subjects and subelite athletes, dietary nitrate supplementation has been reported to improve tolerance to constant power output, high-intensity cycling (1), knee-extensor exercise (2), and running (23). Typically, enhanced exercise tolerance has been reported in exercise trials of 6–15 min in duration. However, it remains unknown whether nitrate supplementation may be ergogenic during shorter duration, higher intensity exercise. Improvements in cycling time trial (TT) performance for 4 and 16.1 km (22) and 10 km (6), with a range of maximal exercise durations of 6–30 min, have also been reported after nitrate supplementation. Because exercise performance in the severe domain is a function of CP and $W'$, collectively these results suggest a beneficial shift in the power-duration relationship (rightward and/or upward) for severe-intensity exercise as a result of nitrate supplementation. Because the CP is associated with a particular metabolic rate (3), the increased ratio of power output to VO$_2peak$ with nitrate (1,6,22,25) indicates that nitrate supplementation might increase the CP. However, recent studies have suggested that nitrate supplementation might specifically affect blood flow and contractile function in Type II muscle fibers (11,16), factors which might, in turn, be expected to increase the $W'$.

Therefore, the purpose of this study was to investigate the effects of dietary nitrate supplementation on 1) the power-duration relationship for severe-intensity exercise (CP and $W'$) and 2) the RMR. We hypothesized that nitrate supplementation would improve exercise tolerance across a range of severe-intensity exercise bouts by increasing the CP and/or $W'$. We also hypothesized that nitrate supplementation would reduce the RMR.

**METHODS**

**Subjects**

Nine habitually active, male subjects (mean ± SD; age = 22 ± 3 yr, height = 180 ± 7 cm, body mass = 77 ± 9 kg; VO$_2peak$ = 54.5 ± 7.5 mL·kg$^{-1}$·min$^{-1}$) volunteered to take part in this study. All procedures used in this study were approved by the institutional ethics committee. The subjects gave their written, informed consent before the commencement of the study, once the experimental procedures, associated risks, and potential benefits of participation had been described. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 4 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Subjects were also asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively, and to consume the same light pre-exercise meal of their choice 4–5 h before testing (see Supplementation section). In addition to this, subjects abstained from using antibacterial mouthwash and chewing gum throughout the study (14). For each subject, all exercise tests were performed at the same time of day (±2 h).

**Experimental Design**

The protocol involved 12 separate visits to the laboratory and consisted of a ramp incremental test at the beginning and end of the study and, for each of the two conditions (experimental and placebo), an RMR test and four separate constant power output trials at different severe-intensity work rates, which were presented in random order. Subjects were given a minimum of 24 h of rest between each visit, with all tests being completed within a 4-wk period. During visit 1, subjects performed a ramp incremental test to exhaustion to assess VO$_2peak$ and gas exchange threshold (GET). After this, the subjects were assigned in a double-blind, randomized, crossover design to consume 500 mL·d$^{-1}$ of nitrate-rich BR or nitrate-depleted BR placebo (PL). During visits 2–6, subjects performed four, severe-intensity, constant power output trials to exhaustion to determine CP and $W'$ and one RMR test. These tests were repeated (visits 7–11) once a washout period of at least 72 h had elapsed. Finally, after a further 72-h washout period, a follow-up ramp incremental test was performed (visit 12) to assess whether the prediction trials had resulted in a training effect.
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

Ramp Incremental Tests

All exercise testing was performed using an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands). During visit 1, subjects completed 3 min of baseline cycling at 20 W and 80 rpm, after which the power output was increased at a rate of 30 W min⁻¹ in a linear fashion until volitional exhaustion was achieved or until the subject was unable to maintain the 80-rpm pedal rate. The height and the configuration of the saddle and handlebars were recorded and reproduced in subsequent tests. Breath-by-breath pulmonary gas exchange data were collected continuously during the incremental test and averaged for the consecutive 10-s periods. VO₂peak was determined as the highest mean VO₂ during any 30-s period. The GET was determined from a cluster of measurements, including 1) the first disproportionate increase in CO₂ production (VCO₂) from visual inspection of individual plots of VCO₂ vs VO₂, 2) an increase in expired ventilation (V̇E/VO₂) with no increase in V̇E/V̇CO₂, and 3) an increase in end-tidal O₂ tension with no fall in end-tidal CO₂ tension.

Supplementation

After the completion of the nonsupplemented visit 1, subjects were assigned in a double-blind, randomized, crossover design to receive a course of dietary supplementation before visits 2–6 and visits 7–11. The supplements were either concentrated nitrate-rich BR (2 x 250 mL·d⁻¹) of BR providing a total of 8.2 mmol nitrate per day; Beet it, James White Drinks, Ipswich, UK) or nitrate-depleted PL (2 x 250 mL·d⁻¹) of BR providing a total of 0.006 mmol nitrate per day; Beet it, James White Drinks). The PL beverage was created by passing the juice, before pasteurization, through a column containing Purolite A520E ion exchange resin, which selectively removes nitrates (22). The PL was similar to the BR in appearance, taste, and smell. Subjects were instructed to consume the beverages in the morning and afternoon of days 1 and 2 of supplementation, then in the morning and 2.5 h before their first laboratory visit on day 3. Subjects continued to consume two 250-mL beverages each day and two on the day of testing (one on waking and the other 2.5 h before commencement of the test) until the four exercise tests and the RMR test were complete. In total, the subjects consumed BR and PL for a minimum of 7 d and a maximum of 12 d. A washout period of at least 72 h separated each supplementation period. Subjects were instructed to follow their normal dietary habits throughout the testing period and to replicate their diet between conditions during the supplementation periods. Subjects were told that supplementation may cause beeturia (red urine) and red stools temporarily but that this side effect was harmless.

Determination of Power-Duration Relationship

To estimate CP and W', four prediction trials were completed during each supplementation period. The power outputs for the trials were equal to 70% Δ (a power output representing GET plus 70% of the interval between the power outputs at GET and VO₂peak), 80% Δ, and 100% peak power, with the power output for the final trial being calculated to obtain a range of times to exhaustion between 2 and 15 min as has been recommended (17,20). This calculated intensity typically approximated 60% Δ. Each prediction trial began with a 3-min baseline period at 20 W. This was followed by an abrupt transition to the appropriate power output. Subjects maintained a cadence of 80 rpm for as long as possible, with tests being terminated when cadence fell to less than 70 rpm for more than 5 s. Strong verbal encouragement was provided during each test, and the time to exhaustion was recorded to the nearest second. Breath-by-breath pulmonary gas exchange data were collected continuously, and blood [lactate] was measured at rest and as soon as possible after the termination of exercise in each trial. Subjects were not informed of their power outputs or performance on any of the tests until the entire experiment had been completed.

RMR Assessment

Upon arrival at the laboratory, subjects were seated and asked to rest for 10 min before the start of the test. Measurements were made in a well-ventilated, quiet laboratory setting at a temperature of 22°C–25°C, with mild ambient lighting throughout all tests. RMR was measured using indirect calorimetry. Breath-by-breath pulmonary gas exchange and ventilation were measured by an open circuit ventilated hood system (Oxycon beta; Mijnhardt, Bunnik, the Netherlands). Data were collected during a 15-min period. The first 5 min of data were discarded, and the remaining 10 min of data were used in subsequent analyses.

Measurements

Before each testing session, the BP of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro; GE Medical Systems, Tampa, FL) while subjects were seated at rest. Subjects were seated in a resting state for 10 min before the measurements. A total of four measurements were recorded, with the mean of the final three measurements being calculated. Mean arterial pressure (MAP) was calculated as 1/3 x systolic pressure + 2/3 x diastolic pressure. The mean systolic, diastolic, and MAP for all sessions (four prediction trials and RMR session) was calculated for both the BR and the PL conditions.

Also, at each test session, after the measurement of BP, a venous blood sample was taken for the determination of plasma [NO₃⁻]. Venous blood samples (~4 mL) were drawn into lithium-heparin tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Within 3 min of collection, samples were centrifuged at 4000 rpm and 4°C for 10 min. Plasma was extracted and immediately frozen at ~80°C for later analysis of [NO₃⁻]. Before and regularly during
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO\textsubscript{3}\textsuperscript{-}. After plasma samples were thawed at room temperature, the [NO\textsubscript{3}\textsuperscript{-}] was determined using a modification of the chemiluminescence technique as described previously by Bailey et al. (1).

During all exercise tests, pulmonary gas exchange and ventilation were measured continuously with subjects wearing a nose clip and breathing through a mouthpiece and impeller turbine assembly (Jaeger Triple V, Hoechberg, Germany). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O\textsubscript{2}) and infrared (CO\textsubscript{2}) analyzers (Oxycron Pro; Jaeger) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-L syringe (Hans Rudolph, Kansas City, MO). Pulmonary gas exchange variables were calculated and displayed breath by breath. The same gas analysis equipment was used during the RMR tests, although the subjects had a clear, ventilated hood system (Oxycron beta; Mijnhardt) placed over their head instead of the aforementioned mouthpiece and nose clip. HR was measured using short-range radio telemetry (model 610; Polar Electro Oy, Kempele, Finland). At rest and after the termination of exercise, fingertip blood samples were collected into a capillary tube and analyzed for [lactate] (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH).

Data Analysis

**CP and W’**. Estimates of CP and W’ from the prediction trials were calculated using three different models (using equations 1–3) as described previously (17,20). The model producing the lowest SE was used in subsequent analysis (17,18). Estimates of CP and W’ were subsequently used to predict the time taken to complete a range of total work done (W) targets (50, 75, 100, 125, 150, 175, 200, 225, 250 kJ). These work done targets were chosen to represent the applicable range of the power-duration relationship within the severe-intensity domain, where performance times would range from approximately 2 to 15 min.

**Oxygen uptake analysis.** The breath-by-breath VO\textsubscript{2} data from each exercise test were initially examined to exclude errant breaths caused by coughing and swallowing, with those values lying more than four SD from the local mean being removed. The first 20 s of data after the onset of exercise (the phase I response) were deleted and a non-linear least-square algorithm was used to fit the data. A biexponential model was used to characterize the VO\textsubscript{2} responses to the severe-intensity exercise bouts as described in the following equation:

\[
\text{VO}_2(t) = \text{VO}_2\text{baseline} + A_1\left[1-e^{-t/TD_{1}/n}\right] + A_2\left[1-e^{-t/TD_{2}/n}\right] \tag{5}
\]

where \(\text{VO}_2(t)\) represents the absolute \(\text{VO}_2\) at a given time \(t\), \(\text{VO}_2\text{baseline}\) represents the mean \(\text{VO}_2\) during the final 90 s of the baseline period; \(A_1, TD_{1}\), and \(n\) represent the amplitude, time delay, and time constant, respectively, describing the phase I increase in \(\text{VO}_2\) above baseline; and \(A_2, TD_{2}\), and \(n\) represent the amplitude of, time delay before the onset of, and time constant describing the development of the \(\text{VO}_2\) slow component, respectively.

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. The end-exercise \(\text{VO}_2\) for all four work rates was defined as the mean \(\text{VO}_2\) measured during the final 15 s of exercise. The same “filtering” technique was used for the breath-by-breath \(\text{VO}_2\) data collected during the RMR tests. A mean value during the 10-min collection period was calculated.

**Statistical analyses.** Differences in plasma [NO\textsubscript{3}\textsuperscript{-}], BP, time to exhaustion, CP, W’ and cardiorespiratory responses, between the conditions were analyzed with paired-samples \(t\)-tests. Additional paired samples \(t\)-tests were performed on the phase II \(\text{VO}_2\) time constants irrespective of exercise intensity. A one-way repeated-measures ANOVA was used to identify differences in CP and W’ estimates between the three models and the plasma [NO\textsubscript{3}\textsuperscript{-}] across visits 1–5 as well as across work rates. A two-way repeated-measures ANOVA (condition × work rate) was used to assess differences in end-exercise \(\text{VO}_2\) and predicted performance times. Significant main effects were further analyzed using simple contrasts with Fisher’s LSD. All data are presented as mean ± SD unless stated otherwise with statistical significance being accepted when \(P < 0.05\).

**RESULTS**

Self-reported compliance to the supplementation regimen was 100%, and no deleterious effects were reported.

**Plasma [NO\textsubscript{3}\textsuperscript{-}] and BP.** ANOVA revealed that plasma [NO\textsubscript{3}\textsuperscript{-}] was significantly elevated for BR compared with PL (\(P < 0.01\)), but there was no difference across time (\(P > 0.05\)) or exercise intensity (\(P > 0.05\)). For PL visits 1–5, the plasma [NO\textsubscript{3}\textsuperscript{-}] was 98 ± 41, 96 ± 29, 75 ± 16, 86 ± 26, and 73 ± 31 nM, respectively; whereas for BR visits 1–5, the plasma [NO\textsubscript{3}\textsuperscript{-}] was 297 ± 98, 262 ± 107, 295 ± 108, 209 ± 86, and 228 ± 135 nM, respectively. The plasma [NO\textsubscript{3}\textsuperscript{-}] was higher (\(P < 0.05\)) in the BR than that in the PL for the RMR visit (BR, 286 ± 113 vs PL, 89 ± 32 nM), 60% Δ (BR, 213 ± 146 vs PL, 69 ± 29 nM), 70% Δ (BR, 223 ± 93 vs PL, 87 ± 28 nM), 80% Δ (BR, 270 ± 131 vs PL, 96 ± 46 nM), and 100% peak (BR, 285 ± 97 vs PL, 87 ± 19 nM). On average, the subjects consumed the BR or the PL supplement for 5 ± 2 d before each of the experimental trials. Across all testing conditions, BR significantly increased plasma [NO\textsubscript{3}\textsuperscript{-}] by 197% when compared with PL (BR, 255 ± 70 vs PL 86 ± 21 nM; \(P < 0.01\)), whereas diastolic BP (BR, 65 ± 5 vs PL, 65 ± 5 mm Hg; \(P > 0.05\)) and MAP (BR, 86 ± 7 vs PL, 84 ± 4 mm Hg; \(P > 0.05\)) were not significantly different between conditions.
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

**Ramp incremental test.** The main experiment resulted in no training effect ($P > 0.05$) upon VO$_2$peak (initial test, $4.13 \pm 0.44$ vs final test, $4.09 \pm 0.56$ mL min$^{-1}$ kg$^{-1}$), peak power output (initial test, $344 \pm 34$ vs final test, $338 \pm 32$ W), or power output at GET (initial test, $129 \pm 30$ vs final test, $125 \pm 20$ W).

**Exercise tolerance, CP, and W'.** Exercise tolerance (expressed as time to exhaustion) across the four severe-intensity prediction trials after PL and BR is displayed in Figure 1. BR significantly improved time to exhaustion for three of the four intensities when compared with PL: at $60\% \Delta$ (BR, 696 vs PL, 593; $P < 0.05$), $70\% \Delta$ (BR, 452 vs PL, 390; $P < 0.05$), and $80\% \Delta$ (BR, 294 vs PL, 263; $P < 0.05$) but not at $100\%$ peak (BR, 182 vs PL, 166; $P = 0.10$). Baseline and end-exercise HR and blood [lactate] were not significantly different between conditions (Table 1).

ANOVA revealed that the estimates of CP and W' derived from the three different models (hyperbolic power-time, power-1/time model, and work-time; equations 1–3) were not significantly different from one another ($P > 0.05$). For the power-1/time model, the values for CP were 218 vs $221 \pm 27$ W for PL and BR, respectively, and the values for W' were 17.8 vs 3.0 and 19.3 vs 4.6 kJ for PL and BR, respectively. For the work-time model, the values for CP were 218 vs 217 vs 28 W for PL and BR, respectively, and the values for W' were 17.7 vs 3.0 and 19.7 vs 4.1 kJ for PL and BR, respectively. For the power-time model, the values for CP were 216 vs 214 vs 27 W for PL and BR, respectively, and the values for W' were 18.5 vs 3.3 and 21.2 vs 4.4 kJ for PL and BR, respectively. The coefficients of variation (CV; SE expressed as a percentage of the parameter estimate) associated with the CP estimates from all models were <5%. However, the CV associated with the W' estimates for the power-time (PL, 10.5% ± 8.1%; BR, 10.8% ± 7.3%), work-time (PL, 9.9% ± 7.7%; BR, 10.5% ± 8.0%), and power-1/time (PL, 8.3% ± 5.9%; BR, 8.3% ± 6.1%) models were typically slightly larger than the arbitrary cutoff points that have been proposed as acceptable (17,18). Overall, the power-1/time model elicited the lowest SE (CP: PL, 6 ± 2 W; BR, 5 ± 4 W; W', PL, 1.4 ± 1.0 kJ; BR, 2.3 ± 2.1 kJ). The CV averaged across CP and W' in both conditions was lowest in the power-1/time model (5.5%) compared with the power-time (6.4%) and work-time models (6.2%), and so, as is the convention (17,18), the estimates from the model associated with the lowest error were used in further analysis.

A representation of the power-duration and power-1/time relationships in BR and PL is presented in Figure 2. For the power-1/time model, BR resulted in small but nonsignificant changes in both CP (BR, 221 vs 27 W for PL, 218 W) and W' (BR, 19.3 vs 4.6 vs PL; 17.8 ± 3.0 kJ). The group mean difference in CP between BR and PL (+1.4%) was smaller than the mean CV associated with the parameter estimates. The CP increased in eight of nine subjects, and in four of these subjects, the increase in CP was greater than the CV associated with the individual CP estimates in BR and PL. The group mean difference in W' between BR and PL (+8.4%) was similar to the mean CV associated with the parameter estimates. The W' increased in six of nine subjects, and in four of these subjects, the increase in W' was greater than the CV associated with the individual W' estimates. When the CP and W' were combined to predict performance in a time-trial scenario (using equation 4) and for work-done targets of 50, 75, 100, 125, 150, 175, 200 and 225 kJ, the ANOVA revealed a significant main effect by supplement ($P < 0.05$) and an interaction effect ($P < 0.05$). Specifically, the performance times were significantly lower in BR compared with PL for all time trials except the shortest one (50 kJ) (Fig. 3).

**Oxygen uptake kinetics.** The pulmonary VO$_2$ parameters derived from the model fit, during each of the prediction trials, are presented in Table 2. VO$_2$ values during the baseline period and at the end of exercise were unchanged with BR compared with PL across all prediction trials. Likewise, compared with PL, BR had no effect upon the phase II

---

**Table 1. Mean ± SD heart rate and blood [lactate], during four different severe-intensity exercise bouts.**

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% Δ</td>
<td>101 ± 11</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>70% Δ</td>
<td>100 ± 9</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>80% Δ</td>
<td>101 ± 9</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>100% peak</td>
<td>81 ± 1.6</td>
<td>86 ± 1.2</td>
</tr>
</tbody>
</table>

**Figure 1.** Group mean ± SD times to exhaustion across four severe-intensity power outputs, after PL (gray bars) and BR (black bars) supplementation. *$P < 0.05$.**
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

FIGURE 2—Effects of BR on the power–duration relationship established from four severe-intensity prediction trials. The group mean ± SE power–duration profiles are shown in panel (A), and the group mean ± SE power–time relationships are shown in panel (B). Responses after PL are represented by the dashed line and open symbols and BR by the black line and closed symbols. Note the rightward-shifted power–duration curve after BR.

time constant, the $\dot{V}O_2$ primary amplitude, or the $\dot{V}O_2$ slow component amplitude for any of the individual prediction trials. However, when the phase II time constant was compared between conditions irrespective of exercise intensity, it was significantly shorter in BR (BR, 22.8 ± 7.4 vs PL, 25.4 ± 7.2 s; P < 0.05). The end-exercise $\dot{V}O_2$ values across all trials were not significantly different from the $\dot{V}O_2$peak attained during the ramp incremental tests.

RMR. RMR was not altered by BR (BR, 0.27 ± 0.06 vs PL, 0.27 ± 0.06 L·min$^{-1}$).

DISCUSSION

The principal original finding of this investigation was that dietary supplementation with nitrate-rich BR significantly improved exercise tolerance in three of four severe-intensity constant power output exercise bouts (ranging between ~4- and 12-min duration), with a trend for improved performance also in the shortest bout (~3-min duration). In contrast to our hypothesis, neither the CP nor the $W$ were significantly improved by BR supplementation.

Nevertheless, the improved exercise tolerance across the severe exercise intensity domain would be expected to result in a significant improvement in performance as predicted by the two-parameter CP model. Another original finding of this study was that BR did not significantly alter RMR.

Effects of nitrate supplementation on plasma $[NO_2^–]$ and BP. Plasma $[NO_2^–]$ was significantly increased after nitrate-rich BR supplementation compared with PL. These findings are consistent with previous research, which has reported elevations in plasma $[NO_2^–]$ after dietary nitrate supplementation (1,14,25,53,36). Importantly, plasma $[NO_2^–]$ was not significantly higher before the later laboratory visits compared with the earlier ones and the elevation in plasma $[NO_2^–]$ in BR compared with PL was similar for all exercise intensities. Also consistent with previous literature (24,41), systolic BP was significantly
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

reduced (~4 mm Hg) with BR compared with PL. Increased NO bioavailability stimulates smooth muscle relaxation via the synthesis of cyclic guanosine monophosphate. It is this NO-mediated smooth muscle relaxation that is considered to be responsible for reductions in BP after nitrate supplementation (24,41).

Effect of nitrate supplementation on exercise tolerance, CP, and W’. A novel finding of the present study was that dietary nitrate supplementation significantly improved exercise tolerance during several severe-intensity exercise bouts. Previous studies (1,23) have reported that nitrate supplementation can enhance exercise tolerance, but these have focused on just one exercise intensity (70%–75% Δ). Interestingly, in these studies, it was reported that, compared with PL, BR increased exercise tolerance by 14%–16%, which is very similar to the 17% improvement at 60% Δ (exercise duration of ~10–11 min) and 16% improvement at 70% Δ (exercise duration of ~7 min) recorded in the present study. Compared with PL, BR enhanced exercise tolerance at higher exercise intensities too; there was a significant 12% improvement at 80% Δ (exercise duration of ~4–5 min) and a nonsignificant (P = 0.10) 10% improvement at 100% peak (exercise duration of ~3 min). This suggests that nitrate supplementation may benefit performance in shorter, higher intensity sports events than have been considered previously.

Although the increased exercise tolerance during constant power output exercise bouts indicates a physiological benefit of nitrate supplementation, it has been proposed that the magnitude of the changes elicited after an intervention can be difficult to interpret because of the shape of the power–duration relationship (42). We therefore used the four constant power output exercise bouts in the BR and PL conditions to calculate the CP and W’ using the power–time model because this produced the lowest error associated with the parameter estimates. Nitrate supplementation resulted in a 1.4% (3 W) increase in CP (which approached statistical significance, P = 0.07) and an 8.4% (1.5 kJ) increase in W’ (not significant).

Although the modest 1.4% improvement in CP and 8.4% increase in W’ do not appear to be substantial and were not statistically significant, when these values are applied to an exercise performance scenario that their potential importance becomes clear. It is important to note that severe-intensity exercise performance is determined by the interplay of the CP and W’, and not by either parameter alone (20). When the two parameters were combined to predict performance according to the two-parameter CP model (equation 4), the time to complete a fixed amount of work was significantly less in BR compared with PL across the applicable range of the power–duration relationship, except for the shortest target work done (50 kJ) where the predicted completion time was approximately 2.5 min (Fig. 3). The improvement in predicted performance is consistent with the experimental data, which showed an increased F_{\text{new}} at the three lowest work rates, but no significant improvement in the shortest trial (100% peak) after BR (Fig. 1). These analyses demonstrate that the apparently small, nonsignificant changes in CP and W’ together result in a significant alteration in predicted endurance performance. The potential benefits highlighted for performance (approximately 2%–3%) are much greater than the 0.6% value suggested to be the smallest “worthwhile” improvement for road TT cyclists (32). Interestingly, the differences between PL and BR in predicted performance (Fig. 3) are very similar to the beneficial effects of nitrate supplementation reported for cycling TT performance previously (4 km TT improved by 2.8% [22], 10 km TT improved by 1.2% [6], and 16.1 km TT improved by 2.7% [22]).

Effect of nitrate supplementation on oxygen uptake kinetics. The improvements in exercise tolerance at any given power output in the present study were evident without any significant changes in the dynamic VO_{2} response to exercise. Previous studies have suggested that the improvements in exercise tolerance and/or performance after nitrate supplementation might be linked to changes in VO_{2} kinetics. For example, Bailey et al. (1) reported that BR resulted in a 23% reduction in the VO_{2} slow component and a 16% improvement in exercise tolerance during constant power output cycle exercise at 70% Δ. As discussed by Burnley and Jones (5), a reduction in the amplitude of the VO_{2} slow component would delay the time before VO_{2\text{peak}} is attained and could therefore contribute to enhanced exercise tolerance. Also, Lansley et al. (22) found that BR increased power output for the same VO_{2} during cycling TT performances, implying improved muscle efficiency. An improved muscle efficiency after nitrate supplementation may be observed either as a lower VO_{2} for the same power output or, conversely, a higher power output for the same VO_{2}. In the present study, the amplitudes of the VO_{2} primary and slow components were not different between BR and PL. Moreover, ANOVA revealed that the end-exercise VO_{2} was neither different between conditions nor different from the VO_{2\text{peak}} measured during ramp incremental exercise. This latter result indicates that VO_{2\text{peak}} is not reduced by nitrate supplementation, which is consistent with some (1,36), but not all (4,27), previous studies.

Interestingly, the VO_{2} phase II time constant was slightly but significantly shorter in BR compared with PL when all data were considered together, irrespective of exercise intensity. A possible mechanism for faster VO_{2} kinetics in BR compared with PL is a preferential distribution of O_{2} delivery to Type II muscle fibers (11) and/or to muscle loci that may be relatively more hypoxic (39). Theoretically, faster VO_{2} kinetics would reduce the contribution of substrate-level phosphorylation energy turnover in the first 1–2 min after the transition to high-intensity exercise and may lead to improved exercise tolerance (5,31). It is not clear, however, whether this small improvement in VO_{2} kinetics (~2.6 s) might have contributed to the slightly higher CP (~3 W) observed with BR compared with PL in the present study.
Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise

It is noteworthy that training and other interventions often result in opposite effects on CP (i.e., increased) and $W'$ (i.e., decreased) (19,37,38). In the present study, we observed small, albeit nonsignificant, increases in both CP and $W'$, which may be a consequence of a multiplicity of effects of nitrate supplementation on muscle and vascular function. It is known that NO can modulate key processes involved in muscle force production, including the ATP cost of actin-myosin interaction and Ca$^{2+}$ handling (13), mitochondrial efficiency (26), and vascular tone and blood flow regulation (15). Exercise above CP elicits a disproportionate recruitment of Type II fibers (9). It has recently been reported that BR supplementation results in a marked increase in hind limb blood flow during exercise in rats after dietary supplementation with BR, with the increased blood flow being preferentially distributed to muscle groups that principally contain Type II fibers (11). It has also been reported that nitrate supplementation increases muscle force production in mice via modulations to intracellular Ca$^{2+}$ handling in fast-twitch fibers (16). Collectively, these modifications may account for the small improvements in CP and/or $W'$ observed in the present study, which in turn resulted in improved exercise tolerance during severe-intensity exercise.

It should be noted that recent studies indicate that nitrate supplementation may be less effective as an ergogenic aid in highly trained endurance athletes, at least when nitrate is ingested acutely and/or longer duration, lower intensity endurance performance is assessed (4,7,43). Therefore, it is not clear whether the results of this present study can be applied to highly trained endurance athletes. Compared with less well-trained subjects, endurance athletes have higher baseline plasma [NO$_3^-$], greater training-related NOS activity, a higher proportion of Type I fibers, and greater mitochondrial and capillary density, all of which may reduce the potential benefits of nitrate supplementation (43). The dose–response relationship, including the optimal amount, duration, and timing of nitrate supplementation, and the interaction of nitrate supplementation with subject training status and exercise intensity/duration are presently not known and are an important focus of ongoing research.

Effect of dietary nitrate on RMR. In contrast to our hypothesis, dietary nitrate supplementation did not change RMR. Previous research has shown that nitrate supplementation can increase the efficiency of mitochondrial respiration (26). Specifically, these authors reported a 19% improvement in oxidative phosphorylation efficiency in skeletal muscle mitochondria harvested after nitrate supplementation. The explanation for these improvements included a reduced proton slippage across the inner mitochondrial membrane, which is believed to account for a substantial amount of resting energy expenditure (26). In the present study, however, nitrate supplementation did not alter RMR. The lack of change in resting VO$_2$ suggests that nitrate supplementation did not alter oxidative phosphorylation efficiency or that the method used (whole-body indirect calorimetry) was not sufficiently sensitive to detect such changes.

In conclusion, short-term dietary supplementation with nitrate-rich BR increases exercise tolerance within the severe-intensity exercise domain. Although statistically nonsignificant, in concert, the small improvements in CP and $W'$ would be expected to conflate into a meaningful improvement in cycling TT performance in subelite cyclists.

The authors thank Beet It Ltd. for providing the beverages used in this study gratis.

The authors have no conflicts of interest to declare. The results of this study do not constitute endorsement by the American College of Sports Medicine.

REFERENCES

Chapter 4: Effects of Nitrate on the Power-Duration Relationship for Severe-Intensity Exercise


Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O\textsubscript{2} uptake dynamics during exercise in hypoxia and normoxia

James Kelly,1 Anni Vanhatalo,1 Stephen J. Bailey,1 Lee J. Wyile,1 Christopher Tucker,1 Stephen List,1 Paul V. Wingard,2 and Andrew M. Jones1

1Sport and Health Sciences, College of Life and Environmental Sciences, St. Luke’s Campus, University of Exeter, Exeter, United Kingdom; and 2University of Exeter Medical School, St. Luke’s Campus, University of Exeter, Heavitree Road, Exeter, United Kingdom

Submitted 13 February 2014; accepted in final form 9 July 2014

Kelly J, Vanhatalo A, Bailey SJ, Wyile LJ, Tucker C, List S, Wingard PG, Jones AM. Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O\textsubscript{2} uptake dynamics during exercise in hypoxia and normoxia. Am J Physiol Regul Integr Comp Physiol. 2014;307:H8020–H8030. First published July 9, 2014; doi:10.1152/ajpregu.00068.2014. We investigated the effects of dietary nitrate (NO\textsubscript{3}\textsuperscript{-}) supplementation on the concentration of plasma nitrite (NO\textsubscript{2}\textsuperscript{-}) and the rate of nitrite uptake (V\textsubscript{NO2}) during hypoxia and normoxia. We also assessed the effects of dietary nitrate supplementation on the rate of metabolic NO\textsubscript{2}\textsuperscript{-} production via the oxidation of L-arginine, which can be catalyzed by nitric oxide synthase (NOS), but may be blunted in the presence of conditions of reduced availability (52). We now widely accept that NO can also be generated via an alternative pathway, whereby inorganic nitrate (NO\textsubscript{3}\textsuperscript{-}) is reduced to nitrite (NO\textsubscript{2}\textsuperscript{-}) and further to NO. This NO\textsubscript{3}\textsuperscript{-}-dependent NO\textsubscript{3}\textsuperscript{-}NO\textsubscript{2}\textsuperscript{-}NO pathway represents a complementary system for NO synthesis spanning a broad range of redox states (49). In addition to being produced endogenously, the body’s NO\textsubscript{3}\textsuperscript{-} stores can be increased via the diet, with green leafy vegetables and beetroot being particularly rich in NO\textsubscript{3}\textsuperscript{-}. Upon ingestion, inorganic NO\textsubscript{3}\textsuperscript{-} is absorbed into the gut and passes into the systemic circulation where ~25% of it is concentrated in the saliva (47–49). Commensal bacteria in the oral cavity then reduce NO\textsubscript{3}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{-} (21). Some saliva NO\textsubscript{2}\textsuperscript{-} is converted into NO when swallowed into the acidic environment of the stomach (7, while the remainder is absorbed, increasing circulating plasma NO\textsubscript{3}\textsuperscript{-} concentration (50). This NO\textsubscript{3}\textsuperscript{-} may be reduced to NO via a number of enzymatic and nonenzymatic pathways (e.g., xanthine oxidoreductase and deoxyxanthine deaminase), which are potentiated in hypoxic environments, such as may be evident in contracting skeletal muscle (55).

NO plays a key role in the physiological response and adaptation to hypoxia. Reduced fraction of O\textsubscript{2} in inspired air results in reductions in arterial O\textsubscript{2} concentration and intracellular partial pressure of O\textsubscript{2} (P\textsubscript{O2}). The development of muscle hypoxia leads to increased metabolic perturbation (46) and reduced functional capacity at altitude (2) and in several disease conditions (22, 34). To restore sufficient O\textsubscript{2} supply, local blood flow is increased via hypoxia-induced vasodilation with NO being implicated as a major mediator of this process (12). NO\textsubscript{3}\textsuperscript{-} may also promote hypoxic vasodilation in an NO-independent manner (16).

Dietary NO\textsubscript{3}\textsuperscript{-} supplementation, in the form of nitrate rich beetroot juice (BR), represents a practical method of increasing circulating plasma NO\textsubscript{3}\textsuperscript{-} (51, 42, 67) and NO\textsubscript{2}\textsuperscript{-} (4, 33, 62). NO\textsubscript{3}\textsuperscript{-} supplementation has been shown to reduce resting blood pressure (3, 33, 42) and oxygen uptake (V\textsubscript{O2}) during submaximal exercise (4, 39, 40, 41, 62, 67) and to improve exercise performance in young, healthy individuals exercising in normoxia conditions (4, 38), but not necessarily in well-trained athletes (2, 6). These changes may be related to NO-mediated alterations in mitochondrial efficiency (30), muscle contractile function (3, 28), and enhanced muscle blood flow, with preferential distribution to type II fibers (23). These physiological alterations could be particularly beneficial when normal O\textsubscript{2} availability (~21%) is reduced. Indeed, NO\textsubscript{3}\textsuperscript{-} supplementation in the form of BR has recently been shown to...
reduce muscle metabolic perturbation during exercise in hypoxia and to restore constant-work rate exercise tolerance and postexercise indices of oxidative function toward values observed in normoxia (64). BR supplementation has also been shown to improve arterial and skeletal muscle oxygenation and extend incremental exercise tolerance (50), and to enhance cycling economy and time-trial performance (51) in hypoxia. However, while these studies suggest that BR can improve physiological responses and exercise performance in hypoxia, it has yet to be determined whether the effects of BR are more pronounced in hypoxia relative to normoxia.

The dose-response and pharmacodynamic relationships of BR supplementation have recently been investigated in normoxia (67), providing a guide to enable optimal timing and dosing of BR intake to elicit peak circulating plasma [NO3] values. However, the kinetics of plasma [NO3] during hypoxic exercise and subsequent recovery, and possible changes elicited by BR supplementation, are presently unknown. It was recently reported that during high-intensity, intermittent running exercise, plasma [NO3] declined significantly during exhaustive exercise and showed a tendency to recover back to baseline following 15 min of passive rest (68). Previous research has reported increases (1, 54) but, more commonly, decreases (6, 19, 26, 42, 63) in plasma [NO3] during exercise. In addition to exercise, the metabolism of NO and its derivat"ives are known to be influenced by intracellular PO2 and the fraction of inspired oxygen (FIO2). In vitro, endothelial NOS (eNOS) expression and eNOS-derived NO production are reduced in hypoxia (25, 53). However, in vivo, eNOS expression and activity can be upregulated or downregulated by hypoxia, with both decreased (58) and increased (44, 48) NO bioavailability being reported in hypoxia. Characterizing the kinetic changes in [NO3] during exercise and recovery at different FIO2 may offer insight into NO metabolism during exercise in normoxia and hypoxia. This understanding may have important implications for athletes exercising in hypoxic environments.

Considering that the NO3− : NO2− : NO pathway is facilitated in hypoxic conditions (48), we reasoned that BR supplementation may modulate the changes in [NO3] during exercise and recovery and may help to ameliorate the negative effects of hypoxia on exercise tolerance. The primary aim of this study was to investigate the effects of BR supplementation on physiological responses (plasma [NO3] dynamics, pulmonary VO2 and muscle oxygenation) and exercise tolerance, in both normoxia and hypoxia. We hypothesized that the reduction of [NO3] during exercise would be greater in hypoxia compared with normoxia and that [NO2] would be higher at the same iso-time during exercise following BR compared with PL supplementation. We also hypothesized that BR supplementation would improve moderate-intensity exercise economy and severe-intensity exercise tolerance in both hypoxia and normoxia, with greater effects being evident in hypoxia.

METHODS

Subjects

Twelve physically active male subjects (means ± SD; age, 22 ± 4 yr; height, 1.80 ± 0.06 m; body mass, 78 ± 6 kg; VO2 peak = 58.3 ± 6.3 mL·kg⁻¹·min⁻¹) volunteered to take part in this study. The protocol and procedures used in this study were approved by the Institutional Research Ethics Committee. All subjects gave written, fully informed consent prior to commencement of the study, once the experimental protocol, associated risks, and potential benefits of participation had been outlined. Subjects were instructed to arrive at the laboratory at least 3 h postprandial and to avoid strenuous exercise in the 24 h preceding each testing session. Subjects were asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively, and to consume the same light preexercise meal of their choice 4–5 h before testing. In addition to this, subjects were asked to abstain from using antibacterial mouthwash and chewing gum for the duration of the study, since this has been shown to blunt the conversion of NO2 to NO3 in the oral cavity (27). Subjects were also instructed not to eat during the duration of the study. All exercise tests were performed at the same time of day (±1 h) for each subject.

Procedures

Subjects were required to attend the laboratory on six occasions over a 4-wk period. All exercise tests were performed using an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During visit 1, subjects completed an incremental test to exhaustion for the determination of the maximal VO2 uptake (VO2max) and the gas exchange threshold (GET). Subjects performed 5 min of baseline cycling at 20 W and 80 rpm, after which the power output was increased at a rate of 30 W/min in a linear fashion until volitional exhaustion. The height and configuration of the saddle and handlebars were recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas exchange data were collected continuously during the incremental test and averaged over 10-s periods. VO2 was determined as the highest mean VO2 during any 30-s period. The GET was determined from a number of measurements, including 1) the first disproportionate increase in CO2 production (VCO2) from visual inspection of individual data; 2) a decrease in inspired oxygen (VI/VO2) with no increase in V[E/WCO2]; 3) Power outputs representing moderate- and severe-intensity exercise for each individual were calculated, taking into account the mean response time for VO2 during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the power output at GET).

All subjects were familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. Visit 2 served as a familiarization to exercising in normobaric hypoxia. Following completion of the familiarization session, subjects were randomly assigned to receive 3 days of dietary supplementation with 140 μg/day of NO3-rich BR or 140 μg/day of NO2-depleted BR concentrate as a placebo (PL), (see supplementation below), prior to the subsequent exercise trials.

During visits 3–6, the subjects completed step-transition, cycling exercise for the determination of pulmonary VO2 and plasma [NO3] kinetics. In total, there were four different experimental conditions: 1) hypoxia-BO (H-HBR); 2) hypoxia-PL (H-PL); 3) normoxia-BO (N-HBR); and 4) normoxia-PL (N-PL). Trial order was randomly assigned in a balanced fashion, such that three subjects started on H-BO, three started on H-PL, three started on N-BO, and three started on N-PL.

Upon arrival at the laboratory, a cannula (Inoty-W, Becton-Dickinson, Madrid, Spain) was inserted into the subject’s antecubital vein to enable frequent blood sampling before, during, and after the exercise protocol. Prior to the exercise protocol, subjects lay in a supine position for 10 min breathing normoxic inspirate. A further 16-min period elapsed with subjects breathing either the hypoxic or normoxic inspirate. The exercise protocol involved 5 min of moderate-intensity cycling at 80% GET, and one bout of severe-intensity cycling at 75% Δ (a power output representing GET plus 75% of the difference between the power outputs at GET and VO2peak (65), which was continued to volitional exhaustion. Each
exercise bout involved an abrupt transition to the target power output initiated from a 20-W baseline, with the three exercise bouts separated by 6 min of passive recovery. The severe-intensity exercise bout was continued until task failure as a measure of exercise tolerance. The time to exhaustion was recorded when the pedal rate fell by >10 rpm below the 80 rpm pedal rate. In these bouts, the subjects were verbally encouraged to continue for as long as possible. Following exhaustion, a further 10-min recovery period elapsed with subjects continuing to breathe either the hypoxic or normoxic Inspirate.

The VO₂ responses for the two moderate bouts were averaged before analysis to reduce breath-to-breath noise and enhance confidence in the parameters derived from the modeling process. Venous blood was sampled preexercise (prior to any exercise and breathing of experimental Inspirate), then during the baseline 20-W cycling preceding the first moderate transition (Mod1BL) and at 1 (Mod1), 3 (Mod3), and 5 (Mod5) min of the first moderate-intensity exercise bout. Further samples were drawn during the 20-W baseline preceding the severe transition (SevBL) and after 1 (Sev1) and 3 (Sev3) min of severe-intensity exercise and at exhaustion (Exh). Finally, samples were drawn during recovery from the severe bout at 1.5 (Rec1.5), 3 (Rec3), and 10 (Rec10) min.

Inspirate

The inspirate was generated using a Hypoxic HYP 100 filter system (Sporting Edge UK, Basingstoke, UK), with the generator supplying the inspirate via an extension conduit to a 150-liter Douglas Bag (Crankle, Birmingham, UK). This acted as a reservoir and mixing chamber and had a separate outlet tube feeding into a two-way breathing valve system (Hans Rudolph, Cranleb). The two-way valve was connected to the mouthpiece, which provided a constant, unidirectional flow rate and ensured that no rebreathing of expired air occurred. The O₂ and CO₂ concentrations of the inspirate was monitored during each test using a Servomex 5200 High Accuracy Paramagnetic O₂ and CO₂ Analyser (Servomex, Crawley, UK). The gas analyzer was calibrated prior to each test with a 16.0% O₂, 8.0% CO₂, and 76.0% N₂ gas mix (BOC Special Gases, Guildford, UK).

For the N-PL and N-TR trials, the Hypoxic HYP 100 generator was switched to normoxic mode (i.e., all O₂ filters were turned off so that no O₂ was removed from the ambient air). However, during the H-PL and H-TR trials, the generator was set to maximum O₂ filtration, which supplied an O₂ of 0.131 ± 0.002, and an FICO₂ of 0.004 ± 0.000.

Supplementation

After completion of the nonsupplemented visits 1 and 2, subjects were assigned in a double-blind, randomized, crossover design to receive a course of dietary NO₂⁻ placebo supplementation before visits 3–6. The supplements were either concentrated, NO₂⁻ rich BR (2 × 70 ml of BR providing 6.4 mmol NO₂⁻ per day; Beet it, James White Drinks, Ipswich, UK) or concentrated, NO₂⁻ depleted PL (2 × 70 ml of PL providing 0.006 mmol NO₂⁻ per day; Beet it, James White Drinks). The PL beverage was created by passing the juice, before pasteurization, through a column containing Parolite A520E ion-exchange resin, which selectively removes nitrate ions. The PL was identical to the BR in appearance, taste, and smell. Subjects were instructed to consume the beverages in the morning and afternoon of days 1 and 2 of supplementation, and then in the morning and 2.5 h before the exercise test on day 3. A washout period of at least 72 h separated each supplementation period. Subjects were instructed to follow their normal dietary habits throughout the testing period and to replicate their diet and timing of supplementation across conditions. Subjects were informed that the supplementation may cause diarrhea (red urine) and red stools temporarily, but that this side effect was harmless.

Measurements

Venous blood samples were drawn into 5-ml lithium-heparin tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). Two hundred microliters of blood was immediately hemolyzed in 200 μl of cold Triton X-100 buffer solution (Triton X-100; Amresco, Solon, OH) and analyzed to determine blood [lactate] and [glucose] (YSI 2300, Yellow Springs Instruments, OH). Blood samples for the determination of plasma [NO₂⁻] and [NO₃⁻] were collected into lithium-heparin tubes and immediately centrifuged at 4,000 rpm and 4°C for 5 min. Plasma was extracted and immediately frozen at −80°C for later analysis of [NO₂⁻] and [NO₃⁻].

Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO₂⁻. Plasma [NO₂⁻] and [NO₃⁻] were analyzed using gas phase chemiluminescence N Oxidation Analyzer (Sievers NOA 280; Analytik, Durham, UK). The concentrations of NO₂⁻ and NO₃⁻ were determined by plotting signal area (mV) against a calibration plot of 23.5 μM to 1 μM sodium nitrite and 100 nM to 10 μM sodium nitrate, respectively. The rate of change in plasma [NO₂⁻] during the severe exercise bout was calculated as the difference between preexercise baseline and exercise [NO₂⁻] values.

During all laboratory exercise tests, pulmonary gas exchange and ventilation were measured continuously with subjects wearing a nose clip and breathing through a mouthpiece and impeller turbine assembly (Triple V; Jaeger, Höchburg, Germany). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O₂) and infrared (CO₂) analyzers (Oxycon Pro, Jaeger, Höchburg, Germany) via a capillary tube connected to the mouthpiece. Pulmonary gas variables were calculated and displayed breath-by-breath. Heart rate (HR) and arterial oxygen saturation (Sao2) were continuously measured during the test protocol using a pulse oximeter device (Rad-87; Masimo, Irvine, CA), which was attached to the subject’s right index finger.

The oxygenation status of the muscles vastus lateralis of the right leg was monitored via near-infrared spectroscopy (NIIR) (NIRO 200, Hamamatsu Photonics KK, Hamamatsu City, Japan) during the exercise protocol, as described previously (4). Deoxyhemoglobin concentration ([HbO₂]), oxyhemoglobin concentration ([HBO₂]), total hemoglobin concentration ([Hbh₂]), and tissue oxygenation index (TOI) were measured.

Data Analysis

The breath-by-breath VO₂ data from each exercise test were initially examined to exclude errant breaths caused by coughing and swallowing with those values being more than four SDs from the mean being removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values, and, for each individual, identical moderate-intensity repetitions were time-aligned to the start of exercise and ensemble-averaged. This approach enhances the signal-to-noise ratio and improves confidence in the parameters derived from the modeling process. The first 20 s of data after the onset of exercise (the phase 1 response) were deleted, and a nonlinear least squares algorithm was used to fit the data thereafter. A single-exponential model was used to characterize the phase II VO₂.
responses to both moderate- and severe-intensity exercise, as described in the following equation:

\[
\dot{V}_O_2(t) = \dot{V}_O_2\text{baseline} + A_e \left[ 1 - e^{-t/T_d}\right]
\]

where \(\dot{V}_O_2(t)\) represents the absolute \(\dot{V}_O_2\) at a given time \(t\); \(\dot{V}_O_2\text{baseline}\) represents the mean \(\dot{V}_O_2\) over the final 60 s of baseline cycling; \(A_e\), \(T_d\), and \(t\) represent the amplitude, time delay, and time constant, respectively, describing the phase II increase in \(\dot{V}_O_2\) above baseline. An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. The end-exercise \(\dot{V}_O_2\) was defined as the mean \(\dot{V}_O_2\) measured over the final 30 s of exercise.

The fitting strategy was subsequently used to identify the onset of any "slow component" in the \(\dot{V}_O_2\) response to severe-intensity exercise, as previously described (56). The fitting window was lengthened iteratively until the exponential model fit demonstrated a discernible departure from the measured response profile. Identification, via visual inspection, of the flat residual plot profile (signifying a good fit to measured data) systematically differing from zero, gave indication of the delayed slow-component onset. The magnitude of the slow component for \(\dot{V}_O_2\) was measured as the difference between the phase II steady-state amplitude and the final \(\dot{V}_O_2\) value, averaged over the last 30 s of exercise.

To obtain information on muscle oxygenation, the [Hb] response to exercise was also modeled, as described previously (4). The [Hb] kinetics for moderate- and severe-intensity exercise were determined using a single-exponential model similar to that described above (Eq. 1), with the exception that the fitting window commenced at the time at which the [Hb] signal increased 1 SD above the baseline mean (18). For moderate-intensity exercise, the fitting window was constrained to the point at which monoexponentiality became distorted, consequent to a gradual fall in [Hb], as determined by visual inspection of the residual plots. For severe-intensity exercise, the [Hb] fast and slow-phase responses were described as described above for the \(\dot{V}_O_2\). The [HbO2], [Hb], and T01 responses were not modeled as they do not approximate an exponential. Rather, the changes in these variables were assessed by determining the [HbO2], [Hb], and TOI at baseline (60 s preceding stop transition), at 120 s and at end-exercise during moderate exercise and at baseline, 60 s, 120 s, and exhaustion for severe exercise.

Statistical Analyses

Differences in the cardiorespiratory, NIRS-derived, pulse-oximetry and exercise tolerance variables between conditions were analyzed using two-way (supplement × \(\dot{V}_O_2\)) repeated-measures ANOVA. Blood metabolites were analyzed via two-way (condition × time) repeated-measures ANOVA, during moderate-intensity exercise, severe-intensity exercise, and recovery from exercise (condition refers to H-BR, H-PL, N-BR, or N-PL). Significant effects were further explored using simple contrasts with Fisher’s least significant difference test. One-tailed paired t-tests were used to compare differences in exercise tolerance between BR and PL treatments in hypoxia and normoxia. Correlations between physiological and performance variables were assessed via Pearson’s product-moment correlation coefficient. All data are presented as means ± SD with statistical significance being accepted when \(P < 0.05\).

RESULTS

Self-reported compliance to the supplementation regimen was 100% and the subjects’ food diaries confirmed that the timing of supplement taken on the morning of the laboratory tests was consistent across the experimental conditions. No deleterious side effects were reported.

Plasma [NO2] and [NO3]

Preexercise, plasma [NO3] was significantly elevated in H-BR compared with H-PL (90 ± 80 vs. 88 ± 56 nM; \(P = 0.02\)) and N-BR relative to N-PL (N-BR: 401 ± 276 vs. N-PL: 61 ± 28 nM; \(P = 0.01\)) but did not differ between H-BR and N-BR (\(P = 0.54\)) or H-PL and N-PL (\(P = 0.66\)). The group mean kinetic profiles of plasma [NO2] during moderate-intensity and severe-intensity exercise and subsequent recovery are presented in Fig. 1.

Plasma [NO3] was significantly elevated at all time points following BR compared with PL in both hypoxia and normoxia, although no differences were evident in the kinetic response during exercise and recovery (data not shown).

Fig. 1. Plasma [NO2] response during moderate-intensity and severe-intensity exercise and recovery following beetroot juice (BR) and placebo (PL) supplementation in normoxia (N) and hypoxia (H). Group means ± SEI plasma. H-BR was greater than H-PL at each time point, and N-BR was greater than N-PL at each time point. \(P < 0.05\) for N-BR compared with H-BR, \(P < 0.05\) compared with moderate baseline. \(P < 0.05\) compared with severe baseline. Where error bars are not visible, the size of the data point exceeds the error.
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O$_2$ uptake dynamics during exercise in hypoxia and normoxia

Moderate exercise. ANOVA revealed there were significant main effects by condition and time on plasma [NO$_2^-$] during moderate-intensity exercise. BR supplementation significantly elevated plasma [NO$_2^-$] across all time points compared with PL in both hypoxic and normoxic conditions (Δ t < 0.05). In N-BR, plasma [NO$_2^-$] was significantly decreased after 5 min of moderate-intensity exercise (Mod5) compared with ModBL (ModBL: 332 ± 184 vs. Mod5: 250 ± 207 nM; P = 0.04). However, the decrease in plasma [NO$_2^-$] in N-BR in H-Bl only showed a trend toward a reduction (ModBL: 306 ± 109 vs. Mod5: 270 ± 125 nM; P = 0.05) and this decline in plasma [NO$_2^-$] from ModBL to Mod5 was not significantly different in H-Br (-7 ± 12 nM/min) compared with N-BR (-11 ± 16 nM/min), H-PL (-4 ± 6 nM/min) compared with N-PL (-2 ± 4 nM/min), H-Br (-7 ± 12 nM/min) compared with N-PL (-2 ± 4 nM/min) or N-Br (-11 ± 16 nM/min) compared with N-PL (-2 ± 4 nM/min).

Severe exercise. There were significant main effects by condition and time and an interaction effect for plasma [NO$_2^-$] during severe-intensity exercise to exhaustion. BR supplementation significantly elevated plasma [NO$_2^-$] across all time points compared with PL in both hypoxic and normoxic conditions (Δ t < 0.05). In N-BR, plasma [NO$_2^-$] significantly decreased after 3 min of severe-intensity exercise (Sev3) and at exhaustion, compared with SevBL (SevBL: 271 ± 177; Sev3: 260 ± 129; P = 0.01; exhaustion: 122 ± 8 nM; P < 0.01). In H-BR, plasma [NO$_2^-$] decreased from SevBL (277 ± 142 nM; P = 0.01) to Sev3 (177 ± 164 nM; P = 0.03) and exhaustion (171 ± 155 nM; P < 0.01). The absolute decline in plasma [NO$_2^-$] from SevBL to exhaustion showed a trend toward being smaller in H-BR (106 ± 50 nM) compared with N-BR (138 ± 79 nM; P = 0.10). In N-PL, plasma [NO$_2^-$] decreased from SevBL (40 ± 23 nM) to exhaustion (19 ± 18 nM; P = 0.02). This decrease was not significant in H-PL (SevBL: 3 ± 45 nM; vs. exhaustion: 37 ± 45 nM/min; P = 0.52). The rate of decline in plasma [NO$_2^-$] was significantly greater from SevBL to exhaustion in H-BR compared with H-PL (H-BR: −50 ± 22 vs. H-PL: −7 ± 10 nM/min; P < 0.01) and in N-BR compared with N-PL (N-BR: −26 ± 19 vs. N-PL: −1 ± 6 nM/min; P < 0.01) but was not different between N-BR and H-BR (P = 0.68) or N-PL and H-PL (P = 0.13) (Fig. 1).

Recovery. During the 10-min recovery from exhaustive exercise, ANOVA revealed significant main effects by condition and time and an interaction effect for plasma [NO$_2^-$] across all time points compared with PL in both hypoxic and normoxic conditions (Δ t < 0.05). In N-BR, plasma [NO$_2^-$] was lower at exhaustion compared with 3 min into the recovery period (P = 0.05), with a significant difference also evident between Rec1.5 and Rec3 (P = 0.01). Plasma [NO$_2^-$] was significantly higher in H-BR compared with N-BR at Rec1.5 (P = 0.04). In N-PL, recovery of plasma [NO$_2^-$] was evident between exhaustion and Rec10 (P = 0.04), with a significant increase in [NO$_2^-$] from Rec3 to Rec10 also evident (P = 0.04). In H-PL, plasma [NO$_2^-$] tended to recover between Rec1.5 and Rec3 (P = 0.06), with a further increase evident between Rec3 and Rec10 (P < 0.01).

Blood [glucose] was significantly reduced in H-BR compared with N-BR at Rec1.5 (H-BR: 4.3 ± 1.0 mM vs. N-BR: 5.5 ± 1.2 mM; P = 0.01), Rec3 (H-BR: 4.5 ± 1.1 mM vs. N-BR: 5.6 ± 1.3 mM; P = 0.02) and Rec10 (H-BR: 4.7 ± 1.0 mM vs. N-BR: 5.3 ± 1.0 mM; P = 0.03). No differences were evident between PL and BR conditions.

Arterial O$_2$ saturation and heart rate. The SaO$_2$ data at rest and during moderate-intensity and severe-intensity exercise are reported in Table 1. Resting SaO$_2$ and HR prior to the administration of inspire were not significantly different between conditions. However, ANOVA revealed a significant main effect by FIO2 following 10 min of breathing the hypoxic or normoxic inspire, with SaO$_2$ being significantly reduced in H-PL compared with N-PL (P < 0.01) and H-BR compared with N-BR (P < 0.01) in the final 30 s of gas inspiration.

Moderate exercise. During moderate-intensity exercise, SaO$_2$ was significantly reduced in both hypoxic conditions compared with the normoxic conditions (both P < 0.01) (Table 1). HR was significantly elevated in both hypoxic conditions compared with the normoxic conditions in the final 30 s of exercise (both P < 0.01), with H-BR being lower than H-PL (P = 0.05) over the entire 6-min duration.

Severe exercise. SaO$_2$ was significantly lower in H-PL compared with N-PL (P < 0.01) and in H-BR compared with N-BR (P < 0.01) at exhaustion following severe-intensity exercise. There were no differences in SaO$_2$ between BR and N-BR.

Table 1. Arterial oxygen saturation and heart rate during rest and in response to moderate- and severe-intensity exercise

<table>
<thead>
<tr>
<th></th>
<th>N-PL</th>
<th>N-BR</th>
<th>H-PL</th>
<th>H-BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting without inspire</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>98 ± 1</td>
<td>99 ± 1</td>
<td>96 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>99 ± 1</td>
<td>99 ± 1</td>
<td>96 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>10-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>96 ± 4</td>
<td>99 ± 1</td>
<td>94 ± 4</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>62 ± 4</td>
<td>61 ± 1</td>
<td>61 ± 1</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>Resting with inspire</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>99 ± 1</td>
<td>99 ± 1</td>
<td>93 ± 8</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>99 ± 1</td>
<td>99 ± 1</td>
<td>93 ± 8</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>10-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>97 ± 3</td>
<td>98 ± 2</td>
<td>87 ± 4</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>97 ± 3</td>
<td>98 ± 2</td>
<td>87 ± 4</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>6-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>81 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>81 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>10-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>81 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>81 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>6-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>81 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>81 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>10-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>99 ± 1</td>
<td>99 ± 1</td>
<td>93 ± 8</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>99 ± 1</td>
<td>99 ± 1</td>
<td>93 ± 8</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>6-min period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO$_2$ (%)</td>
<td>97 ± 3</td>
<td>98 ± 2</td>
<td>87 ± 4</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>97 ± 3</td>
<td>98 ± 2</td>
<td>87 ± 4</td>
<td>88 ± 4</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. HR, heart rate; N-PL, normoxic placebo; N-BR, normoxic beet root juice; H-PL, hypoxia placebo (NO$_3^-$-depleted beet root juice); H-BR, hypoxia beet root juice; *P < 0.05 compared to H-PL. **P < 0.05 compared to N-BR. †P < 0.05 compared to N-PL.
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O$_2$ uptake dynamics during exercise in hypoxia and normoxia

PL in either hypoxia or normoxia. Also, there were no differences in HR between conditions (Table 1).

$V_\text{O}_2$ Kinetics

Pulmonary $V_\text{O}_2$ responses across the four experimental conditions are presented in Figs. 2 and 3, and the parameters derived from the model fits are summarized in Table 2.

**Moderate exercise.** ANOVA revealed a significant main effect by supplement and an interaction effect on the $V_\text{O}_2$ response to moderate-intensity exercise. The $V_\text{O}_2$ in the final 30 s of exercise in H-BR was significantly lower compared with H-PL ($P = 0.02$) and N-PL ($P = 0.01$). BR supplementation also resulted in a reduced $V_\text{O}_2$ during baseline (20 W) exercise in hypoxia compared with PL ($P = 0.02$). The $V_\text{O}_2$ phase II $\tau$ tended to be increased (i.e., slower kinetics) in hypoxia compared with normoxia ($P = 0.07$). Post hoc analyses revealed that the $V_\text{O}_2$ phase II $\tau$ was smaller (i.e., faster kinetics) in H-BR compared with H-PL ($P = 0.04$).

**Severe exercise.** During severe-intensity exercise, the $V_\text{O}_2$ slow-component amplitude ($P < 0.01$) and $V_\text{O}_2$ at exhaustion ($P < 0.01$) were significantly reduced in both hypoxia inspirate in both PL and BR (Table 2). In hypoxia, BR tended to further reduce the end-exercise $V_\text{O}_2$ compared with H-PL ($P = 0.07$), while BR had no effect upon end-exercise $V_\text{O}_2$ in normoxia.

* NIRS

The [Hb], [HBO$_2$], [Hb$_{sat}$], and TOI values measured during moderate- and severe-intensity exercise are shown in Table 3.

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Pulmonary $O_2$ uptake ($V_\text{O}_2$) responses during a step-increment to a moderate-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles (○), with the PL responses being shown as open circles (●). The dashed vertical line denotes the abrupt "step" transition from baseline to moderate-intensity cycling exercise. Error bars indicate the SE. A: group mean response to moderate-intensity exercise in normoxia ($\sim$ 21% Fio$_2$). B: group mean response to moderate-intensity exercise in hypoxia ($\sim$ 13.2% Fio$_2$). *$P < 0.05$ compared with H-PL.
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O\textsubscript{2} uptake dynamics during exercise in hypoxia and normoxia

P = 0.50). The increase in severe-intensity exercise tolerance was correlated with the reduction in moderate steady-state VO\textsubscript{2} following BR supplementation in hypoxia (r = -0.96; P < 0.01).

**DISCUSSION**

Consistent with previous findings, the decline of plasma [NO\textsubscript{2} \textsuperscript{-}] during exercise was greater following BR compared with PL supplementation. However, in contrast to our experimental hypothesis, the decline of plasma [NO\textsubscript{2} \textsuperscript{-}] during exercise was similar or slightly smaller in hypoxia compared with normoxia. Nonetheless, 3 days of BR supplementation significantly speeded VO\textsubscript{2} kinetics and lowered the steady-state VO\textsubscript{2} during moderate-intensity cycle exercise in hypoxia, but not normoxia. Furthermore, BR supplementation improved severe-intensity exercise tolerance in hypoxia (P < 0.05), but not normoxia (P > 0.05). These findings suggest that BR is more effective at improving exercise economy and exercise tolerance in hypoxia than normoxia.

**Effects of BR Supplementation on the Kinetic Profile of Plasma [NO\textsubscript{2} \textsuperscript{-}]**

Plasma [NO\textsubscript{2} \textsuperscript{-}] increased significantly following BR supplementation compared with PL, at rest and prior to administration of the inspire. These findings are consistent with previous research, which has consistently reported elevations in plasma [NO\textsubscript{2} \textsuperscript{-}] (3, 4, 33, 51, 62, 67), following BR supplementation.

Previous studies have suggested that baseline plasma [NO\textsubscript{2} \textsuperscript{-}] and/or the change in the concentrations of this metabolite during exercise may be associated with exercise performance (19, 53, 61, 68). This study is the first to characterize [NO\textsubscript{2} \textsuperscript{-}] dynamics during and following exercise of different intensities in hypoxia and normoxia with and without NO\textsubscript{2} \textsuperscript{-} supplementation. The results suggest that the metabolism of NO and its derivatives are altered by exercise and NO\textsubscript{2} \textsuperscript{-} supplementation and, to a lesser extent, F\textsubscript{O\textsubscript{2}}. The interpretation of these data is not straightforward, however, NO\textsubscript{2} can be reduced in vivo to bioactive NO\textsubscript{2} and further to NO (47), and this reduction of NO\textsubscript{2} to NO is expected to be facilitated in hypoxia (13). However, NO\textsubscript{2} is also an oxidation product of NO generation via the NOS pathway (30) with NOS [NO\textsubscript{3} \textsuperscript{-}] providing a sensitive marker of NO production through NOS (43). Therefore, the dynamics of plasma [NO\textsubscript{2} \textsuperscript{-}] over the exercise bouts is

```
Table 2. Pulmonary oxygen uptake kinetics in response to moderate- and severe-intensity exercise in hypoxic and normoxic conditions

<table>
<thead>
<tr>
<th></th>
<th>N-PL</th>
<th>N-BR</th>
<th>H-PL</th>
<th>H-BR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO\textsubscript{2}, ml/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1102 ± 156</td>
<td>1090 ± 145</td>
<td>1167 ± 123</td>
<td>1055 ± 135</td>
</tr>
<tr>
<td>End exercise</td>
<td>1970 ± 251</td>
<td>1908 ± 240</td>
<td>2040 ± 247</td>
<td>1905 ± 275</td>
</tr>
<tr>
<td>Phase II, t, s</td>
<td>22 ± 10</td>
<td>17 ± 8</td>
<td>31 ± 11</td>
<td>24 ± 13</td>
</tr>
<tr>
<td>Primary amplitude</td>
<td>886 ± 210</td>
<td>895 ± 256</td>
<td>882 ± 284</td>
<td>849 ± 208</td>
</tr>
<tr>
<td><strong>Severe-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO\textsubscript{2}, ml/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1212 ± 179</td>
<td>1205 ± 158</td>
<td>1244 ± 125</td>
<td>1193 ± 177</td>
</tr>
<tr>
<td>End exercise</td>
<td>4814 ± 470</td>
<td>4721 ± 434</td>
<td>4396 ± 360</td>
<td>3751 ± 249</td>
</tr>
<tr>
<td>Phase II, t, s</td>
<td>30 ± 6</td>
<td>28 ± 9</td>
<td>35 ± 14</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>Primary amplitude</td>
<td>2716 ± 396</td>
<td>2636 ± 486</td>
<td>2450 ± 497</td>
<td>2264 ± 386</td>
</tr>
<tr>
<td>Slow component amplitude</td>
<td>886 ± 235</td>
<td>881 ± 259</td>
<td>902 ± 250</td>
<td>301 ± 274</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. *P < 0.05 compared to H-PL. #P < 0.05 compared to N-PL.

AJP-Regul Integr Comp Physiol • doi:10.1152/ajprega.00668.2014 • www.ajprega.org
```

55
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary $O_2$ uptake dynamics during exercise in hypoxia and normoxia

Table 3. Near-infrared spectroscopy-derived muscle [HbB], [HbO2], [HbD], and TOI dynamics during moderate- and severe-intensity exercise

<table>
<thead>
<tr>
<th></th>
<th>N-PL</th>
<th>N-BR</th>
<th>H-PL</th>
<th>H-BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate-intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[HbB], AU</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.5</td>
<td>6.5</td>
<td>11.5</td>
<td>10.5</td>
</tr>
<tr>
<td>120 s</td>
<td>11.8</td>
<td>11.7</td>
<td>18.8</td>
<td>17.8</td>
</tr>
<tr>
<td>End exercise</td>
<td>12.8</td>
<td>11.7</td>
<td>20.2</td>
<td>18.2</td>
</tr>
<tr>
<td>Time constant, s</td>
<td>23.7</td>
<td>19.2</td>
<td>22.9</td>
<td>23.7</td>
</tr>
<tr>
<td>Amplitude</td>
<td>5.4</td>
<td>6.4</td>
<td>8.5</td>
<td>7.6</td>
</tr>
</tbody>
</table>

| [HbO2], AU           |      |      |      |      |
| Baseline             | 2.6  | 3.6  | 4.5  | 2.7  |
| 120 s                | 1.2  | 2.6  | 2.4  | 2.8  |
| End exercise         | 4.5  | 5.5  | 0.5  | 2.9  |

| [HbD], AU            |      |      |      |      |
| Baseline             | 1.0  | 1.0  | 1.0  | 1.0  |
| 120 s                | 1.0  | 1.0  | 1.0  | 1.0  |
| End exercise         | 1.0  | 1.0  | 1.0  | 1.0  |

| TOI, AU              |      |      |      |      |
| Baseline             | 65.3 | 65.4 | 61.4 | 63.4 |
| 120 s                | 61.5 | 60.6 | 52.5 | 54.6 |
| End exercise         | 62.7 | 61.7 | 52.6 | 54.6 |

Severe-intensity exercise

| [HbB], AU            |      |      |      |      |
| Baseline             | 5.5  | 5.5  | 10.6 | 10.5 |
| 120 s                | 19.3 | 18.3 | 25.2 | 24.3 |
| End exercise         | 22.1 | 21.7 | 23.1 | 21.8 |
| Time constant, s     | 13.5 | 11.5 | 11.5 | 12.6 |
| Primary amplitude    | 14.0 | 14.0 | 14.9 | 14.0 |
| Slow phase amplitude | 3.2  | 3.2  | 2.2  | 2.2  |

| [HbO2], AU           |      |      |      |      |
| Baseline             | 7.2  | 8.7  | 6.3  | 5.8  |
| 120 s                | 4.7  | 3.5  | 9.4  | 10.3 |
| End exercise         | 7.9  | 7.7  | 11.2 | 12.2 |

| [HbD], AU            |      |      |      |      |
| Baseline             | 1.0  | 1.0  | 1.0  | 1.0  |
| 120 s                | 1.0  | 1.0  | 1.0  | 1.0  |
| End exercise         | 1.0  | 1.0  | 1.0  | 1.0  |

| TOI, AU              |      |      |      |      |
| Baseline             | 70.5 | 69.2 | 64.4 | 64.4 |
| 120 s                | 52.2 | 51.1 | 44.9 | 44.9 |
| End exercise         | 48.1 | 47.9 | 41.9 | 41.9 |

Data are presented as means ± SD. Deoxygenated hemoglobin concentration ([HbD], oxygenated hemoglobin concentration ([HbO2]), total hemoglobin concentration ([HbB]), and tissue oxygenation index (TOI) are shown. $p < 0.05$ compared to N-BR, $p < 0.05$ compared to N-PL, AU arbitrary units.

It is likely reflective of the dynamic balance between NOS-derived NO and NO2 reduction to NO. In the present study, plasma $[NO_2^-]$ declined during both moderate- and severe-intensity exercise (Fig. 1) with the magnitude and rate of plasma $[NO_2^-]$ decline being significantly greater in the BR trials compared with PL trials, in both normoxia and hypoxia. These findings suggest that the reduction of NO2 to NO outweighed the synthesis of NO through NOS during exercise.

The rate of plasma $[NO_2^-]$ decline over the 5-min moderate-intensity bout was not significantly different between N-BR and H-BR, and N-PL and H-PL. However, following 5 min of moderate-intensity exercise, plasma $[NO_2^-]$ had fallen significantly below ModBL in N-BR; whereas, there was only a trend for a lower plasma $[NO_2^-]$ in H-BR. Similarly, the rate of plasma $[NO_2^-]$ decline over the severe-intensity exercise bout was not significantly different between N-BR and H-BR or N-PL and H-PL, but the absolute fall in plasma $[NO_2^-]$ tended to be less in H-BR than in N-BR, in spite of a longer exercise duration in N-BR. These results are contrary to our hypothesis and suggest that, in hypoxia, the contribution of NOS to NO production (30), and subsequently to the regulation of muscle perfusion and matching of $O_2$ supply and demand (Ref. 12) during recovery. Following BR supplementation, the recovery profile of plasma $[NO_2^-]$ was slightly different between normoxia and hypoxia. Plasma $[NO_2^-]$ was higher in H-BR than N-BR following 1.5 min of recovery, although the difference between Ehd and 1.5 Srec was not different between conditions. It is important to note that differences in plasma $[NO_2^-]$ dynamics between hypoxia and normoxia were not substantial either during exercise or in recovery.

Effects of BR Supplementation on the Physiological Response to Moderate-Intensity Exercise

BR supplementation significantly reduced the $O_2$ cost of submaximal cycle exercise in hypoxia. $V_{O_2}$ during baseline cycling in H-BR was reduced by 10% compared with H-PL and by 4% compared with N-PL. Furthermore, a 7% reduction in the end-exercise (steady-state) $V_{O_2}$ was found in H-BR compared with H-PL. These findings are consistent with previous studies that have reported reductions in submaximal cycling $V_{O_2}$ in varying severities of hypoxia. For example, Maschelein et al. (50) reported a 4% reduction in steady-state $V_{O_2}$ with an $F_{O_2}$ of 0.11 during cycle exercise at 45% peak $V_{O_2}$, and Muggeridge et al. (51) reported a ~6–8% reduction in steady-state $V_{O_2}$ at an $F_{O_2}$ of 0.15 during cycle exercise at 60% of maximum work rate, following BR supplementation. A reduction in muscle metabolic perturbation (i.e., slower rates of change of muscle pH and phosphate creatine (PCr) and inorganic phosphate concentrations) during severe-intensity knee-extensor exercise in hypoxia has also been reported following BR supplementation (64).

In the present study, the $V_{O_2}$ phase II $\tau$ during moderate-intensity exercise was reduced by BR supplementation in hypoxia. This finding is consistent with a recent study in older individuals, where the $V_{O_2}$ mean response time was speeded with BR supplementation (32). This may be related to the slower $V_{O_2}$ kinetics that is typically found in older individuals and the potential to abate this through enhancing muscle $O_2$ delivery (57), via increasing NO bioavailability. Similarly, hypoxia tended to slow $V_{O_2}$ kinetics in the young healthy participants in the present study. Specifically, the phase II $\tau$ tended to be slowed in hypoxia compared with normoxia (from ~22 to ~31 s; Table 2). This observation is consistent with previous reports of slower $V_{O_2}$ kinetics in hypoxia (29, 59). BR supplementation speeded the phase II $\tau$ in hypoxia toward values recorded in normoxia, thereby helping to reverse the detrimental effect of a reduced $E_{O_2}$ on $V_{O_2}$ kinetics. These findings are consistent with a recent study that showed muscle PCr recovery kinetics, which reflect the maximal rate of mitochondrial ATP resynthesis and are influenced by $O_2$ availability, were speeded by BR supplementation in hypoxia (64).
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary $O_2$ uptake dynamics during exercise in hypoxia and normoxia

These data suggest that, in addition to reducing $O_2$ demand during exercise (50, 51, and present study), BR may enhance skeletal muscle $O_2$ availability in hypoxia. In contrast to some (3, 4, 14, 40, 41, 62), but not all (5, 8, 32, 66), previous studies, 3 days of BR supplementation did not significantly reduce $V_O_2$ during submaximal exercise in normoxia. Previous studies have typically reported reductions in steady state $V_O_2$ of ~3-5% following several days of NO$_3^-$ supplementation (40, 40, 62). The mechanistic bases for this lower $O_2$ cost of exercise have been suggested to include improved mitochondrial efficiency (39) and/or reductions in the ATP cost of muscle force production (3), which may be linked to enhanced $Ca^{2+}$-related muscle contractility (28). NO is involved in the regulation of mitochondrial $O_2$ consumption, and it is well established that NO has a strong affinity for cytochrome-c oxidase (COX) (9). It has been suggested that competition for the COX binding site between NO and $O_2$ may be responsible, in part, for the reduced $O_2$ cost of exercise following NO$_3^-$ supplementation (40, 41), with this initiating a signaling cascade resulting in mitochondrial protein changes, which collectively enhance respiratory chain efficiency (39).

Interestingly, hypoxia, per se, may also result in an acute, reversible inhibition of COX (10). The combination of hypoxia and BR supplementation may, therefore, make it more likely for these effects to be manifest. It is also noteworthy that reductions in $V_O_2$ during moderate-intensity exercise were recently reported to be evident following acute supplementation (8 mmol NO$_3^-$ (4 × 70 ml BR shots), tended to be evident with 8.4 mmol NO$_3^-$ (2 × 70 ml BR shots), but were not evident with 4.2 mmol NO$_3^-$ (1 × 70 ml BR shot) (67). It is, therefore, possible that an insufficient NO$_3^-$ dose was consumed immediately prior to the tests to significantly influence the $V_O_2$ response to exercise in normoxia in the present study. Furthermore, the individual differences in the $V_O_2$ response to exercise in normoxia evident in the current study, may have also contributed to the lack of statistically significant effects. It may be concluded that BR supplementation can (3, 4, 14, 40, 41, 62), but does not always (present study, 5, 8, 32, 66), alter the $O_2$ cost of exercise in normoxia.

Indices of muscle oxygenation measured with NIRS were altered as a result of the manipulation of $P_O_2$ during moderate-intensity exercise but BR supplementation did not significantly influence this response. Consistent with a previous study (50), [HbO$_2$] was greater in hypoxia indicating that muscle fractional $O_2$ extraction was increased, while [HbO$_2$] and TOI were significantly reduced in hypoxia compared with normoxia. Although not significant, BR supplementation tended to ameliorate the negative effects of hypoxia upon TOI during moderate-intensity exercise in the current study (a 5.6% increase in TOI), in a similar fashion to that reported by Masschelein et al. (50) (a 4% increase in TOI). These effects are consistent with observations that the arterial-venous [NO$_3^-$] difference is associated with limb vasodilation and increased skeletal muscle blood flow during exercise performed in hypoxia (20). The trend for an improved TOI with BR supplementation indicates better muscle oxygenation (24), which may have been responsible for the speeding of the VO$_2$ kinetics observed in hypoxia. Consistent with a possible improvement in oxygenation status, the typical compensatory rise in HR in hypoxia was attenuated by BR compared with PL during moderate-intensity exercise. Specifically, HR was 5–6 beats/min lower in the H-BR compared with the H-PL condition. There were no differences between H-BR and H-PL in indices of muscle oxygenation or HR during severe-intensity exercise.

Whether the reduction in cardiac work (lower HR) and metabolic requirement (lower $V_O_2$) with BR observed in the present study might translate into enhanced performance during prolonged low-intensity exercise at altitude remains to be determined. Furthermore, older age and a number of disease conditions, including peripheral arterial disease, diabetes, COPD, and anemia, are associated with tissue hypoxia. A reduced $O_2$ cost of moderate-intensity exercise (i.e., walking) and reduced muscle metabolic perturbation during physical activity may improve the quality of life in individuals with these diseases (34, 64). However, further research is required to explore the effects of BR supplementation on health and functional capacity in patient populations.

**Effects of BR Supplementation on the Physiological Response to Severe-Intensity Exercise**

The end-exercise $V_O_2$ was significantly reduced during severe-intensity exercise in hypoxia compared with normoxia. Moreover, [HbO$_2$] and TOI of the m. vastus lateralis were significantly reduced, while [HbO$_2$] and HR were significantly increased in hypoxia compared with normoxia, consistent with previous findings (50). There was a trend toward a reduction in end-exercise $V_O_2$ with BR compared with PL supplementation in hypoxia of ~6%. This finding indicates the $V_O_2$peak may be reduced by NO$_3^-$ supplementation and is consistent with some (6, 42) but not all previous studies (4, 33, 62) conducted in normoxia.

Tolerance to severe-intensity cycle exercise in hypoxia in the present study was significantly improved (9%, $P < 0.05$) following BR supplementation. This finding is consistent with earlier studies that reported that BR supplementation increased exercise tolerance during constant-work-rate (64) and incremental (50) exercise protocols and enhanced cycling time-trial performance (51) in hypoxia. However, in contrast to previous findings (3, 4, 8, 33, 37), we found no effect of BR supplementation on exercise tolerance in normoxia. An interesting observation in the present study was the significant correlation between the reduction in steady-state $V_O_2$ and the improvement in exercise tolerance following BR supplementation in hypoxia ($r = -0.96$). Therefore, the lack of effect on $V_O_2$ during submaximal exercise in normoxia following BR supplementation may explain the lack of effect on exercise tolerance. Further research is required to address the physiological bases for responders and nonresponders to dietary nitrate supplementation.

**Perspectives and Significance**

This study provides the first description of the influence of $P_O_2$ and BR supplementation on plasma [NO$_3^-$] dynamics during moderate- and severe-intensity exercise and subsequent recovery in humans. The greater rate of decline of plasma [NO$_3^-$] during exercise following BR compared with PL supplementation suggests that elevating plasma [NO$_3^-$] prior to exercise may promote NO production through the nitrate-nitrite-NO pathway. In hypoxia, but not normoxia, BR supplementation reduced the $O_2$ cost of moderate-intensity exercise, speeded VO$_2$ kinetics, and improved severe-intensity exercise.
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary \( \text{O}_2 \) uptake dynamics during exercise in hypoxia and normoxia

Tolerance. These findings may have important implications for individuals exercising at altitude.

ACKNOWLEDGMENTS

We thank Sarah Jackman, Sinéad McDougall, Matthew Black, and Jamie Blackwell for technical support, and Boet Il Ltd. for providing the beverages used in this study, grants.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

11. Casey DF, Maden BD, Curry TJ, Eisenach JH, Wilkins BW, Joiner MA. 
Chapter 5: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary $O_2$ uptake dynamics during exercise in hypoxia and normoxia
Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults

James Kelly, Jonathan Fulford, Anni Vanhatalo, Jamie R. Blackwell, Olivia French, Stephen J. Bailey, Mark Gilchrist, Paul G. Winyard, and Andrew M. Jones

1Sport and Health Sciences, College of Life and Environmental Sciences; 2Peninsula National Institute for Health Research Clinical Research Facility, Peninsula Medical School; and 3Peninsula Medical School, University of Exeter, St. Luke’s Campus, Exeter, United Kingdom

Submitted 5 September 2012; accepted in final form 18 November 2012

Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults. Am J Physiol Regul Integr Comp Physiol. 304: R73–R83, 2013. First published November 21, 2012; doi:10.1152/ajpregu.00406.2012.—Dietary nitrate ($NO_3^−$) supplementation has been shown to reduce resting blood pressure and alter the physiological response to exercise in young adults. We investigated whether these effects might also be evident in older adults. In a double-blind, randomized, crossover study, 12 healthy, older (60–70 yr) adults supplemented their diet for 3 days with either nitrate-rich concentrated beetroot juice (BR; $2\times 70$ m/d, $−9.6$ mm/d $NO_3^−$) or a nitrate-depleted beetroot juice placebo (PL; $2\times 70$ m/d; $−0.01$ mm/d $NO_3^−$). Before and after the intervention periods, resting blood pressure and plasma [$nitrite$] were measured, and subjects completed a battery of physiological and cognitive tests. Nitrate supplementation significantly increased plasma [$nitrite$] and reduced resting systolic (BR: $115\pm 9$ vs. PL: $120\pm 6$ mmHg; $P<0.05$) and diastolic (BR: $70\pm 5$ vs. PL: $73\pm 5$ mmHg; $P<0.05$) blood pressure. Nitrate supplementation resulted in a speeding of the $V_{O2}$ mean response time (BR: $25\pm 7$ vs. PL: $28\pm 7$ s; $P<0.05$) in the transition from standing rest to treadmill walking, although in contrast to our hypothesis, the $O_{2}$ cost of exercise remained unchanged. Functional capacity (6-min walk test), the muscle metabolic response to low-intensity exercise, brain metabolite concentrations, and cognitive function were also not altered. Dietary nitrate supplementation reduced resting blood pressure and improved $V_{O2}$ kinetics during treadmill walking in healthy older adults but did not improve walking or cognitive performance. These results may have implications for the enhancement of cardiovascular health in older age.

nitrate; nitrite; nitric oxide; blood pressure; exercise performance; cognitive performance; $O_2$ uptake kinetics

The beneficial effects of a vegetable-rich diet upon cardiovascular health (27) and longevity (79) have been well described. These positive effects have been attributed, in part, to inorganic nitrate ($NO_3^−$), which is particularly rich in leafy greens and beetroot. The $NO_3^−$ anion itself is inert, and any biological effects are likely to be the result of its conversion to the nitrite anion ($NO_2^−$) in the mouth via facultative anaerobic bacteria on the surface of the tongue (25). When swallowed, $NO_2^−$ can be further converted into nitric oxide ($NO$) (9), but it is clear that some $NO_2^−$ enters the circulation. The subsequent reduction of $NO_2^−$ to $NO$ and other reactive nitrogen intermediates is facilitated in hypoxia (11). The production of NO via nitric oxide synthase (NOS) is impaired in hypoxia and, thus, it has been proposed that the $NO_3^−$-$NO_2^−$-NO pathway represents a complementary system for NO generation across a wide range of redox states (53). NO is an essential physiological signaling molecule with numerous functions in the body, including the regulation of blood flow, muscle contractility, glucose and calcium homeostasis, and mitochondrial respiration and biogenesis (17, 21, 70).

There is now substantial evidence that dietary NO supplementation, either in the form of sodium nitrate (NaNO_3) or beetroot juice, can significantly increase plasma [$NO_3^−$] and reduce resting blood pressure in young adults (3, 49, 76, 81). Moreover, dietary NO supplementation may have positive effects upon the physiological response to exercise (5, 50). Supplementation with NaNO_3 (0.1 mmol·kg$^{-1}$·day$^{-1}$, Ref. 50) or beetroot juice (0.5 l/d, containing 5.5 mmol/day of NO$^−$; Ref. 4) resulted in a significant reduction in oxygen uptake ($V_{O2}$) during submaximal cycling. In a recent placebo-controlled study, we reported that beetroot juice supplementation significantly reduced the $O_{2}$ cost of treadmill walking and improved exercise tolerance in healthy young adults (47). These results are remarkable because the $V_{O2}$-work rate relationship has traditionally been considered to be independent of age, health status, and aerobic fitness (36). The reduction in the $O_{2}$ cost of moderate-intensity exercise following dietary NO$^−$ supplementation may be a result of a reduced ATP cost of muscle force production (5) and/or enhanced mitochondrial efficiency (51).

The availability of the NOS substrate L-arginine, and especially the NOS cofactor tetrahydrobiopterin, is lower in older age (23), which together with lower plasma [$NO_3^−$] (68), a sensitive marker of NOS activity (42), suggests that NO synthesis through the NOS-NO pathway might be impaired with the process of aging. In addition, superoxide ($O_2^−$) production is increased with aging, which would also be expected to lower NO bioavailability, given the rapid reaction between $O_2^−$ and NO to form peroxynitrite (37). Given the positive association between NO and vascular health (34), these aging-related perturbations to NO metabolism might contribute toward the endothelial dysfunction (46, 52) and arterial hypertension (26) that develop with old age. Therefore, it is feasible that dietary NO$^−$ supplementation might enhance NO bioavailability and vascular function in older adults.

The aging process is associated with a number of functional and structural changes to the cardiovascular and muscular systems that may perturb O$2$ delivery and utilization. For instance, the ability to increase cardiac output (45) and skeletal
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults

During exercise is attenuated with increasing age. Moreover, the distribution of blood flow in the microcirculation, capillary density, and capillary hemodynamics (7, 18, 30, 59, 60, 65), as well as mitochondrial volume density and oxidative function (15, 16) are compromised with aging. There is evidence that $V_{O_2}$ kinetics in the transition from a lower to a higher metabolic rate is slowed in older compared with younger adults (3, 14, 22) and that this may be related to a limitation in muscle $O_2$ delivery (66). The reduction in maximal oxidative phosphorylation capacity in aged muscle (15, 16, 28) might also contribute toward the slower $V_{O_2}$ kinetics. Since dietary NO$_3^-$ supplementation has been shown to increase muscle blood flow (19) and the maximal rate of oxidative ATP production (51), it is possible that dietary NO$_3^-$ supplementation might speed $V_{O_2}$ kinetics in older adults. Faster $V_{O_2}$ kinetics would be expected to reduce metabolic perturbation and fatigue development in the transition from a lower to a higher metabolic rate and may, thus, enhance exercise tolerance. The influence of NO$_3^-$ supplementation on $V_{O_2}$ kinetics in older adults has yet to be determined.

Increased NO bioavailability might also enhance brain blood flow and cognitive function in older age. In addition to brain shrinkage in senescence (71), the capacity of the brain to produce ATP via oxidative phosphorylation decreases (10) and, in combination with chronic ischemia of white matter (63), this results in a decline of cognitive function. Furthermore, age-related mitochondrial dysfunction has been associated with the neuronal loss, which is a feature of neurodegenerative diseases (13). Recent studies suggest that NO plays a key role in cerebral vasodilation and blood flow (64), neurotransmission, and the coupling of neural activity to local cerebral blood flow (62). Therefore, dietary NO$_3^-$ supplementation may have the potential to modify cerebrovascular physiology and enhance cognitive function. Indeed, Presley et al. (63) recently reported that dietary nitrate improves regional white matter perfusion in older adults in areas of the brain that are involved in executive functioning and speculated that this may offset the influence of aging on cognitive decline and dementia (32).

The purpose of the present study, therefore, was to assess whether the physiological effects of dietary NO$_3^-$ supplementation reported previously in young adults are also evident in older adults. An additional purpose was to use $^1$H magnetic resonance spectroscopy (MRS) brain-scanning techniques to investigate whether NO$_3^-$ supplementation can influence concentrations of key metabolites in the brain, which have been strongly related to cognitive health and whether this translates into improved cognitive function. We hypothesized that dietary supplementation with NO$_3^-$-rich beetroot juice would reduce resting blood pressure, speed $V_O_2$ kinetics, and lower the $O_2$ cost of treadmill walking, and improve functional capacity and cognitive function in healthy older adults.

METHODS

Subjects

Twelve older adults (six male and six female) volunteered to participate in this study (mean ± SD; males: age 64 ± 4 yr, height 175 ± 6 cm, body mass 71 ± 9 kg; females: age 63 ± 2 yr, height 163 ± 6 cm, body mass 67 ± 14 kg). All subjects were ostensibly healthy and were not taking medication. None of the subjects was a tobacco smoker or user of dietary supplements. Subjects were screened prior to participation to ensure their suitability for the study. The study was approved by the Institutional Research Ethics Committee. All subjects gave their written, informed consent before the commencement of the study, once the experimental procedures, associated risks, and potential benefits of participation had been described. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Subjects were also asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively. All tests were performed at approximately the same time of day (±2 h) for each subject.

Procedures

Subjects were required to attend the laboratory on six occasions over a 6-wk period. During visit 1, subjects provided a venous blood sample for determination of plasma NO$_3^-$, and resting blood pressure (BP) was measured. The subjects then completed a submaximal ramp incremental treadmill exercise test to determine gas exchange threshold (GET). All treadmill tests were performed in a well-ventilated laboratory at 20–22°C on a flat-belt treadmill (PPS-55 Sport, Woodway, Weil am Rhein, Germany) set at a 1% grade (35). Initially, subjects completed 3 min of baseline walking exercise at 1 km/h, after which the belt speed was increased by 1 km/h every minute. Subjects were instructed to exercise until they were breathing heavily, the exercise became challenging, or that the treadmill speed was uncomfortably fast for them to continue. Alternatively, if the subject's heart rate (HR) reached a predetermined value (80% of age-predicted maximum), the exercise test was terminated. The breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental test and averaged over consecutive 10-s periods. The GET was determined from a cluster of measurements, including 1) the first disproportionate increase in CO$_2$ production ($V_{CO_2}$) from visual inspection of individual plots of $V_{CO_2}$ vs. $V_O_2$, 2) an increase in expired ventilation ($V_{E}/V_{O_2}$ with no increase in $V_{E}/V_{CO_2}$, and 3) an increase in end-tidal $O_2$ tension with no fall in end-tidal CO$_2$ tension. Subsequently, treadmill speeds that would require 80% of the GET (moderate-intensity exercise) were calculated, with account taken of the mean response time for $V_O_2$ during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the treadmill speed at GET). During visit 1, subjects were also given a cognitive training session to familiarize them with the process, format, and required responses to all computer-based cognitive tests that were to be utilized during the study. Following this, subjects were assigned in a double-blind, randomized, crossover design to consume 140 ml/day of NO$_3^-$-rich BR or NO$_3^-$-depleted beetroot juice (PL) for 2.5 days prior to each of their subsequent laboratory visits. The subjects were instructed to follow their normal dietary habits throughout the experimental period and asked to record and replicate their diet as closely as possible between conditions during each of the 2.5-day supplementation period. Subjects were also requested to abstain from using antibacterial mouthwash and chewing gum throughout the study since this can markedly reduce the concentration of oral bacteria responsible for the reduction of NO$_3^-$ to NO$_2^-$ (29).

During visits 2 and 3, venous blood samples were drawn, and resting BP was measured. The subjects were then asked to complete step-transition, walking exercise tests on a treadmill for the determination of pulmonary V$O_2$ dynamics. The protocol involved two 6-min bouts of moderate-intensity walking (at 80% GET). Each exercise bout involved an abrupt transition to the target speed initiated from a slow walking baseline (1 km/h), with the two exercise bouts separated by 10 min of passive recovery. Following the step-exercise tests, 10 min of passive recovery was taken before the completion of a 6-min walk test (6MWT) to assess functional capacity. The 6MWT was completed following the appropriate guidelines and standardizations, as suggested in the American Thoracic Society Statement: Guidelines
for the 6MWT (2) with total distance covered being recorded. The test was completed on a straight, flat track. Both the subject and the researcher were blind as to which supplement was being tested, and any encouragement during the test was standardized. HR was recorded throughout both the treadmill exercise tests and the 6MWT. After a further 10-min passive recovery, subjects were asked to complete a number of computer-based cognitive function tests, which assessed the impact of the supplementation on the speed and accuracy of cognitively demanding tasks. There were three cognitive tests in total.

**Serial subtractions.** The original verbal Serial Sevens subtraction test has been employed in a number of other studies and is included as part of the Mini-Mental State Examination for dementia screening. The current study utilized a modified, 4-min, computerized version of the serial subtraction task (67), which was made up of 2 min of serial 3s followed by 2 min of serial 7s subtractions. Before each 2-min section, a standard instruction screen requested the subject to count backward in 3s or 7s, as quickly and as accurately as possible, using the keyboard’s linear number key to enter each response. The instructions also made it clear to subjects that if they were to make a mistake they should carry on subtracting from the new incorrect number. A random starting number between 800 and 999 was presented on the computer screen, which was cleared by the entry of the first response. Subsequently each three-digit response was represented on the screen by three asterisks. Performance data (total number of subtractions and number of errors) were calculated for the serial 3s and 7s responses separately. In the case of an incorrect response, subsequent responses were scored as positive if they were correct in relation to the new number.

**Rapid visual information processing.** This task has been used previously to study the cognitive effects of psychotropic drugs. The subject was asked to monitor a continuous series of single digits to identify targets of three consecutive odd or three consecutive even digits. The digits were presented on the computer screen at a rate of 100/min in pseudo-random order, and the participant was required to respond to the detection of a target string by pressing the space bar as quickly as possible. The task was continuous and lasted 5 min in total, with 5 correct target strings being presented per minute. The subjects were scored for the number of target strings correctly detected, average reaction time (ms) for correct detections, and number of false alarms.

**Number recall.** The subjects were initially presented with a three-digit number on the screen and given 3 s to learn the number. The number was then removed, and subjects were prompted to recall the number verbally to the researcher in either forward or backward form. After 12 three-digit numbers, the subject was presented with 12 four-digit numbers, then 12 five-digit numbers, and so on. The time given to subjects to learn the number increased in a linear fashion, on the order of one additional second per one additional number. The task was terminated when the subject gave three consecutive incorrect backward responses and three consecutive incorrect forward responses. Subjects were scored for number of correct forward responses, number of correct backward responses and given a combined total.

**Visit 4** was performed with no supplementation and acted as a familiarization session for subjects to the exercise protocols that were to be performed in **visit 5** and **6**.

During visits 5 and 6, subjects were required to complete a single-leg, knee-extension exercise test while lying prone in the bore of a 1.5 T superconducting MR scanner (Phillips Gyroscan Clinical Interan). Subjects were familiarized with the dimensions of the scanner and the knee-extension exercise in a purpose-built mock scanner during visit 4. The exercise protocol consisted of unilateral knee extensions with the right leg using a custom-built non-ferromagnetic ergometer. The foot was fastened securely with Velcro straps to a padded foot brace, which was connected to the ergometer load basket via a simple rope and pulley system. Knee extensions over −0.22 m were completed at a constant frequency, set in unison with the magnetic pulse sequence (40 pulses/min), to ensure the quadriceps muscles were positioned in the same phase of contraction during each MR pulse acquisition. The subjects were visually and audibly cued via a display consisting of two vertical bars, one that moved at a constant frequency of 0.67 Hz and one that monitored foot movement via a sensor in the ergometer pulley system. Because we used a pulse-acquire sequence during the exercise protocol that was pulse acquired, the signal originates from the muscle and is, therefore, relatively insensitive to a subject’s movement. Even so, to prevent displacement of the quadriceps relative to the MRS coil during the exercise, Velcro straps were fastened over the subject’s legs, hips, and lower back.

Following an initial 2-min rest period, subjects performed a 4-min low-intensity exercise bout to assess the muscle metabolic response. This bout was repeated after a 6-min rest period. A further 4-min rest period was followed by two bouts of high-intensity exercise of 24-s duration, which were separated by a 4-min rest period. The intensity of these 24-s exercise bouts was carefully selected to ensure a significant depletion of muscle [PCr] without a significant reduction of pH relative to baseline values. Following the exercise, participants were asked to lie still in a supine position in the bore of the scanner for ~45 min, with their head comfortably secured within an 8-channel SENSE head coil. 1H MRS brain measurements of N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), myo-inositol (mi) concentrations and apparent diffusion coefficients (ADC) of both white and gray matter were recorded.

**Supplementation Protocol**

After completion of the nonsupplemented visit 1, subjects were assigned in a double-blind, randomized, crossover design to receive 2.5 days of dietary supplementation prior to visits 2, 3, 5, and 6. The supplements were either concentrated NO3-rich BR (2 × 70 ml/day, organic beetroot juice, each containing ~4.8 mmol NO3, Beet it, James White Drinks, Ipswich, UK) or NO3-depleted PL (2 × 70 ml/day, organic beetroot juice containing ~0.01 mmol NO3, Beet it, James White Drinks, Ipswich, UK). The PL beverage was created by passage of the juice, before pasteurization, through a column containing Purafil AE520E ion exchange resin, which selectively removes NO3 ions (47). The PL was similar to the BR in appearance, taste, and smell. Subjects were instructed to consume one of the 70-ml beverages in the morning and the other in the afternoon of day 1 and 2 of supplementation, and then in the morning and 2.5 h prior to their visit on day 3 of supplementation. At least 72 h washout period separated each supplementation period, and subjects were instructed to maintain their normal daily activities and food intake throughout the study. Subjects were warned that supplementation may cause bacteria (red urine) and red stools temporarily but that this side effect was harmless.

**Measurements**

Prior to each testing session, blood pressure of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa, FL). Subjects were seated at rest for 10 min prior to the measurements. A total of four measurements were recorded, with the mean of the final three measurements being calculated. Mean arterial pressure (MAP) was calculated as $1/3 \times$ systolic pressure + $2/3 \times$ diastolic pressure. The mean of the systolic, diastolic, and MAP measurements made in the two BR- and PL-supplemented sessions (treadmill walking exercise session and MR scanner session) was calculated.

Plasma [NO3] was used as a biomarker for NO availability (42, 52). To obtain plasma [NO3], venous blood samples (~4 ml) were drawn into lithium-heparin tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Within 3 min of collection, samples were centrifuged at 4,000 rpm and 4°C for 10 min. Plasma was extracted and immediately frozen at −80°C for later analysis of [NO3]. Prior to, and regularly during analysis, all glassware, utensils, and surfaces
were rinsed with deionized water to remove any residual NO$_3^-$. After plasma samples were thawed at room temperature, they were initially deproteinized using cold ethanol precipitation. The ethanol was chilled to 0°C, and then 0.4 ml of cooled ethanol was combined with 0.2 ml of plasma. Samples were then vortexed and centrifuged at 14,000 rpm for 5 min, with the supernatant being removed. The [NO$_3^-$] of the deproteinized plasma samples was determined using a modification of the chemiluminescence technique (4).

During all laboratory exercise tests, pulmonary gas exchange and ventilation were measured continuously with subjects wearing a nose clip and breathing through a mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O$_2$) and infrared (CO$_2$) analyzers (Oxycon Pro, Jaeger, Hoechberg, Germany) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a three-liter syringe (Hans Rudolph, Kansas City, MO). Pulmonary gas exchange variables were calculated and displayed breath-by-breath. HR was measured using short-range radio-telemetry (model 610; Polar Electro Oy, Kempele, Finland).

During the MRS exercise measurements, subjects lay in the prone position, inside a whole body scanner. A 6-cm $^{13}$P transmitt/receive surface coil was placed within the subject bed in a way that it was centered over the quadriceps muscle of the right leg. Initially, fast-field echo images were acquired to determine whether the muscle was correctly positioned in relation to the coil. This was aided by the placement of cod liver oil capsules (yielding high-intensity signal points within the image) adjacent to the coil, enabling its orientation relative to the muscle volume under examination to be assessed. A number of preacquisition procedures were performed to optimize the signal from the muscle. Tuning and matching of the coil were carried out, enabling maximal energy transfer between the coil and muscle. An automatic shimming protocol was undertaken within a volume that defines the quadriceps, enhancing homogeneity of the local magnetic field. Throughout all exercise and rest periods, data were acquired every 1.5 s with a spectral width of 1,500 Hz and 1,000 data points. Phase cycling with four phase cycles was employed, which led to the acquisition of a spectrum every 6 s. The resulting spectra were quantified using peak fitting, assuming prior knowledge, using the JMRUI (version 3) software package (61) employing the Advanced Method for Accurate, Robust, and Efficient Spectra (AMARES) fitting algorithm (75). Spectra were fitted assuming the presence of the following peaks: $P_j$ phosphodiester, PC, α-ATP (2 peaks, amplitude ratio 1:1), γ-ATP (2 peaks, amplitude ratio 1:1), and β-ATP (3 peaks, amplitude ratio 1:2:1).

Absolute metabolite values were established via a technique similar to that described previously (40). Prior to the exercise protocols, spatially localized spectroscopy was undertaken to determine the relative signal intensities obtained from a phosphoric acid source and $P_j$ from the subject’s right quadriceps. A subsequent unsaturated scan was used to compare the signals obtained from the phosphoric acid standard with an external $P_j$ solution, where the localized volume sampled within the muscle was the same dimensions and distance from the coil as the $P_j$ solution. This allowed the calculation of muscle $P_j$ concentration, following corrections for relative coil loading. Subsequently, absolute values of [PCr] and ATP concentrations were calculated via the ratio of PCr to P$_j$ and P$_j$ to ATP. Intracellular pH was calculated using the chemical shift of the $P_j$ spectral peak relative to the PCr peak (72). In addition to this, ADP concentration ([ADP]) was calculated as described previously (39).

$^1$H MRS was performed using an eight-channel SENSE head coil in the left frontal white matter and the occipital-parietal gray matter in single voxels of $2 \times 2 \times 2$ cm$^3$. Following automated shimming and pulse angle determination, a point-resolved spectroscopy (PRESS) sequence was undertaken with an echo time of 33 ms and a repetition time (TR) of 2,000 ms with 512 samples acquired and a bandwidth of 1,000 Hz. In each region, the sequence was repeated twice, once with, and once without, water suppression. For the water suppression sequence, an excitation pulse was applied at the water frequency with an 80-Hz window, prior to the PRESS sequence, which consisted of 128 repetitions averaged together with 16 phase cycles. For the nonwater-suppressed sequence, no pulse was applied and 32 repetitions were averaged with 16 phase cycles. Quantification was undertaken in JMRUI (ver. 3) employing the AMARES fitting algorithm (75). For the water-suppressed sequence, the residual water peak was removed via a Hankel Lanczos Singular Values Decomposition filter prior to peak fitting, from which the areas of the NAA, Cr, CH$_3$, and ml peaks were calculated. Subsequently, once a correction had been made for the relative number of averages employed in the water-suppressed and nonwater-suppressed sequences, ratios of NAA: water, Cho:water, Cr:water, ml:water, NAA:Cr, and NAA: (Cr + CH)$_3$ were calculated. In addition to this, diffusion images were acquired using an eight-channel SENSE head coil with a single-shot echo-planar imaging sequence with 15 directions and b values of 0 and 800 s/mm$^2$. Images were acquired at an axial-oblique orientation with a TR of 11,000 ms, an echo time of 66 ms, an in-plane resolution of $2 \times 2$ mm$^2$, and a slice thickness of 2 mm. Regions of interest were selected in the anterior cingulated gyrus, the dorsolateral prefrontal cortex, and the subcortical white matter of the frontal lobe, and ADC were calculated using the $b = 0$ and isotropic $b = 800$ s/mm$^2$ images, such that $\text{ADC} = - (1/800) \ln (SNR)$, where $S$ is the signal intensity in the selected ROI for the $b = 800$ s/mm$^2$, and $SNR$ is the image intensity for the corresponding $b = 0$ image.

**Data Analysis**

**Oxygen uptake.** The breath-by-breath VO$_2$ data from each test were initially examined to exclude errant breaths caused by coughing and swallowing, with these values lying more than 4 SDs from the local mean being removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values, and, for each individual, identical repetitions were time-aligned to the start of exercise and ensemble-averaged. This approach enhances the signal-to-noise ratio and improves confidence in the parameters derived from the modeling process (82). A nonlinear least-squares algorithm was applied to the data. With only two transitions and a relatively low-response amplitude, however, we elected to describe the overall VO$_2$ kinetics using the mean response time (MRT), which was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise. An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. We then calculated the oxygen deficit (OSD) as the product of the VO$_2$ response amplitude (baseline to exercise steady-state) and the MRT. VO$_2$baseline was defined as the mean VO$_2$ measured over the final 90 s of the baseline period. The end-exercise VO$_2$ was defined as the mean VO$_2$ measured over the final 30 s of exercise.

The mean baseline VO$_2$, V$\text{O}_2$, and respiratory exchange ratio (RER) values were calculated over the final 60 s preceding the start of exercise, and the mean end-exercise values were calculated over the final 30 s of exercise.

**Muscle Metabolites**

**Low intensity.** To enhance the signal-to-noise ratio of the [PCr], [Pj], [ADP], and [pH] responses, individual subject transitions to low-intensity exercise were time-aligned to the onset of exercise (0 s), averaged, and interpolated generating a single, second-by-second response.

**High intensity.** To describe the rate of PCr recovery, a time constant was determined by fitting a single-exponential function to the [PCr] measured after the 24-s exercise bout.

---

Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, O$_2$ uptake kinetics, and muscle and cognitive function in older adults
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults

Statistical Analyses

Differences in plasma [NO$_3^-$], BP, exercise performance, and cardio-respiratory, and muscle metabolic, cognitive function, and brain metabolic responses between the conditions were analyzed with two-tailed, paired-samples t-tests, with statistical significance being accepted when $P < 0.05$. Values are expressed as means ± SD.

RESULTS

Twelve participants completed all blood sample, walking exercise, leg extension exercise, and cognitive test sessions. Of the 12 $^{31}$P-MRS data sets, 10 were of suitable quality to include in subsequent data analysis. Ten participants completed the $^1$H-MRS brain scans.

Plasma [NO$_3^-$] and BP

PL supplementation resulted in no significant change in plasma [NO$_3^-$] relative to the nonsupplemented control condition. In contrast, BR supplementation elevated plasma [NO$_3^-$] by 50% relative to control (CON: 206 ± 59 vs. BR: 1,037 ± 627 nM, $P < 0.01$) and by 418% compared with PL (PL: 248 ± 182 mM, $P < 0.01$).

BR supplementation significantly reduced systolic BP relative to control (CON: 125 ± 9 vs. BR: 115 ± 9 mmHg, $P < 0.01$) and compared with PL (120 ± 6 mmHg, $P < 0.05$). Diastolic BP was also significantly reduced with BR ingestion compared with control (CON: 74 ± 7 vs. BR: 70 ± 5 mmHg, $P < 0.01$) and compared with PL (73 ± 5 mmHg, $P < 0.05$). MAP was significantly reduced following BR supplementation relative to both control (CON: 91 ± 7 vs. BR: 85 ± 5 mmHg, $P < 0.01$) and PL (88 ± 4 mmHg, $P < 0.05$).

Moderate-Intensity Walking

The pulmonary $V_{O_2}$ responses to a step transition to moderate-intensity treadmill exercise in both the PL and BR conditions are presented in Fig. 1, and the parameters derived from the model fit are presented in Table 1. There was no significant difference in $V_{O_2}$ between PL and BR during the baseline walking period. The amplitude of the pulmonary $V_{O_2}$ response was not different between the two conditions (PL: 477 ± 200 vs. BR: 464 ± 200 ml/min) and the steady-state $V_{O_2}$ measured over the final 30 s of moderate-intensity walking was also unchanged (PL: 979 ± 269 vs. BR: 977 ± 250 ml/min). However, relative to PL, BR supplementation reduced the $V_{O_2}$ MRT (PL: 28 ± 7 vs. BR: 25 ± 7 s, $P < 0.05$), and the $O_2$ deficit (PL: 225 ± 132 vs. BR: 192 ± 137 ml, $P = 0.07$). Baseline and end-exercise $V_{CO_2}$, $V_e$, RER, and HR were not significantly different between conditions (Table 1).

Table 1. Pulmonary gas exchange, ventilation, and heart rate during moderate-intensity exercise following placebo and beetroot juice supplementation

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Beetroot</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{O_2}$</td>
<td>Baseline, ml/min</td>
<td>518 ± 104</td>
</tr>
<tr>
<td></td>
<td>Primary amplitude, ml/min</td>
<td>477 ± 200</td>
</tr>
<tr>
<td></td>
<td>End exercise, ml/min</td>
<td>979 ± 269</td>
</tr>
<tr>
<td></td>
<td>Mean response time, s</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>$O_2$ deficit, ml</td>
<td>225 ± 132</td>
<td>192 ± 137</td>
</tr>
<tr>
<td>$V_e$</td>
<td>Baseline, l/min</td>
<td>152 ± 39</td>
</tr>
<tr>
<td></td>
<td>End exercise, l/min</td>
<td>250 ± 7.4</td>
</tr>
<tr>
<td>Respiratory exchange ratio</td>
<td>Baseline</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>End exercise</td>
<td>0.89 ± 0.05</td>
</tr>
<tr>
<td>HR</td>
<td>Baseline, bpm</td>
<td>78 ± 9</td>
</tr>
<tr>
<td></td>
<td>End exercise, bpm</td>
<td>95 ± 12</td>
</tr>
<tr>
<td>Amplitude, bpm</td>
<td>17 ± 12</td>
<td>15 ± 7</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. *Significant difference, $P < 0.05$.

Functional Capacity

Compared to PL, BR did not significantly alter functional capacity as measured by total distance covered in the 6MWT (PL: 667 ± 86 vs. BR: 682 ± 89 m, $P > 0.05$).

Low-Intensity Knee-Extension Exercise

Muscle metabolite concentration changes in response to low-intensity exercise are reported in Table 2 and Fig. 2. There

Table 2. Muscle metabolic responses during low-intensity exercise following placebo and beetroot juice supplementation

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Beetroot</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PCr]</td>
<td>Baseline, mM</td>
<td>32.0 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>240 s, mM</td>
<td>25.8 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>Amplitude, mM</td>
<td>6.2 ± 2.5</td>
</tr>
<tr>
<td>[ATP]</td>
<td>Baseline, mM</td>
<td>3.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>240 s, mM</td>
<td>7.9 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Amplitude, mM</td>
<td>4.2 ± 1.1</td>
</tr>
<tr>
<td>[ADP]</td>
<td>Baseline, μM</td>
<td>7.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>240 s, μM</td>
<td>22.5 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>Amplitude, μM</td>
<td>15.1 ± 8.6</td>
</tr>
<tr>
<td>pH</td>
<td>Baseline</td>
<td>7.03 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>240 s</td>
<td>7.07 ± 0.04</td>
</tr>
<tr>
<td>Δ Baseline - 240 s</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, O₂ uptake kinetics, and muscle and cognitive function in older adults

Table 3. Muscle metabolic responses during high-intensity exercise following placebo and beetroot juice supplementation

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Beetroot</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PCr]</td>
<td>30.0 ± 4.2</td>
<td>30.6 ± 5.5</td>
</tr>
<tr>
<td>Baseline, mM</td>
<td>21.9 ± 4.1</td>
<td>23.2 ± 5.5</td>
</tr>
<tr>
<td>24 s, mM</td>
<td>8.1 ± 2.7</td>
<td>7.3 ± 2.8</td>
</tr>
<tr>
<td>Recovery τ, s</td>
<td>35 ± 10</td>
<td>37 ± 15</td>
</tr>
<tr>
<td>[P]</td>
<td>2.9 ± 0.9</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>Baseline, mM</td>
<td>8.9 ± 2.1</td>
<td>8.9 ± 1.9</td>
</tr>
<tr>
<td>24 s, mM</td>
<td>6.9 ± 1.7</td>
<td>5.8 ± 1.5</td>
</tr>
<tr>
<td>[ADP]</td>
<td>8.0 ± 3.3</td>
<td>8.0 ± 2.2</td>
</tr>
<tr>
<td>Baseline, μM</td>
<td>37 ± 13.6</td>
<td>34.1 ± 9.4</td>
</tr>
<tr>
<td>24 s, μM</td>
<td>29.4 ± 12.3</td>
<td>26.1 ± 10.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.99 ± 0.03</td>
<td>7.00 ± 0.02</td>
</tr>
<tr>
<td>Baseline</td>
<td>7.00 ± 0.03</td>
<td>7.00 ± 0.03</td>
</tr>
<tr>
<td>Δ Baseline - 240 s</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.04</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.

between the two conditions (PL: 35 ± 10 vs. BR: 37 ± 15 s; P > 0.05).

Cognitive Performance

Performance results from the cognitive function tests are presented in Table 4. Cognitive performance on the Serial Subtraction test was not different between PL or BR supplementation for serial 3s (PL: 29 ± 8 vs. BR: 26 ± 14, P > 0.05) or serial 7s (PL: 16 ± 9 vs. BR: 16 ± 10, P > 0.05). Likewise, no significant differences between PL and BR supplementation were found during the Rapid Visual Information Processing test: correct target IDs (PL: 21 ± 4 vs. BR: 23 ± 4, P > 0.05), errors (PL: 9 ± 17 vs. BR: 9 ± 16, P > 0.05), and average response time (PL: 599 ± 199 vs. BR: 674 ± 194 ms, P > 0.05). There were no significant differences in number recall performance data between PL and BR supplementation: forward correct (PL: 29 ± 8 vs. BR: 27 ± 8, P > 0.05), backward

![Graph](image1.png)

Fig. 2: Group mean muscle metabolic responses to low-intensity, leg-extension exercise following PL and BR supplementation. The change in muscle [PCr] (A), [ADP] (B), and [P] (C) from rest to steady state were unaffected by BR supplementation. The dotted vertical line denotes the abrupt “step” transition from rest to low-intensity, leg-extension exercise.

were no significant differences in the baseline or end-exercise [P], [ADP], or pH between the two conditions. Although the magnitude of PCr depletion was reduced by ~15% following BR supplementation compared with PL (PL: 6.2 ± 2.5 vs. BR: 5.3 ± 3.0), this difference was not statistically significant.

[PCr] Recovery Kinetics

Muscle metabolite concentration changes in response to the 24-s bout of high-intensity exercise are reported in Table 3, with the PCr depletion and subsequent recovery being illustrated in Fig. 3. Reductions in muscle [PCr], from resting baseline, following high-intensity exercise were not different between the two conditions (PL: 8.1 ± 2.7 vs. BR: 7.3 ± 2.8 mM; P > 0.05). The end-exercise pH was also not significantly different from the resting baseline (PL: 7.00 ± 0.03 vs. BR: 7.00 ± 0.03; P > 0.05). The [PCr] recovery τ was not different

![Graph](image2.png)

Fig. 3: Group mean intramuscular [PCr] response to 24-s high-intensity, leg-extension exercise and subsequent recovery. [PCr] responses following BR are represented as solid circles, with the PL responses being shown as open circles.
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults

Table 4. Cognitive performance tests following placebo and beetroot juice supplementation

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Beetroot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serial subtraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’s, correct responses in 2 min</td>
<td>29 ± 8</td>
<td>26 ± 14</td>
</tr>
<tr>
<td>7’s, correct responses in 2 min</td>
<td>16 ± 9</td>
<td>16 ± 10</td>
</tr>
<tr>
<td>Rapid visual information processing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correct target LD’s</td>
<td>21 ± 4</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Errors</td>
<td>9 ± 17</td>
<td>9 ± 16</td>
</tr>
<tr>
<td>Average response time, ms</td>
<td>599 ± 199</td>
<td>674 ± 194</td>
</tr>
<tr>
<td>Number recall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forwards correct</td>
<td>29 ± 8</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>Backwards correct</td>
<td>22 ± 7</td>
<td>21 ± 7</td>
</tr>
<tr>
<td>Total correct</td>
<td>51 ± 14</td>
<td>48 ± 14</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.

correct (PL: 22 ± 7 vs. BR: 21 ± 7, $P > 0.05$) and total correct (PL: 51 ± 14 vs. BR: 48 ± 14, $P > 0.05$).

Brain Metabolic Concentrations

A summary of the effects of BR supplementation upon resting brain metabolite concentrations and apparent diffusion coefficients is presented in Table 5. Resting concentration ratios of NAA:water, Cr:water, Ch:water, ml:water, NAA:Cr, NAA:Ch, and NAA:Cr+Ch in both left frontal white matter and occipito-parietal grey matter were not significantly different between the two conditions. Likewise, there were no differences between PL and BR in apparent diffusion coefficients from the anterior cingulate gyrus, the dorsolateral prefrontal cortex, and the subcortical white matter of the frontal lobe, suggesting BR did not modulate diffusive characteristics in the brain.

DISCUSSION

The principal original findings of this investigation were that, consistent with our hypotheses, short-term (2.5 days) dietary $NO_3^-$ supplementation in the form of concentrated beetroot juice (which elevated plasma [nitrate] four-fold) significantly reduced resting blood pressure and the $O_2$ mean response time during walking exercise in a healthy senescent population. These findings are important, as they provide evidence that dietary supplementation with a natural food product may act as a valuable intervention in preventing hypertension and speeding $O_2$ kinetics in older adults. However, in contrast to our hypotheses, $NO_3^-$ supplementation did not significantly alter the steady-state $O_2$ cost of walking, functional walking performance, the muscle metabolic response to low-intensity exercise, brain metabolite concentrations, or cognitive function.

Effects of Nitrate Supplementation on Plasma [NO$_3^-$] and BP

Following supplementation with $NO_3^-$-rich BR, plasma [NO$_3^-$] was increased to 418% of the PL value. These findings are consistent with previous studies that reported significant elevations in plasma [NO$_3^-$] following dietary NO$_3^-$ supplementation (29, 50, 76). CON plasma [nitrate] values in the present study population were similar to those found in young adults. This was surprising because lower [nitrate] values may be expected in an older population (68). Moreover, it might be expected that the effect of NO$_3^-$ supplementation on plasma [NO$_3^-$] might be smaller in older compared with younger adults due to age-related changes in oral bacterial colonization (63). However, elevation of plasma [NO$_3^-$] in the current study was somewhat greater than that found in previous research with younger adults (4, 29, 50, 76, 81) but similar to that reported previously in older healthy subjects (57) and peripheral arterial disease patients (41).

It is possible that increased plasma [NO$_3^-$] might augment NO bioavailability, thereby compensating for the expected age-dependent reduction in endothelial NOS activity (68). Increased extracellular NO promotes smooth muscle relaxation via the synthesis of cyclic guanosine monophosphate from guanosine triphosphate. Previous studies have revealed significant reductions in systolic and diastolic BP as a result of this NO-related smooth muscle relaxation (49, 81). Likewise, in the present study, we found significant reductions in systolic blood pressure ($-5$ mmHg), diastolic blood pressure ($-3$ mmHg), and mean arterial pressure ($-3$ mmHg) following ingestion of the NO$_3^-$-rich BR, relative to the NO$_3^-$-depleted PL. Supplementation with the NO$_3^-$-depleted PL did not significantly reduce diastolic BP or mean arterial pressure relative to the control condition, which may suggest that the NO$_3^-$ in BR, rather than other compounds found in BR, including antioxidants (83), were principally responsible for the lowering of resting BP. On the other hand, PL did have a small but significant effect on systolic BP relative to control, which may indicate that NO$_3^-$ is not the only bioactive compound in BR, which contributes to the lowering of BP. The present study indicates that BP can be reduced via the systemic reduction of NO$_3^-$-derived NO$_3^-$ in healthy older adults in a similar fashion to that reported previously in young adults (81). This finding is in contrast to a recent study in which dietary nitrate supplementation increased plasma nitrate and nitrite values but did not alter BP in older adults (57). The reason for this difference

Table 5. ¹H-MRS and ADC brain scan data following placebo and beetroot juice supplementation

<table>
<thead>
<tr>
<th>¹H-MRS Brain Scan</th>
<th>Placebo</th>
<th>Beetroot</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA:Water</td>
<td>2.065 ± 0.273</td>
<td>2.141 ± 0.213</td>
</tr>
<tr>
<td>Cr:Water</td>
<td>1.024 ± 0.111</td>
<td>1.050 ± 0.090</td>
</tr>
<tr>
<td>Ch:Water</td>
<td>0.568 ± 0.117</td>
<td>0.559 ± 0.085</td>
</tr>
<tr>
<td>ml:Water</td>
<td>0.757 ± 0.193</td>
<td>0.805 ± 0.102</td>
</tr>
<tr>
<td>NAA:Cr</td>
<td>2.031 ± 0.294</td>
<td>2.048 ± 0.245</td>
</tr>
<tr>
<td>NAA:Ch</td>
<td>3.985 ± 1.054</td>
<td>3.908 ± 0.696</td>
</tr>
<tr>
<td>NAA:Cr+Ch</td>
<td>1.315 ± 0.235</td>
<td>1.337 ± 0.162</td>
</tr>
<tr>
<td>White matter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAA:Water</td>
<td>1.575 ± 0.263</td>
<td>1.657 ± 0.180</td>
</tr>
<tr>
<td>Cr:Water</td>
<td>0.899 ± 0.140</td>
<td>0.940 ± 0.078</td>
</tr>
<tr>
<td>Ch:Water</td>
<td>1.016 ± 0.108</td>
<td>0.992 ± 0.149</td>
</tr>
<tr>
<td>ml:Water</td>
<td>0.950 ± 0.226</td>
<td>1.065 ± 0.337</td>
</tr>
<tr>
<td>NAA:Cr</td>
<td>1.761 ± 0.118</td>
<td>1.742 ± 0.143</td>
</tr>
<tr>
<td>NAA:Ch</td>
<td>1.546 ± 0.177</td>
<td>1.692 ± 0.401</td>
</tr>
<tr>
<td>NAA:Cr+Ch</td>
<td>0.821 ± 0.069</td>
<td>0.850 ± 0.109</td>
</tr>
<tr>
<td>ADC, 10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsolateral prefrontal cortex</td>
<td>0.782 ± 0.033</td>
<td>0.783 ± 0.050</td>
</tr>
<tr>
<td>Anterior cingulate gyrus</td>
<td>0.753 ± 0.137</td>
<td>0.790 ± 0.101</td>
</tr>
<tr>
<td>Frontal lobe (deep white matter)</td>
<td>0.817 ± 0.052</td>
<td>0.841 ± 0.073</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. MRS, magnetic resonance spectroscopy; NAA, N-acetyl aspartate; Cr, creatine; Ch, choline; ml, myo-inositol; ADC, apparent diffusion coefficient.
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, O\textsubscript{2} uptake kinetics, and muscle and cognitive function in older adults

is unclear. Our results suggest that a high NO\textsubscript{3} diet may benefit cardiovascular health in older adults.

**Effects of Nitrate Supplementation on the Physiological Responses to Walking**

A novel finding of the present study was the small but significant speeding of VO\textsubscript{2} kinetics following the onset of exercise subsequent to dietary NO\textsubscript{3} supplementation. Faster VO\textsubscript{2} kinetics would be expected to reduce the reliance on nonoxidative metabolic processes across the transition from a lower to a higher metabolic rate and, therefore, to reduce muscle metabolic perturbation (i.e., changes in substrates or metabolites that have been associated with fatigue development; Ref. 36, 82). In the present study, the O\textsubscript{2} deficit was reduced by 15% following NO\textsubscript{3} supplementation, as a function of the faster VO\textsubscript{2} kinetics. Whether the small speeding of VO\textsubscript{2} kinetics that we observed is of functional relevance remains unclear, however, given that we did not find differences in 6MWT performance. Previous studies with young adults have not found faster VO\textsubscript{2} kinetics following NO\textsubscript{3} supplementation (5, 47, 76). Older adults typically have slower VO\textsubscript{2} kinetics (3, 14, 22) and are more likely to evince a speeding of VO\textsubscript{2} kinetics following interventions designed to enhance muscle O\textsubscript{2} delivery (66) than their younger counterparts. The MRT for VO\textsubscript{2} kinetics for the older subjects tested in the present study was surprinsingly fast (i.e., ~28 s). This may be due to both the exercise modality that we employed (i.e., walking) and the fact that our subjects were physically active. Given that NO\textsubscript{3} supplementation did not significantly alter the maximal rate of oxidative ATP resynthesis (see **Effects of Nitrate Supplementation on Muscle [PCr] Recovery**), it is possible that the faster VO\textsubscript{2} kinetics that we observed was linked to enhanced muscle vasodilatation and blood flow, which offset an O\textsubscript{2} delivery limitation to VO\textsubscript{2} kinetics in our older subjects.

No effects on the O\textsubscript{2} cost of walking were evident in the present study, which is in contrast to results reported in younger adults (47) and to the body of literature, which indicates that NO\textsubscript{3} supplementation improves exercise efficiency (4, 51, 76). It is unclear why older adults may respond differently to younger adults with respect to the influence of NO\textsubscript{3} supplementation on the O\textsubscript{2} cost of exercise. However, the lack of significant change in walking economy is consistent with the lack of change in muscle metabolic responses that we observed (see **Effects of Nitrate Supplementation on Muscle Metabolism During Low-Intensity Exercise**).

**Effects of Nitrate Supplementation on Functional Capacity**

Dietary NO\textsubscript{3} supplementation has been reported to improve high-intensity exercise tolerance (4, 5, 47), and time-trial performance (12, 48) in athletic young adults. In the present study, we assessed the functional capacity of our older subjects using the 6MWT. There was no significant difference in 6MWT performance between PL and BR. However, there was a 2.2% mean increase in total distance covered in the BR condition, which is similar to the improvements in performance reported for 4 km and 16.1 km (~2.7%; Ref. 48) and 10 km (~1.0%; Ref. 12) cycling time-trials. A speeding of the VO\textsubscript{2} kinetics, as was observed in the present study following NO\textsubscript{3} supplementation, would be expected to improve performance in certain physical tasks. It is unclear why NO\textsubscript{3} supplementation did not result in a significant improvement in 6MWT performance in the present study. It is possible that the 6MWT lacks the sensitivity to detect small improvements in functional capacity consequent to an acute intervention (24), especially in the physically active subjects in the present study. Future investigations into the influence of NO\textsubscript{3} supplementation on functional capacity in older adults might usefully employ a more comprehensive battery of physical performance tests.

**Effects of Nitrate Supplementation on Muscle Metabolism During Low-Intensity Exercise**

In the present study, the fall in muscle [PCr] during low-intensity knee-extensor exercise was not significantly attenuated following NO\textsubscript{3} supplementation. However, the magnitude of [PCr] depletion was reduced by 15%, on average. In an earlier study in young adults we reported that NO\textsubscript{3} supplementation significantly reduced the amplitude of [PCr] depletion during low-intensity exercise (5). The linear relationship observed between VO\textsubscript{2} and intramuscular [PCr], both before and after NO\textsubscript{3} supplementation, suggested that the reduction in the O\textsubscript{2} cost of exercise was subsequent to enhanced efficiency within the muscle contractile apparatus. It is unclear why the fall in muscle [PCr] was significantly spared in younger adults (5) but not older adults (present study). Interindividual variability may have precluded the attainment of statistical significance in the present study. Alternatively, the lower ATP cost of muscle contraction in older adults (74) may have served to reduce the impact of NO\textsubscript{3} supplementation on muscle contractile efficiency.

**Effects of Nitrate Supplementation on Muscle [PCr] Recovery**

The rate at which intramuscular [PCr] recovers immediately following exercise is thought to reflect the maximal rate of oxidative synthesis of ATP, with limited contribution from glycolysis (38). An increased rate of [PCr] recovery would suggest improvements in maximal oxidative rate as a function of increased mitochondrial volume and oxidative enzyme activity or, in the event of tissue hypoxia, O\textsubscript{2} supply (1). In the present study, NO\textsubscript{3} supplementation did not significantly alter muscle [PCr] recovery kinetics, consistent with our previous findings in young adults (47).

**Effects of Nitrate Supplementation on Brain Metabolite Concentrations and Cognitive Performance**

The amino acid N-acetylaspartate (NAA) found in neurons in the adult central nervous system (58) has been suggested to be a marker of neuronal viability (54). NAA has been shown to be closely related to mitochondrial activity in ATP production and O\textsubscript{2} consumption (6), which suggests an association between [NAA] and metabolic efficiency in the brain (73). Previous studies have shown that [NAA] is associated with both intellectual and neuropsychological (84) measures of cognition in young adults. In the present study, we considered whether NO\textsubscript{3} supplementation may provide beneficial effects upon metabolic efficiency and blood flow within the brain, in a similar fashion to what has been reported within skeletal muscle (50, 77). However, there were no significant differences in [NAA] following NO\textsubscript{3} supplementation. Likewise,
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, O$_2$ uptake kinetics, and muscle and cognitive function in older adults

ml, a carbohydrate found in the brain that is elevated in patients with Alzheimer’s disease and mild cognitive impairment (33, 43), was not affected by the NO$_3^-$ supplementation. Moreover, NO$_3^-$ supplementation did not alter the concentrations of Cr or Ch in the brain, both of which are considered important in neurological health, energy metabolism, and cognitive ability (56, 78). It is well documented that chronic ischemia and poor cerebral perfusion, specifically to the white matter, is associated with cognitive decline and dementia (69).

It was recently shown that an elevated dietary NO$_3^-$ intake increased cerebral blood flow to the anterior cingulated gyrus, the dorsolateral prefrontal cortex and subcortical and deep white matter of the frontal lobes in a population of older adults (63). We were unable to identify changes to apparent diffusion coefficients in the aforementioned regions despite providing a larger NO$_3^-$ dose to our subjects (24.6 mmol over 2.5 days) compared with Presley et al. (63) (12.4 mmol over 2 days). A possible explanation for this discrepancy is that the subjects in these studies were, on average, 10 yr older than the subjects we studied, increasing the likelihood that blood flow to these specific brain areas was diminished.

Given the previous report that increased dietary NO$_3^-$ intake increased brain blood flow in older adults (63), we assessed the influence of NO$_3^-$ supplementation on cognitive function. Specifically, measures of attention, concentration, information processing, and working memory were completed using validated cognitive function tests. However, we could not discern significant effects of NO$_3^-$ supplementation on cognitive function. A lack of effect of NO$_3^-$ supplementation on cognitive function might not be considered surprising given that there were no significant changes in NMR parameters of cerebral functionality or metabolism.

**Experimental Considerations**

Although we have attributed the reductions in resting BP and VO$_2$ MRT during the transition to walking exercise to an increased NO$_3^-$ intake, we appreciate that BR contains a number of other compounds that may influence physiological function in humans at rest and during exercise. Specifically, betaine has been linked to improving muscular endurance, strength, and power (31, 55) and can be found in beetroot. Likewise, the polyphenols, queretin and resveratrol, which are found in beetroot have, in some instances, been reported to increase aerobic capacity and stimulate mitochondrial biogenesis (20, 44). Although we do not rule out the potential for NO$_3^-$ to operate synergistically with these compounds, the unchanged plasma [NO$_3^-$], diastolic BP, MAP, and VO$_2$ response following PL supplementation suggests that NO$_3^-$ is the key “bioactive” compound in BR. Nevertheless, the reduced systolic BP following PL supplementation compared with the control condition may suggest that other components of beetroot juice, such as antioxidants (83), might also contribute to the BP-lowering effect of BR in older adults.

While we were successful in recruiting a cohort of older adults to this study (mean age of 64 yr), the subjects tended to be physically active and were interested in the health benefits of diet and exercise. In this regard, they may have been unrepresentative of their age group (for example, they had a very fast VO$_2$ MRT), and this may have reduced the likely impact of NO$_3^-$ supplementation on functional capacity assessed with the 6MWT, regional brain blood flow, and cognitive function. That is, there may have been limited opportunity for NO$_3^-$ supplementation to positively influence physical or cognitive function because our subjects were not yet sufficiently impaired. Moreover, the 6MWT might not have been the most sensitive or appropriate test for these physically fit older adults. Physical and cognitive decline is likely accelerated beyond ~70 yr of age (46, 69), and our results do not discount the possibility that NO$_3^-$ supplementation may be beneficial in older, more impaired individuals (e.g., Ref. 63). It is also pertinent to note that the dietary intervention in the present study was short-term. Longer-term NO$_3^-$ supplementation should be required to enhance vascular structure and function (68), which may, in turn, improve the matching of O$_2$ delivery to metabolic rate (7, 18) and enhance metabolic control. Future studies should consider the possible benefits of longer-term NO$_3^-$ supplementation in senescent subjects with greater physical and cognitive impairment.

**Perspectives and Significance**

Short-term (2.5 days) dietary NO$_3^-$ supplementation resulted in a four-fold increase in plasma [nitrite] and significant reductions in resting blood pressure in normotensive older adults. These results suggest that NO$_3^-$ supplementation may have potential in reducing the risk of hypertension and cardiovascular disease in older adults. The VO$_2$ kinetics was accelerated during treadmill walking, although this did not translate into enhanced performance during a 6MWT. Indices of brain metabolism and cognitive performance were not significantly altered. The results suggest that increased dietary NO$_3^-$ intake may provide a practical therapeutic and/or prophylactic intervention for reducing the risk of hypertension and improving VO$_2$ kinetics in older adults. Whether this may translate into improved functional capacity in functionally impaired older adults should be considered in subsequent research.

**ACKNOWLEDGMENTS**

We thank Prof. Nigel Benjamin for assistance with the production of the placebo beetroot juice and Beet It for providing the beverages used in this study for free.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults
Chapter 6: Effects of short-term dietary nitrate supplementation on blood pressure, $O_2$ uptake kinetics, and muscle and cognitive function in older adults


Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O₂ uptake kinetics during moderate- and high-intensity cycle exercise

Abstract

Purpose: The purpose of this study was to assess the effects of dietary nitrate (NO₃⁻) supplementation on muscle metabolism and pulmonary O₂ uptake (V̇O₂) kinetics during cycle exercise. Methods: In a double-blind, randomised, crossover study, eight healthy males supplemented their diet with either 140 ml·d⁻¹ of NO₃⁻-rich beetroot juice (8.4 mmol NO₃⁻; BR) or 140 ml·d⁻¹ of nitrate-depleted beetroot juice (PL) for 3-days prior to moderate and severe-intensity cycle exercise trials. Plasma samples were collected and pulmonary V̇O₂ was measured at rest and during exercise and muscle biopsies were sampled before and immediately after the exercise bouts. Results: Muscle [NO₃⁻] and plasma [NO₂⁻] and [NO₃⁻] were higher (P<0.05) in BR compared to PL. Neither baseline nor end-exercise V̇O₂ were different between BR and PL for either moderate-intensity or severe-intensity exercise (P>0.05). Muscle [ATP], [PCr], [lactate], [HAD] and pH before and at the end of exercise were not different between conditions (P>0.05). The time-to-exhaustion during severe-intensity exercise was not different between conditions (BR: 140 ± 57 vs. PL: 133 ± 60 s, P>0.05). However, a significant order effect was evident (visit 2: 123 ± 57 vs. visit 3: 151 ± 57 s: P<0.05). Importantly, the Δ in muscle [NO₃⁻] following BR compared to PL was positively correlated with the Δ in severe-intensity exercise tolerance (r = 0.67; P<0.05). Conclusions: NO₃⁻ supplementation elevated plasma [NO₃⁻] and [NO₂⁻], and muscle [NO₃⁻], but this did not lead to changes in V̇O₂ or muscle metabolic responses during moderate- and severe-intensity exercise at the group mean level. However, the higher muscle [NO₃⁻] with BR compared to PL was correlated with an extended time to exhaustion, suggesting that muscle [NO₃⁻] may be linked to performance during severe-intensity exercise.
Introduction

Following the onset of exercise, an immediate increase in ATP turnover and an exponential rise in oxygen (O₂) consumption are evident within the contracting muscle cells. This disparity in the rates of muscle ATP utilization and ATP supply via oxidative phosphorylation obligates a compensatory energy contribution from substrate-level phosphorylation (Poole et al., 2008). Pulmonary O₂ uptake (VO₂), which provides a close representation of muscle VO₂ (Grassi et al., 1996, Krustrup et al., 2009), attains a ‘steady-state’ within 120-180 s following the onset of moderate-intensity exercise (below the gas exchange threshold; GET) (Whipp et al., 1982). However, during heavy (above GET but below critical power) and severe-intensity (above critical power) exercise, an additional phase of the VO₂ response, the VO₂ ‘slow component’, is evident which delays and/or prevents the attainment of a steady state. The development of the VO₂ slow component is closely related to accelerated muscle PCr (Rossiter et al., 2002) and glycogen utilization (Krustrup et al., 2004), and to the accumulation of fatigue associated metabolites (H⁺, Pᵢ, ADP). Interventions that alter the VO₂ response during exercise and modulate the rate at which the body’s energy stores are depleted and fatiguing metabolites are accumulated are therefore likely to have important implications for exercise tolerance (Jones & Burnley, 2009).

Dietary nitrate (NO₃⁻) supplementation in the form of NO₃⁻ salts or NO₃⁻-rich beetroot juice (BR) can have beneficial cardiovascular and metabolic effects. These effects have been attributed to the conversion of the relatively inert NO₃⁻ anion to bioactive nitrite (NO₂⁻) and nitric oxide (NO). NO is a gaseous, lipophilic free radical involved in a variety of mammalian physiological processes, including mitochondrial respiration and biogenesis, muscle contractility and the regulation of blood flow (Cooper et al., 1999; Dejam et al., 2004; Nisoli et al., 2006). NO is produced via the oxidation of L-arginine, a complex O₂-dependent process that is catalysed by a family of nitric oxide synthase (NOS) enzymes and requires several substrates (Alderton et al., 2001). NO is also produced via the NO₃⁻-NO₂⁻-NO pathway by which ingested NO₃⁻ rapidly absorbed from the gut enters the enterosalivary circulation and is concentrated in the saliva (Lundberg et al., 2008). Anaerobic bacteria, residing in crypts on the dorsum of the tongue, reduce the NO₃⁻ to NO₂⁻ (Duncan et al., 1995). Once swallowed into the acidic environment of the stomach, some of this
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary $\hat{V}O_2$ uptake kinetics during moderate- and high-intensity cycle exercise

Salivary NO$_2^-$ is further converted to NO (Benjamin et al., 1994), with the remainder being absorbed to increase circulating plasma NO$_2^-$ concentration ([NO$_2^-$]). This circulating NO$_2^-$ may be converted into NO via a number of enzymatic and non-enzymatic pathways (Cosby et al., 2003; Godber et al., 2000).

Inorganic NO$_3^-$ ingestion has been reported to reduce resting blood pressure (Bailey et al., 2010; Kapil et al., 2010; Kelly et al., 2013; Larsen et al., 2006; Webb et al., 2008) and modify the physiological response to exercise (Bailey et al., 2009; Larsen et al., 2007). These modifications include reductions in the $O_2$ cost of moderate- (Bailey et al., 2009; Larsen et al., 2007; Wylie et al., 2013a) and severe-intensity constant-work-rate exercise (Lansley et al., 2011a). NO$_3^-$ supplementation can also speed the $\hat{V}O_2$ phase II time constant ($\tau$) (Kelly et al., 2013) and reduce the amplitude of the $\hat{V}O_2$ slow component (Bailey et al., 2009). Improvements in time to exhaustion during constant-work-rate exercise (Bailey et al., 2009; Kelly et al., 2013, Lansley et al., 2011a) and increased performance in intense intermittent exercise (Wylie et al., 2013b), time trials (Bond et al., 2012; Cermak et al., 2012; Lansley et al., 2011b; Mugggeridge et al., 2014) and incremental exercise (Masschelein et al., 2012, Vanhatalo et al., 2010) have been reported following NO$_3^-$ supplementation. The effects of NO$_3^-$ supplementation may be more limited in elite athletes compared to recreationally active individuals (Bescos et al., 2012, Wilkerson et al., 2012). The proposed mechanisms behind the physiological benefits and ergogenic effects of NO$_3^-$ supplementation include NO-mediated modifications in muscle contractile function (Bailey et al., 2010; Hernández et al., 2012), mitochondrial efficiency (Larsen et al., 2011) and improved muscle blood flow, with preferential distribution to type II fibres (Ferguson et al., 2013).

Research has consistently reported that NO$_3^-$ supplementation significantly increases circulating plasma [NO$_3^-$] (Kapil et al., 2010, Larsen et al., 2010) and [NO$_2^-$] (Bailey et al., 2009; Kelly et al., 2013; Vanhatalo et al., 2010; Wylie et al., 2013a). However, it is currently unknown whether dietary NO$_3^-$ supplementation can increase [NO$_3^-$] concentrations within skeletal muscle tissue, and whether this is important in eliciting the biological effects previously reported. Therefore the aim of this study was to assess the influence of dietary NO$_3^-$ supplementation on skeletal muscle [NO$_3^-$] as well as pulmonary $\hat{V}O_2$ and the muscle metabolic responses to constant-work-rate moderate- and severe-intensity exercise. These data may enable better understanding of the mechanisms by which
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O$_2$ uptake kinetics during moderate- and high-intensity cycle exercise

dietary NO$_3^-$ supplementation can affect muscle energetics, exercise economy and exercise tolerance. It was hypothesised that: 1) muscle [NO$_3^-$] would be elevated; 2) the magnitude of muscle PCr degradation and metabolite accumulation would be reduced; 3) the O$_2$ cost of moderate-intensity cycle exercise would be reduced; and 4) exercise tolerance during severe-intensity cycle exercise would be improved, as a result of NO$_3^-$ supplementation.

Methods

Subjects

Eight physically active male subjects (mean ± SD; age = 26 ± 3 yrs, height = 1.78 ± 0.05 m, body mass = 84 ± 7 kg, V$\acute{O}$$_2$peak = 52.3 ± 4.2 mL·kg$^{-1}$.min$^{-1}$) volunteered to take part in this study. The study was approved by the Institutional Research Ethics Committee. All subjects gave written, informed consent prior to commencement of the study, once the experimental protocol, associated risks, and potential benefits of participation had been outlined. Subjects were instructed to arrive at the laboratory in a fully hydrated and rested state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Subjects were asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively, and to consume the same light pre-exercise meal of their choice 3 h before testing. In addition to this, subjects were asked to abstain from using antibacterial mouthwash and chewing gum for the duration of the study since this has been shown to blunt the conversion of NO$_3^-$ to NO$_2^-$ in the oral cavity (Govoni et al., 2008). All exercise tests were performed at the same time of day (± 2 h) for each subject.

Procedures

Subjects were required to attend the laboratory on three occasions over a 2-wk period. All exercise tests were performed using an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands). During visit 1, subjects completed a ramp incremental test to exhaustion for the determination of V$\acute{O}$$_2$peak and gas exchange threshold (GET). Subjects performed 3 min of baseline cycling at 20 W and 80 rpm, after which the work rate was increased at a rate of 30 W·min$^{-1}$ in a linear fashion until volitional exhaustion was achieved or until the subject was unable to maintain the 80 rpm pedal rate. The height and configuration of the saddle and handlebars were recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental test and averaged over 10-s periods. V$\acute{O}$$_2$peak was
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary $O_2$ uptake kinetics during moderate- and high-intensity cycle exercise
determined as the highest mean $\dot{V}O_2$ during any 30-s period. The GET was determined from 1) the first disproportionate increase in $CO_2$ production ($\dot{V}CO_2$) from visual inspection of individual plots of $\dot{V}CO_2$ and $\dot{V}O_2$ and 2) an increase in expired ventilation ($\dot{V}E/\dot{V}O_2$) with no increase in $\dot{V}E/\dot{V}CO_2$. All subsequent work rates were calculated with account taken of the mean response time for $\dot{V}O_2$ during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the work rate at the GET).

Following *visit 1*, subjects were randomly assigned to receive 3-days of dietary supplementation (See ‘Supplementation’ below), prior to the subsequent exercise trials. During *visits 2 and 3* subjects completed step-transition, cycling exercise for the determination of pulmonary $\dot{V}O_2$, plasma $[NO_2^-]$ and $[NO_3^-]$ kinetics, muscle metabolite concentrations and exercise tolerance. Upon arrival at the laboratory, a cannula (Insyte-W™ Becton-Dickinson, Madrid, Spain) was inserted into the subject’s antecubital vein to enable frequent blood sampling before, during and after the exercise protocol. Skin incisions were made under local anaesthesia in preparation for subsequent muscle biopsies of the *m. vastus lateralis*.

The exercise protocol involved one 10-min bout of moderate-intensity cycling at 80% GET and one bout of severe-intensity cycling at 75% $\Delta$ (a work rate representing GET plus 75% of the difference between the work rates at GET and $\dot{V}O_{2peak}$) to exhaustion. Each exercise bout involved an abrupt transition to the target work rate initiated from a 3-min, 20 W baseline, with the exercise bouts separated by 27-min of passive recovery. After 6 min of severe-intensity exercise, subjects stopped cycling for 1 min in order for a muscle biopsy to be obtained (see later). Following this, the exercise bout continued until task failure as a measure of exercise tolerance. The time to exhaustion was recorded when the pedal rate fell by $>10$ rpm below 80 rpm. In these bouts, the subjects were verbally encouraged to continue for as long as possible. Blood was sampled during the baseline cycling preceding the moderate transition and after 1, 2, 3, 6 and 10 min of moderate-intensity exercise. Further samples were drawn during the baseline preceding the severe transition and after 1, 2, 3 and 6 min of severe-intensity exercise, immediately before the resumption of severe-intensity exercise, and at exhaustion. All subjects were able to maintain the severe-intensity exercise for the 6 min duration apart from one who completed 5 min 35 s on each occasion. Finally, samples were drawn during recovery from the severe exercise bout at 1, 3 and 5-mins.

75
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O₂ uptake kinetics during moderate- and high-intensity cycle exercise

Supplementation

After completion of the non-supplemented visit 1, subjects were assigned in a double-blind, randomized, crossover design to receive a course of dietary NO₃⁻ supplementation before visits 2 and 3. The supplements were either NO₃⁻-rich BR (2 x 70 mL·d⁻¹ of BR providing ~8.4 mmol NO₃⁻ per day; Beet it, James White Drinks, Ipswich, UK) or NO₃⁻-depleted PL (2 x 70 mL·d⁻¹ of PL providing ~0.006 mmol NO₃⁻ per day; Beet it, James White Drinks, Ipswich, UK). The PL beverage was created by passage of the juice, before pasteurization, through a column containing Purolite A520E ion exchange resin, which selectively removes NO₃⁻ ions. The PL was similar to the BR in appearance, taste and smell. Subjects were instructed to consume the beverages in the morning and afternoon of days 1 and 2 of supplementation, then in the morning and 2.5 h before the exercise trial on day 3. A washout period of at least 72 h separated each supplementation period. Subjects were instructed to follow their normal dietary habits throughout the testing period and to replicate their diet between conditions during supplementation periods. Subjects were informed that the supplementation may cause beeturia (red urine) and red stools temporarily but that this side effect was harmless.

Measurements

Muscle samples were obtained from the medial part of the m. vastus lateralis under local anaesthesia (1% lidocaine) using the Bergstrom needle biopsy technique with suction (Bergstrom, 1962). Muscle samples were taken at 5 different time points during the protocol: at rest (Rest), following 10 min of moderate-intensity exercise (Mod), following 6 min of severe-intensity exercise (Sev), following a 45 s recovery period (Rec) and at exhaustion (Exh). Biopsy samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis. Wet muscle samples were weighed on scales (XP6U Ultra-Microbalance, Mettler Toledo Ltd, Leicester UK) located in a -20°C cold cabinet, before undergoing a freeze-drying process. Samples were dissected to remove blood, fat, and connective tissue. Approximate 2 mg aliquots of isolated muscle fibres were weighed on a fine balance (XP6U Ultra-Microbalance, Mettler Toledo Ltd, Leicester UK) and stored in 500 µL microcentrifuge tubes at -80°C. Prior to metabolite analysis, 200 µL of 3 M perchloric acid was added to ~2 mg dry weight muscle tissue. Following a short centrifuge and 30 min incubation on ice, 170 µL was transferred (without fibres) to a fresh microcentrifuge tube, and 255 µL cooled 2 M potassium bicarbonate (KHCO₃) was added.
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O₂ uptake kinetics during moderate- and high-intensity cycle exercise

This was centrifuged, and the supernatant was analysed for pH, concentrations of ATP, PCr, lactate and 3-Hydroxyacyl CoA dehydrogenase (HAD) as previously described (Lowry & Passonneau, 1972). The supernatant was also analysed for the concentration of NO₃⁻ by gas phase chemiluminescence, as described below.

Blood samples were drawn into 5-mL lithium-heparin tubes (Vacutainer, Becton-Dickinson, New Jersey, USA). 200 μL of blood was immediately haemolysed in 200 μL of cold Triton X-100 buffer solution (Triton X-100, Amresco, Salon, OH) and analysed to determine blood [lactate] and [glucose] (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining whole blood was then centrifuged at 4000 rpm for 8 min (Hettich EBA 20, Germany) before plasma was extracted and analysed for [K⁺] and [Na⁺] (9180 Electrolyte Analyzer, F. Hoffman-La Roche, Basel, Switzerland). Blood samples for the determination of plasma [NO₂⁻] and [NO₃⁻] were collected into lithium heparin tubes and immediately centrifuged at 4000 rpm and 4 °C for 8 min. Plasma was extracted and immediately frozen at -80 °C for later analysis of [NO₂⁻] and [NO₃⁻].

Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO₂⁻. Plasma NO₂⁻ and NO₃⁻ were analysed by gas phase chemiluminescence analysis. This initially required NO₂⁻ and NO₃⁻ to be reduced to NO gas. For reduction of NO₂⁻, undiluted plasma was injected into a glass purge vessel containing 5 ml of glacial acetic acid and 1 ml of NaI solution. For NO₃⁻ reduction, plasma samples were deproteinised in an aqueous solution of zinc sulphate (10% w/v) and 1 M sodium hydroxide, prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). Quantification of NO was enabled by the detection of light emitted during the production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentrations of NO₂⁻ and NO₃⁻ were determined by plotting signal area (mV) against a calibration plot of 25 nM to 1 μM sodium nitrite and 100 nM to 10 μM sodium nitrate, respectively.

During all laboratory exercise tests, pulmonary gas exchange and ventilation were measured continuously with subjects wearing a nose clip and breathing through a mouthpiece and impeller turbine assembly (Jaeger Triple V, Hoechburg, Germany). The inspired and expired gas volume and gas concentration signals were continuously sampled.
at 100 Hz, the latter using paramagnetic (O₂) and infrared (CO₂) analysers (Oxycon Pro, Jaeger, Hoechburg, Germany) via a capillary line connected to the mouthpiece. The gas analysers were calibrated using a three-liter syringe (Hans Rudolph, Kansas City, MO, US). Pulmonary gas exchange variables were calculated and displayed breath-by-breath. HR was measured using short-range radio telemetry (model 610; Polar Electro Oy, Kempele, Finland).

Data analysis

The breath-by-breath \( \dot{V}O_2 \) data from each exercise test were initially examined to exclude errant breaths caused by coughing and swallowing with those values lying more than four SD from the local mean being removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values. The first 20 s of data after the onset of exercise (the phase I response) were deleted, and a non-linear least squares algorithm was used to fit the data thereafter. A single-exponential model was used to characterize the phase II \( \dot{V}O_2 \) responses to both moderate- and severe-intensity exercise, as described in the following equation:

\[
\dot{V}O_2(t) = \dot{V}O_2_{baseline} + A_p [1 - e^{-(t-TD_p/\tau_p)}]
\]

where \( \dot{V}O_2 \) \((t)\) represents the absolute \( \dot{V}O_2 \) at a given time \( t \); \( \dot{V}O_2_{baseline} \) represents the mean \( \dot{V}O_2 \) in the baseline period; \( A_p, TD_p, \) and \( \tau_p \) represent the amplitude, time delay and time constant, respectively, describing the phase II increase in \( \dot{V}O_2 \) above baseline. An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. \( \dot{V}O_2_{baseline} \) was defined as the mean \( \dot{V}O_2 \) measured over the final 60 s of baseline cycling. The end-exercise \( \dot{V}O_2 \) was defined as the mean \( \dot{V}O_2 \) measured over the final 30 s of exercise.

The fitting strategy was subsequently used to identify the onset of any ‘slow component’ in the \( \dot{V}O_2 \) response to severe-intensity exercise as previously described (Rossiter et al., 2001). The fitting window was lengthened iteratively until the exponential model-fit demonstrated a discernible departure from the measured response profile. Identification, via visual inspection, of the flat residual plot profile (signifying a good fit to measured data) systematically differing from zero, gave indication of the delayed slow component onset. The magnitude of the slow component for \( \dot{V}O_2 \) was measured as the difference between the end-exercise \( \dot{V}O_2 \) and the primary amplitude.
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary $O_2$ uptake kinetics during moderate- and high-intensity cycle exercise

**Statistical Analyses**

Differences in plasma [NO$_2^-$] and [NO$_3^-$], muscle [NO$_3^-$], exercise tolerance and cardio-respiratory responses between the conditions were analysed with two-tailed, paired-samples t-tests. Differences in blood and muscle metabolites between conditions were assessed using two-way (supplement x time) repeated measures ANOVA. Significant effects were further explored using simple contrasts with Fisher’s LSD. Correlations between physiological and performance variables were assessed via Pearson’s product-moment correlation coefficient. All data are presented as mean ± SD unless stated otherwise. Statistical significance was accepted when $P < 0.05$.

**Results**

Self-reported compliance to the supplementation regimen was 100%, and no deleterious effects were reported.

**Blood Variables**

Blood [glucose] and [lactate] and plasma [sodium] and [potassium] were not significantly different between BR and PL supplementation (Table 1).

The response profiles of plasma [NO$_3^-$] and [NO$_2^-$] are presented in Figure 1 and Figure 2, respectively. There were significant main effects by supplement on plasma [NO$_3^-$] ($P<0.05$) and by supplement and time on plasma [NO$_2^-$] (all $P<0.05$). At resting baseline, BR significantly elevated plasma [NO$_3^-$] (BR: 238 ± 79 µM) compared to PL (11 ± 4 µM, $P<0.05$). [NO$_3^-$] was greater in BR than PL at each measurement time point (Figure 1). At rest, BR supplementation elevated plasma [NO$_2^-$] when compared to PL (BR: 493 ± 287 nM, vs. PL: 43 ± 34 nM, $P<0.05$). [NO$_2^-$] was greater in BR than PL at each measurement time point (Figure 2). Following 10 min of moderate-intensity exercise, [NO$_2^-$] was significantly reduced in PL to 26 ± 21 nM ($P<0.05$) and showed a trend for a reduction in BR to 411 ± 293 nM ($P=0.07$) from baseline. During severe-intensity exercise to exhaustion, [NO$_2^-$] declined from baseline to 15 ± 11 nM in PL and to 174 ± 95 nM in BR ($P<0.05$).
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O\textsubscript{2} uptake kinetics during moderate- and high-intensity cycle exercise

**Muscle [NO\textsubscript{3}]-**

The effect of dietary NO\textsubscript{3} supplementation on muscle [NO\textsubscript{3}] is illustrated in Figure 3. ANOVA revealed a main effect for supplementation on muscle [NO\textsubscript{3}] (P<0.05), which tended to be higher at rest (BR: 23.6 ± 9.2 vs. PL: 17.3 ± 8.9 nmol/mg DW; P=0.14) and following 10 min of moderate-intensity exercise (BR: 31.5 ± 12.0 vs. PL: 17.2 ± 11.4 nmol/mg DW; P=0.08). Muscle [NO\textsubscript{3}] was higher in BR compared to PL following 6 min of severe-intensity exercise (BR: 28.4 ± 12.7 vs. PL: 16.8 ± 8.0 nmol/mg DW; P<0.05) and 45 s after the exercise (BR: 32.0 ± 11.4 vs. PL: 13.4 ± 4.0 nmol/mg DW; P<0.05). Muscle [NO\textsubscript{3}] remained unchanged in both BR and PL across all time-points (P>0.05). On average, muscle [NO\textsubscript{3}] was 72% greater in BR compared to PL across the entire protocol (Figure 3).

**Muscle Metabolites and pH**

Muscle [ATP], [PCr], [lactate] and pH were not significantly different between BR and PL trials during either moderate- or severe-intensity exercise. ANOVA revealed there was a main effect for time on muscle [ATP], [PCr], [lactate] and pH (Table 2).

**\(\dot{V}_O\textsubscript{2}\) Kinetics and HR**

Group mean pulmonary \(\dot{V}_O\textsubscript{2}\) responses to moderate- and severe-intensity exercise in BR and PL were not different (Table 3). During moderate-intensity exercise, baseline \(\dot{V}_O\textsubscript{2}\) (BR: 1180 ± 155 vs. PL: 1183 ± 201 ml/min; P>0.05) and end-exercise \(\dot{V}_O\textsubscript{2}\) (BR: 1824 ± 272 vs. PL: 1830 ± 252 ml/min; P>0.05) were unchanged in BR compared to PL, as were the primary amplitude (BR: 645 ± 153 vs. PL: 648 ± 167 ml/min; P>0.05) and the phase II \(\tau\) (BR: 18 ± 6 vs. PL: 20 ± 9 s; P>0.05). In the response to severe-intensity exercise, baseline \(\dot{V}_O\textsubscript{2}\) (BR: 1234 ± 270 vs. PL: 1226 ± 114 ml/min; P>0.05), end-exercise \(\dot{V}_O\textsubscript{2}\) (BR: 4248 ± 326 vs. PL: 4309 ± 385 ml/min; P>0.05), the primary amplitude (BR: 2353 ± 264 vs. PL: 2331 ± 241 ml/min; P>0.05), the phase II \(\tau\) (BR: 31 ± 9 vs. PL: 30 ± 6 s; P>0.05), and the \(\dot{V}_O\textsubscript{2}\) slow component amplitude (BR: 661 ± 101 vs. PL: 752 ± 171 ml/min; P>0.05) were not different in BR compared to PL. Heart rate was not different between BR and PL during either moderate- or severe-intensity exercise.
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O\textsubscript{2} uptake kinetics during moderate- and high-intensity cycle exercise

**Exercise Tolerance**

Exercise tolerance was measured as time to exhaustion during severe-intensity exercise preceded by 6 min of severe-intensity exercise and 1-min rest, and no difference was observed between BR and PL (140 ± 57 s vs. 133 ± 60 s, \( P>0.05 \)). There was a significant order effect, such that exercise tolerance was better in visit 3 (151 ± 57 s) compared to visit 2 (123 ± 57 s; \( P<0.05 \)), irrespective of the supplement taken.

Additional analyses revealed that participants who consumed PL before BR generally evidenced better physiological and exercise performance on visit 3 compared to visit 2 (considered hereafter as the PL→BR group), whereas participants who consumed BR before PL generally experienced worse physiological and exercise performance on visit 3 compared to visit 2 (considered hereafter as the BR→PL group). These grouped data are presented in Table 4. The PL→BR group had a significantly greater increase in plasma [NO\textsubscript{2}\textsuperscript{-}] following BR compared to the BR→PL (PL→BR: 665 ± 189 nM vs. BR→PL: 236 ± 43 nM; \( P<0.05 \)). Steady-state \( \dot{V}_\text{O}_2 \) during moderate-intensity exercise was reduced in the PL→BR group whereas the BR→PL group had an increase in \( \dot{V}_\text{O}_2 \) (PL→BR: -94 ± 65 vs. BR→PL: 108 ± 65 ml/min; \( P<0.05 \)) following BR supplementation (Figure 4). Exercise tolerance was improved in the PL→BR group (+35 ± 29 s), whilst a decrease was evident in the BR→PL group (-21 ± 11 s; \( P<0.05 \)) following BR supplementation. Although not statistically significant, the PL→BR group tended to have a greater increase in muscle [NO\textsubscript{3}\textsuperscript{-}] compared to the BR→PL group (Table 4).

The change in muscle [NO\textsubscript{3}\textsuperscript{-}] following BR supplementation compared to PL was positively correlated with the change in severe-intensity exercise tolerance (\( r = 0.67; P<0.05; n = 8 \)) (Figure 5). The change in steady-state \( \dot{V}_\text{O}_2 \) during moderate-intensity exercise following BR supplementation compared to PL was negatively correlated with the change in severe-intensity exercise tolerance (\( r = -0.66; P<0.05; n = 8 \)) (Figure 5).
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary \( \text{O}_2 \) uptake kinetics during moderate- and high-intensity cycle exercise

Table 7.1. Blood [lactate] and [glucose] and plasma [sodium] and [potassium] responses during moderate- and severe-intensity cycling, following BR and PL.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lactate (mM)</th>
<th>Glucose (mM)</th>
<th>Sodium (mM)</th>
<th>Potassium (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BR</td>
<td>PL</td>
<td>BR</td>
<td>PL</td>
</tr>
<tr>
<td>Rest</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>4.4 ± 0.7</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>1 min</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>2 min</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>4.2 ± 0.6</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>3 min</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>6 min</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>4.1 ± 0.5</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>10 min</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>4.1 ± 0.5</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>BR</td>
<td>PL</td>
<td>BR</td>
<td>PL</td>
</tr>
<tr>
<td>1 min</td>
<td>0.9 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>4.5 ± 0.4</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>2 min</td>
<td>1.3 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>4.3 ± 0.3</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>3 min</td>
<td>2.3 ± 1.0</td>
<td>2.0 ± 0.6</td>
<td>4.3 ± 0.3</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>6 min</td>
<td>3.7 ± 1.2</td>
<td>3.9 ± 1.4</td>
<td>4.1 ± 0.5</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>7 min</td>
<td>9.1 ± 2.2</td>
<td>8.8 ± 3.3</td>
<td>4.1 ± 0.6</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>Exh</td>
<td>9.1 ± 2.2</td>
<td>8.6 ± 2.5</td>
<td>4.2 ± 0.7</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>BR</td>
<td>PL</td>
<td>BR</td>
<td>PL</td>
</tr>
<tr>
<td>1 min</td>
<td>12.1 ± 2.8</td>
<td>10.8 ± 3.1</td>
<td>4.7 ± 0.8</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>2 min</td>
<td>11.7 ± 3.0</td>
<td>11.0 ± 2.6</td>
<td>5.0 ± 1.0</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>3 min</td>
<td>12.2 ± 2.2</td>
<td>11.9 ± 2.4</td>
<td>6.0 ± 0.9</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>5 min</td>
<td>12.4 ± 2.3</td>
<td>11.8 ± 1.9</td>
<td>5.8 ± 1.0</td>
<td>6.0 ± 1.1</td>
</tr>
</tbody>
</table>
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary $O_2$ uptake kinetics during moderate- and high-intensity cycle exercise

Table 7.2. Muscle [ATP], [PCr], [lactate], [HAD] and pH responses during moderate and severe-intensity cycling, following BR and PL. * = significantly different from ‘Rest’ ($P < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>ATP (mmol/kg DW)</th>
<th>PCr (mmol/kg DW)</th>
<th>lactate (mmol/kg DW)</th>
<th>pH</th>
<th>HAD (µmol/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BR</td>
<td>PL</td>
<td>BR</td>
<td>PL</td>
<td>BR</td>
</tr>
<tr>
<td>Rest</td>
<td>32.0 ± 7.1</td>
<td>35.6 ± 9.4</td>
<td>65.2 ± 11.3</td>
<td>67.4 ± 10.8</td>
<td>8.7 ± 6.1</td>
</tr>
<tr>
<td>Mod</td>
<td>29.6 ± 7.1*</td>
<td>31.9 ± 7.5*</td>
<td>53.9 ± 16.2*</td>
<td>57.1 ± 15.7*</td>
<td>8.2 ± 3.3</td>
</tr>
<tr>
<td>Sev</td>
<td>22.6 ± 4.2*</td>
<td>23.8 ±7.1*</td>
<td>18.8 ± 17.9*</td>
<td>17.4 ± 17.6*</td>
<td>93.8 ± 32.2*</td>
</tr>
<tr>
<td>Exh</td>
<td>21.4 ± 7.2*</td>
<td>24.2 ± 19*</td>
<td>12.7 ± 9.7*</td>
<td>17.2 ±17.8*</td>
<td>95.0 ± 17.7*</td>
</tr>
</tbody>
</table>
Table 7.3. Oxygen uptake kinetics in response to moderate- and severe-intensity exercise, following PL and BR supplementation.

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate-intensity exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\dot{V}O_2$ Baseline, ml/min</td>
<td>1183 ± 201</td>
<td>1180 ± 155</td>
</tr>
<tr>
<td>$\dot{V}O_2$ End Exercise, ml/min</td>
<td>1830 ± 252</td>
<td>1824 ± 272</td>
</tr>
<tr>
<td>Phase II Time Constant, s</td>
<td>20 ± 9</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Primary amplitude, ml/min</td>
<td>648 ± 167</td>
<td>645 ± 153</td>
</tr>
<tr>
<td><strong>Severe-intensity exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\dot{V}O_2$ Baseline, ml/min</td>
<td>1226 ± 114</td>
<td>1234 ± 270</td>
</tr>
<tr>
<td>$\dot{V}O_2$ End Exercise, ml/min</td>
<td>4309 ± 385</td>
<td>4248 ± 326</td>
</tr>
<tr>
<td>Phase II time constant, s</td>
<td>30 ± 6</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>Primary amplitude, ml/min</td>
<td>2331 ± 241</td>
<td>2353 ± 264</td>
</tr>
<tr>
<td>Slow Component Amplitude, ml/min</td>
<td>752 ± 171</td>
<td>661 ± 101</td>
</tr>
</tbody>
</table>
Table 7.4. Physiological and performance changes when participants considered as ‘responders’ and ‘non-responders’. * = significantly different between the two groups \((P < 0.05)\). PL→BR, participants who consumed PL on visit 2 and BR on visit 3; BR→PL, participants who consumed BR on visit 2 and PL on visit 3.

<table>
<thead>
<tr>
<th></th>
<th>Δ Plasma NO₂⁻ ((nM))</th>
<th>Δ Muscle ([\text{NO}_3^-]) ((\text{nmol/mg DW}))</th>
<th>Δ Moderate-intensity (\dot{V}O_2) ((\text{ml/min}))</th>
<th>Δ Exercise Tolerance ((s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Responders’ ((n = 4)) (\text{PL→BR})</td>
<td>1</td>
<td>821</td>
<td>0</td>
<td>-83</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>868</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>406</td>
<td>2.2</td>
<td>-181</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>565</td>
<td>27.1</td>
<td>-114</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>665 ± 189 *</td>
<td>8.72 ± 10.78</td>
<td>-94 ± 65 *</td>
</tr>
<tr>
<td>‘Non Responders’ ((n = 4)) (\text{BR→PL})</td>
<td>1</td>
<td>184</td>
<td>1.9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>208</td>
<td>0</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>294</td>
<td>17</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>256</td>
<td>-3.91</td>
<td>127</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>236 ± 43</td>
<td>3.84 ± 8.05</td>
<td>108 ± 65</td>
</tr>
</tbody>
</table>
Figure 7.1. Plasma [NO$_3^-$] response during moderate- and severe-intensity exercise and recovery following BR (A & C) and PL (B & D). Error bars indicate the SE. BR was greater than PL at each time point. * $P < 0.05$ BR compared to PL; † $P < 0.05$ compared to baseline.
Figure 7.2. Plasma [NO$_2^-$] response during moderate- and severe-intensity exercise and recovery following (A & C) and PL (B & D). Error bars indicate the SE. BR was greater than PL at each time point. *$P < 0.05$ BR compared to PL; †$P < 0.05$ compared to baseline.
Figure 7.3. Muscle [NO$_3^-$] response during moderate- and severe-intensity exercise following BR (A & C) and PL (B & D). Error bars indicate the SE. BR was greater than PL at each time point. * $P < 0.05$ BR compared to PL.
Figure 7.4. Pulmonary oxygen uptake (\(\dot{V}O_2\)) responses of A) the BR→PL group and B) the BR→PL group during a step increment to a moderate-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles, with the PL responses being shown as open circles. The dotted vertical line denotes the abrupt ‘step’ transition from baseline to moderate-intensity cycle exercise.
Figure 7.5. Pearson product-moment correlation coefficient between A) the change in muscle $[^{\text{[NO}_3\text{-]}}]$ following BR (BR-PL; nmol/mg DW) and the change in exercise tolerance following BR (BR-PL; s); and B) the change in steady-state $\dot{V}O_2$ following BR (BR-PL; ml/min) and the change in exercise tolerance following BR (BR-PL; s).
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary \( \text{O}_2 \) uptake kinetics during moderate- and high-intensity cycle exercise

**Discussion**

The principal original finding of this study, consistent with our hypothesis, was that muscle \([\text{NO}_3^-]\) was elevated following \( \text{NO}_3^- \) supplementation. In contrast to our hypothesis, \( \text{NO}_3^- \) supplementation had no significant effect on the group mean \( \dot{\text{V}} \text{O}_2 \) or muscle metabolic responses to moderate- or severe-intensity exercise. Severe-intensity exercise tolerance was not significantly different between BR and PL conditions. However, an order effect was evident such that severe-intensity exercise tolerance was significantly greater in *visit 3* compared to *visit 2*. Further analysis indicated that this could be explained, in part, by variability in participant responsiveness to \( \text{NO}_3^- \) supplementation, with the change in muscle \([\text{NO}_3^-]\) being significantly correlated to the change in severe-intensity exercise tolerance.

**Effects of \( \text{NO}_3^- \) Supplementation on Blood Variables**

At rest, plasma \([\text{NO}_2^-]\) and \([\text{NO}_3^-]\) were elevated significantly following \( \text{NO}_3^- \) supplementation, compared with PL. These findings are consistent with previous research which has consistently reported elevations in plasma \([\text{NO}_2^-]\) (Bailey *et al.*, 2009; Kelly *et al.*, 2013; Vanhatalo *et al.*, 2010) and \([\text{NO}_3^-]\) (Kapil *et al.*, 2010; Larsen *et al.*, 2010; Wylie *et al.*, 2013a) following BR supplementation. The response profile of plasma \([\text{NO}_2^-]\) during severe-intensity exercise was similar to data recently reported (Wylie *et al.*, 2013b), with \([\text{NO}_2^-]\) declining from baseline to exhaustion during severe-intensity exercise. Interestingly, no changes in \([\text{NO}_3^-]\) were observed during the severe-intensity exhaustive bout in the current study, while \([\text{NO}_3^-]\) increased significantly in the previous report (Wylie *et al.*, 2013b). The ability of an individual to ‘utilize’ \([\text{NO}_2^-]\) during exercise may be important in improving severe-intensity exercise performance (Dreissigacker *et al.*, 2010; Wylie *et al.*, 2013b). The percentage reduction of \([\text{NO}_2^-]\) in the current study was similar in BR and PL conditions (≈60%), although the absolute change in \([\text{NO}_2^-]\) was greater in BR. Similarly, there were no differences in blood [lactate], [glucose], [Na+] or [K+] between BR and PL in the present study.

**Effects of \( \text{NO}_3^- \) Supplementation on Muscle Variables**

This is the first investigation to report the effects of \( \text{NO}_3^- \) supplementation on the \([\text{NO}_3^-]\) of skeletal muscle. Muscle \([\text{NO}_3^-]\) was elevated by 72% following BR compared to PL, indicating that in addition to increasing circulating plasma \([\text{NO}_3^-]\), supplementation also
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O\textsubscript{2} uptake kinetics during moderate- and high-intensity cycle exercise

increases muscle tissue [NO\textsubscript{3}]. This finding contributes to our understanding of the potential mechanistic bases behind the effects of NO\textsubscript{3} supplementation on the physiological responses to exercise. Unfortunately, due to the much lower tissue concentrations of NO\textsubscript{2} compared to NO\textsubscript{3}, it was not possible to ascertain the influence of NO\textsubscript{3} supplementation on skeletal muscle [NO\textsubscript{2}] in the present study.

Using \textsuperscript{31}P-MRS, Bailey et al. (2010) reported that during low-intensity single-leg knee-extensor exercise, NO\textsubscript{3} supplementation resulted in reduced ATP turnover for the same work rate, with a smaller reduction in muscle PCr and less accumulation of ADP and P\textsubscript{i}. Similar effects were observed during high-intensity exercise and the positive changes in muscle metabolic and pulmonary V\textsubscript{O}\textsubscript{2} responses were associated with an improved tolerance to high-intensity exercise (Bailey et al., 2010). It is important to note that in the study of Bailey et al. (2010), the pulmonary V\textsubscript{O}\textsubscript{2} and muscle metabolic (assessed non-invasively by \textsuperscript{31}P-MRS) responses were measured on separate occasions. An important strength of the present study is that the V\textsubscript{O}\textsubscript{2} and muscle metabolic (measured via biopsy) responses were measured simultaneously during cycle ergometer exercise. In contrast to our hypothesis, NO\textsubscript{3} supplementation did not significantly alter the muscle metabolic response to exercise relative to placebo. As would be expected, the overall lack of significant change in muscle [ATP], [PCr], [lactate], [creatine] and pH during exercise between the BR and PL conditions was associated with no significant changes in the group mean V\textsubscript{O}\textsubscript{2} response or exercise tolerance. The sampling, handling and analytical processes involved in the muscle biopsy procedure may provide a heightened risk of measurement error compared to previous research utilising \textsuperscript{31}P-MRS techniques (Bailey et al., 2010; Vanhatalo et al., 2011).

Effects of NO\textsubscript{3} Supplementation on Pulmonary O\textsubscript{2} Uptake

In the present study, there were no changes in the group mean V\textsubscript{O}\textsubscript{2} response to moderate- or severe-intensity constant-work-rate cycling exercise. Previous research has reported significant reductions in baseline (Lansley et al., 2011a) and steady-state V\textsubscript{O}\textsubscript{2} (Bailey et al., 2009; Larsen et al., 2007, Vanhatalo et al., 2010) during moderate-intensity exercise following NO\textsubscript{3} supplementation. Furthermore, reductions in the V\textsubscript{O}\textsubscript{2} slow component (Bailey et al., 2009) and speeding of the phase II \(\tau\) (Kelly et al., 2013) have been observed in response to severe-intensity exercise as a result of NO\textsubscript{3} supplementation. These findings are thought to be consequent to modulations to the ATP cost of muscle force production.
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O$_2$ uptake kinetics during moderate- and high-intensity cycle exercise (Bailey et al., 2009), altered intracellular calcium handling (Hernández et al., 2012) and/or preferential distribution of blood flow to type II muscle fibres (Ferguson et al., 2013). However, other studies have reported no significant difference in the VO$_2$ response to exercise following NO$_3^-$ supplementation (Bescos et al., 2012; Wilkerson et al., 2012).

Effects of NO$_3^-$ Supplementation on Exercise Tolerance

Tolerance to severe-intensity constant-work-rate exercise was not significantly altered following NO$_3^-$ supplementation in the current study, which is contrary to several previous studies (Bailey et al., 2009; Kelly et al., 2013; Lansley et al., 2011a; Vanhatalo et al., 2011). However, other studies have reported no improvement in incremental (Bescos et al., 2011) and time-trial (Bescos et al., 2012; Cermak et al., 2012; Peacock et al., 2012) protocols following NO$_3^-$ supplementation. Those studies that report limited effects of NO$_3^-$ supplementation have usually tested highly trained subjects and/or employed an acute NO$_3^-$ supplementation regimen (Bescos et al., 2012; Cermak et al., 2012; Wilkerson et al., 2012). There have been several studies suggesting the notion of ‘responders’ and ‘non-responders’ to NO$_3^-$ supplementation which may be related to the training status of the participants; that is, aerobically-trained subjects appear to benefit less from NO$_3^-$ supplementation than subjects who are less well trained (Wilkerson et al., 2012). Compared to less well-trained subjects, endurance athletes are known to have higher baseline plasma [NO$_2^-$] (Bescos et al., 2011; Jungersten et al., 1997; Schena et al., 2002), greater training-related NOS activity (McAllister et al., 2006; McConnell et al., 2007), a higher proportion of type I fibres, and greater mitochondrial and capillary density (Jensen et al., 2004), all of which may limit the potential for NO$_3^-$ supplementation to benefit performance (Wilkerson et al., 2012). However, the participants in the present study were recreationally active, with a mean VO$_{2peak}$ of ~52 ml·kg$^{-1}$·min$^{-1}$. Furthermore, the baseline plasma [NO$_2^-$] was similar to values that have been reported previously in recreationally-active individuals and the dose and timing of NO$_3^-$ supplementation utilized in the current study has previously been shown to be effective in this population (Bailey et al., 2009; Kelly et al., 2013).

Experimental Considerations

Although there was no significant difference in exercise tolerance between BR and PL, there was a significant improvement from visit 2 to visit 3 suggesting either a learning effect or a training effect. Whilst all subjects were comfortable within the laboratory setting
Chapter 7: Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O$_2$ uptake kinetics during moderate- and high-intensity cycle exercise

and had completed cycle ergometry protocols previously, some subjects had not experienced the muscle biopsy procedure before and it is possible that this contributed to the improved performance in *visit 3* compared to *visit 2*.

While this issue complicates data interpretation, it is interesting that, upon further inspection, subjects who consumed PL before BR evidenced an improved exercise performance in *visit 3*, whereas those subjects who consumed BR before PL had an impaired exercise performance in *visit 3*. Compared to the BR→PL group, the PL→BR group had: a significantly greater increase in plasma [NO$_2^-$]; a significantly reduced steady-state V̇O$_2$ during moderate-intensity exercise; and a significantly improved severe-intensity exercise tolerance (Table 4). Moreover, although not statistically significant, the PL→BR group had more than twice the muscle [NO$_3^-$] (8.72 vs. 3.84 nmol/mg DW) compared to the BR→PL group. There were no significant differences in V̇O$_{2peak}$ (as assessed in the initial ramp incremental test) or baseline plasma [NO$_2^-$] between the two groups. Irrespective of the order effect in our study, these results suggest that when BR supplementation successfully elevates plasma [NO$_2^-$], beneficial physiological effects such as a reduced steady-state V̇O$_2$ and improved severe-intensity exercise performance can arise.

Consistent with this interpretation, an important novel finding in the present study was that the change in muscle [NO$_3^-$] following BR supplementation was positively and significantly correlated with the change in severe-intensity exercise tolerance. Also, the change in steady-state V̇O$_2$ during moderate-intensity exercise following BR supplementation was negatively correlated with the change in severe-intensity exercise tolerance. This is consistent with a recent study in which we reported that BR supplementation reduced steady-state V̇O$_2$ during moderate-intensity exercise and increased severe intensity exercise tolerance in hypoxia, with these two variables being significantly correlated (Kelly *et al.*, 2014). Collectively, these results suggest that improved skeletal muscle efficiency consequent to greater NO bioavailability (as inferred from greater muscle [NO$_3^-$]) following BR supplementation may promote improved exercise performance. Therefore, while, overall, NO$_3^-$ supplementation did not influence muscle metabolism, V̇O$_2$ or exercise tolerance in the present study, perhaps due to the existence of an ‘order effect’, the data do indicate that an elevation in muscle [NO$_3^-$] has the potential to lower V̇O$_2$ during moderate-intensity exercise and to enhance severe-intensity exercise tolerance.
In conclusion, short-term dietary supplementation with NO₃⁻-rich beetroot juice increases plasma [NO₂⁻] and [NO₃⁻] as well as muscle [NO₃⁻]. Overall, BR supplementation had no significant effect on the muscle metabolic or VO₂ responses during moderate- or severe-intensity exercise, or severe-intensity exercise tolerance. However, a clear conclusion was obscured by the existence of an order effect, and further analysis indicated a significant relationship between the change in muscle [NO₃⁻] and the change in severe-intensity exercise tolerance following BR supplementation. Additional work is required to clarify the inter-relationships between skeletal muscle NO bioavailability (and its malleability via dietary supplementation), metabolic and mechanical efficiency, and fatigue resistance and performance.
Chapter 8: General Discussion

From the identification of NO as a gas by Joseph Priestly in 1772, to the Nobel Prize winning discovery of the endothelial-derived relaxation factor by Furchgott, Ignarro and Murad in the 1980s, and on to modern day scientific research, NO has attracted a wide variety of interest. For much of the time following its discovery, NO was thought of simply as an atmospheric pollutant, but findings over the last 30 years have identified NO as a major signaling molecule with a plethora of functions within the human body. As previously discussed, NO is produced endogenously by NOS enzymes which catalyze the oxidation of L-arginine and also via the NO₃⁻ - NO₂⁻ - NO pathway. Increasing dietary consumption of NO₃⁻ has been shown to elevate the bioavailability of NO and subsequently have a number of beneficial physiological effects. Among others, these include lowering blood pressure, reducing the O₂ cost of exercise and improving exercise tolerance. Scientific interest in the effects of NO₃⁻ supplementation has soared in recent years with many research articles being published.

Research questions addressed

The aim of this thesis was to address the ergogenic and therapeutic qualities of NO₃⁻ supplementation.

1) Does dietary NO₃⁻ supplementation modulate the power-duration relationship for severe-intensity exercise in young, healthy, recreationally active males?

2) How does dietary NO₃⁻ supplementation affect NO metabolism during exercise and can it have beneficial effects on exercise tolerance in hypoxic conditions?

3) Are the beneficial effects of dietary NO₃⁻ supplementation elicited in young, healthy participants also evident in a healthy, older population?

4) How does dietary NO₃⁻ supplementation influence skeletal muscle [NO₃⁻], pulmonary VO₂ and muscle metabolic responses to constant-work-rate moderate- and severe-intensity exercise. Does this help us to understand the mechanistic bases of previously reported improvements in exercise efficiency and exercise tolerance?

Summary of main findings

Influence of NO₃⁻ supplementation on the power-duration relationship

Novel findings from Chapter 4 included significant improvements in exercise tolerance following BR compared to PL at 60% Δ, 70% Δ and 80% Δ with a trend for improvement
at 100% peak power. Critical power and $W'$ remained statistically unchanged as a result of BR although the small, non-significant increments in both caused the hyperbolic power-duration relationship to shift up and rightward. The $\dot{V}O_2$ phase II time constant was slightly but significantly shorter (~10%) in BR compared to PL when all data were considered together, irrespective of exercise intensity. It was also found that BR had no effects upon resting metabolic rate. Data from this study suggests that NO$_3^-$ supplementation increased exercise tolerance across the severe-intensity exercise domain. Although statistically non-significant, in concert, the small improvements in CP and $W'$ would be expected to conflate into a meaningful improvement in cycling TT performance in sub elite cyclists.

Having established that NO$_3^-$ supplementation could improve severe-intensity exercise tolerance in young, healthy individuals, we wanted to establish whether manipulation of FIO$_2$ would alter the efficacy of the supplementation and whether the metabolism of NO and its derivatives would be affected by exercise-intensity and/or FIO$_2$.

Influence of NO$_3^-$ supplementation in hypoxia

Chapter 5, for the first time, characterized the kinetic profile of plasma [NO$_2^-$] during moderate- and severe-intensity exercise in hypoxia and normoxia. Results demonstrated that the rate of decline of plasma [NO$_2^-$] was greater during exercise following BR compared to PL, while FIO$_2$ had a lesser effect upon the decline of [NO$_2^-$]. In hypoxia, but not normoxia, BR supplementation reduced the O$_2$ cost of moderate-intensity exercise, speeded $\dot{V}O_2$ kinetics, and improved severe-intensity exercise performance. These findings may have important implications for individuals exercising at altitude as well as elderly and clinical populations when O$_2$ delivery can be impaired.

This data led us to investigate whether the beneficial effects of NO$_3^-$ supplementation observed in young healthy individuals in hypoxic conditions might also be observed in a healthy older population, where age-related declines in tissue oxygenation may be present.

Influence of NO$_3^-$ supplementation in an older population

Novel data from Chapter 6 indicated that BR supplementation reduced resting systolic, diastolic and mean arterial pressure compared to PL, in an older population (60-70 yrs). BR supplementation also reduced the $\dot{V}O_2$ mean response time and the O$_2$ deficit compared to PL, in response to moderate-intensity walking exercise. During low-intensity knee extensor exercise, the magnitude of PCR depletion was reduced by 15% following BR compared PL,
although this finding was non-significant. All other parameters including measures of exercising muscle metabolism, functional and cognitive capacity were unaltered following NO$_3^-$ supplementation. These findings suggest that NO$_3^-$ supplementation may provide a therapeutic intervention for reducing the risk of hypertension and improving VO$_2$ kinetics in older adults.

In order to provide further insight into the mechanistic bases underpinning the beneficial effects of NO$_3^-$ observed in Chapters 4, 5 and 6, Chapter 7 aimed to assess the effects of [NO$_3^-$] supplementation upon muscle [NO$_3^-$]. Chapter 7 also aimed to assess any changes in the VO$_2$, blood and muscle metabolic responses to moderate- and severe-intensity exercise, following NO$_3^-$ supplementation

**Influence of NO$_3^-$ supplementation on muscle metabolism**

Chapter 7 provided new insight into the effects of BR and showed that muscle [NO$_3^-$] was 72% greater in BR compared to PL, on average, across the entire protocol. Group pulmonary VO$_2$ and heart rate responses to moderate- and severe-intensity cycle exercise remained unchanged following BR, as did exercise tolerance to severe-intensity exercise. However, a clear conclusion was obscured by the presence of an order effect. Further analyses revealed a significant positive correlation between the change in muscle [NO$_3^-$] and the change in exercise tolerance, as well as a significant negative correlation between the change in moderate-intensity VO$_2$ and exercise tolerance. This study highlighted relationships between skeletal muscle NO bioavailability (and its malleability via dietary supplementation), metabolic efficiency, and fatigue resistance and performance.

The main focus of this series of experimental chapters was to investigate the ergogenic and therapeutic effects of dietary NO$_3^-$ supplementation. A wide range of physiological and performance parameters were assessed following dietary supplementation with NO$_3^-$-rich beetroot juice in varying environmental conditions and subject populations. In order to assess the ergogenic and therapeutic effects of NO$_3^-$ supplementation, it was imperative to elucidate whether NO$_3^-$ supplementation had stimulated NO production via the NO$_3^-$-NO$_2^-$-NO pathway.
Evidence of increased NO bioavailability

A consistent finding across all four experimental chapters, and in line with previous research (Bailey et al., 2009; Vanhatalo et al., 2010; Larsen et al., 2010; Kapil et al., 2010; Wylie et al., 2013a; Kelly et al., 2013a) was that NO\textsuperscript{3-} supplementation elevated circulating plasma [NO\textsubscript{3-}] and [NO\textsubscript{2-}]. Chapter 5 and 7 assessed changes in plasma [NO\textsubscript{3-}] and found a significant elevation following NO\textsubscript{3-} supplementation. This elevated concentration of circulating NO\textsubscript{3-} promotes an increase in circulating [NO\textsubscript{2-}], which is demonstrated in all chapters. The 197% and 400% increase in plasma [NO\textsubscript{2-}] in Chapter 4 and Chapter 5, respectively, were comparable to the elevations reported in previous studies (Bailey et al., 2009; Bailey et al., 2010; Lansley et al., 2010). The percentage elevations seen in Chapter 7 (~1000%) were somewhat higher than previously reported. Absolute [NO\textsubscript{2-}] following NO\textsubscript{3-} supplementation were comparable to previous research but [NO\textsubscript{2-}] following placebo were particularly low. Interestingly, resting control plasma [NO\textsubscript{2-}] in older adults in Chapter 6 was similar to that reported in young adults. This was surprising as lower [NO\textsubscript{2-}] values may have been expected in an older population (Sindler et al., 2011). Moreover, it may have been expected that the increase in plasma [NO\textsubscript{2-}] following NO\textsubscript{3-} supplementation might be smaller in older compared with younger adults due to age-related changes in the oral microbiome (Presley et al., 2011). However, the increase in plasma [NO\textsubscript{2-}] in Chapter 6 was greater (418%) than that found in previous research with younger adults (Bailey et al., 2009; Govoni et al., 2008; Larsen et al., 2007; Vanhatalo et al., 2010; Webb et al., 2008), but similar to that reported previously in older healthy subjects (Miller et al., 2012) and peripheral arterial disease patients (Kenjale et al., 2011). Despite the disparity in magnitude of change evidenced across the four studies, plasma [NO\textsubscript{3-}] and [NO\textsubscript{2-}] are clearly and consistently elevated following NO\textsubscript{3-} supplementation.

In Chapters 4 and 6 increases in plasma [NO\textsubscript{2-}] were coupled with reductions in systolic blood pressure. It is thought that increased plasma [NO\textsubscript{2-}] augments the bioavailability of NO which mediates smooth muscle relaxation and results in reductions in blood pressure reported (Archer et al., 1994). The effect of NO\textsubscript{3-} supplementation on blood pressure will be discussed in more detail in a later section.

Chapter 7 was the first investigation to report the effects of NO\textsubscript{3-} supplementation, in the form of beetroot juice on [NO\textsubscript{3-}] of human skeletal muscle tissue. The study reported that muscle [NO\textsubscript{3-}] was elevated by 72% following NO\textsubscript{3-} supplementation compared to placebo.
Chapter 8: General Discussion

This indicates that in addition to increasing circulating plasma $[NO_3^-]$, supplementation also stimulates muscle tissue $NO_3^-$ uptake.

In combination, this information may suggest that the bioavailability of NO was increased following $NO_3^-$ supplementation. $[NO_2^-]$ and $[NO_3^-]$ were assessed in order to provide an indication of how $NO_3^-$ supplementation can influence NO bioavailability. It is appreciated that increased $[NO_2^-]$ and $[NO_3^-]$ alone are not directly indicative of increases in systemic NO production and that measurement of cGMP would have provided further insight into $[NO]$. However, these measures have previously been used to provide estimations of NO bioavailability and are considered to be a practical and sensitive biomarker of NO status.

‘Utilization’ of $NO_3^-$ and $NO_2^-$ during exercise

The metabolism or ‘utilization’ of $NO_3^-$ and $NO_2^-$ during exercise was also assessed during Chapters 5 and 7. Chapter 7 characterized the kinetic profile of $NO_3^-$ and $NO_2^-$ during severe-intensity exercise in normoxic conditions. Results demonstrated that a ~60% depletion of $[NO_2^-]$ was evident at exhaustion following severe-intensity exercise in both BR and PL, although the absolute change in $[NO_2^-]$ was greater in BR. Previous research had reported a significantly larger percentage decrease of $[NO_2^-]$ in BR (54%) compared to PL (20%) (Wylie et al., 2013b). No changes in $[NO_3^-]$ were evident in Chapter 7 as a result of the severe-intensity exercise bout, which was in contrast to the previous study (Wylie et al., 2013b). Research suggests that the change in plasma $[NO_2^-]$ during exercise may be related to exercise performance (Dreissigacker et al., 2010), and suggests that the ability of an individual to ‘utilize’ $NO_2^-$ during exercise may be important to improving exercise performance. The absence of an increase in $NO_3^-$ during exercise and a percentage reduction of $[NO_2^-]$ similar to that seen in PL, may provide an explanation for the lack of ergogenic effect of $NO_3^-$ supplementation seen in Chapter 7.

Chapter 5 investigated the metabolism of $NO_2^-$ during moderate- and severe-intensity exercise in hypoxia and normoxia. The results of this study suggest that the metabolism of NO and its derivatives are altered by $NO_3^-$ supplementation and, to a lesser extent, $FIO_2$. However, interpretation of these data was not straightforward. $NO_3^-$ can be reduced in vivo to bioactive $NO_2^-$ and further to NO (Lundberg et al., 2011) and the reduction of $NO_2^-$ to NO is facilitated in hypoxic environments (Castello et al., 2006). However, $NO_2^-$ is also an oxidation product of NO generation via the ‘conventional’ NOS pathway (Ignarro et al., 1993).
Chapter 8: General Discussion

In Chapter 5, plasma [NO$_2$] declined during both moderate- and severe-intensity exercise. The magnitude and rate of decline of [NO$_2$] was significantly greater during exercise following BR compared to PL in both normoxia and hypoxia, perhaps suggesting increased utilization of the elevated NO$_2^-$ stores present following NO$_3^-$ supplementation. Following 5-min of moderate-intensity exercise, [NO$_2$] had fallen significantly below pre-exercise baseline in N-BR. In H-BR, only a trend in the fall of [NO$_2$] was evident from baseline. During severe-intensity exercise, the rate of plasma [NO$_2$] decline was not significantly different between conditions, but the absolute fall in plasma [NO$_2$] tended to be less in H-BR compared to N-BR. In concert, these data may suggest that in hypoxia, the contribution of NOS to NO production and subsequently to the regulation of muscle perfusion and matching of O$_2$ supply and demand may be slightly greater (Casey et al., 2010). It is important to note that differences in plasma [NO$_2$] dynamics between hypoxia and normoxia were not substantial, either during exercise or in recovery.

The current thesis has contributed to the understanding of how NO$_3^-$ supplementation can increase NO bioavailability. Specifically, Chapter 7 highlighted, for the first time, that NO$_3^-$ supplementation can significantly increase skeletal muscle [NO$_3$], while Chapter 5 was the first published research to characterize the effect of exercise intensity and FIO$_2$ on the kinetic response of plasma [NO$_2$] and [NO$_3$] during and in recovery from exercise.

**Ergogenic effects of NO$_3^-$ supplementation**

**Exercise tolerance**

In line with one of the overarching aims of this thesis, to assess the ergogenic effects of NO$_3^-$ supplementation; three of the four experimental chapters assessed tolerance to severe-intensity exercise whilst the fourth measured functional capacity using a validated walking test. Chapter 4 identified that NO$_3^-$ supplementation significantly improved tolerance to constant work rate cycle exercise during 3 different severe-intensities (60%Δ; ↑ 17%, 70%Δ; ↑ 16% and 80%Δ; ↑ 12%) and resulted in a non-significant, 10% improvement at 100% peak. This was the first study to assess the effects of NO$_3^-$ supplementation upon exercise tolerance at intensities above and below 70-75%Δ. The magnitudes of these improvements were consistent with previous research (Bailey et al., 2009) and would suggest a beneficial, effect of NO$_3^-$ supplementation. In Chapter 5, tolerance to severe-intensity (75%Δ) cycle exercise in hypoxia was significantly improved (9%). This finding is in line with previous studies which have reported that NO$_3^-$ supplementation increases
exercise tolerance during constant work rate (Vanhatalo et al., 2011) and incremental exercise protocols (Masschelein et al., 2012) as well as enhancing cycling time trial performance (Muggeridge et al., 2014) in hypoxia.

In contrast to previous findings (Bailey et al., 2009; Lansley et al., 2011a; Breese et al., 2013), Chapter 5 found no effect of BR supplementation on tolerance to severe-intensity (75%Δ) exercise in normoxia. Chapter 6 demonstrated that NO₃⁻ supplementation had no significant effects upon functional capacity in an older population. There was, however, a 2.2% mean increase in total distance covered in a 6MWT following NO₃⁻ supplementation, which is similar to the improvements in performance reported for 4km and 16.1km (~2.7%; Lansley et al., 2011a) and 10km (~1.0%; Cermak et al., 2012) cycling time trials in younger adults.

Finally, Chapter 7 identified that tolerance to severe-intensity (75%Δ) constant work rate cycle exercise in normoxia was not significantly altered, at the group level, following NO₃⁻ supplementation. These data are contrary to most existing literature (Bailey et al., 2009; Lansley et al., 2011a; Vanhatalo et al., 2011) although some studies have reported no improvement in incremental (Bescos et al., 2011) and time-trial protocols (Bescos et al., 2012; Cermak et al., 2012; Peacock et al., 2012). However, Chapter 7 did reveal that the change in muscle [NO₃⁻] following NO₃⁻ supplementation was positively and significantly correlated with the change in severe-intensity exercise tolerance. Chapter 7 was the first study to report muscle [NO₃⁻], in humans, following NO₃⁻ supplementation. Further work is required to clarify the inter-relationships between changes in muscle [NO₃⁻] and changes in severe-intensity exercise tolerance following NO₃⁻ supplementation.

Combining the findings of this thesis with existing literature, it would be fair to suggest that dietary NO₃⁻ supplementation could be utilized as an ergogenic aid in extending severe-intensity exercise tolerance in normoxic (Bailey et al., 2009; Lansley et al., 2011a; Breese et al., 2013) and, in particular, hypoxic conditions (Vanhatalo et al., 2011; Muggeridge et al., 2014; Masschelein et al., 2012).

Underlying mechanisms behind improvements in exercise tolerance may include better exercise efficiency, up- and rightward shifting of the power-duration relationship, speeded \(\dot{V}O_2\) kinetics and altered muscle metabolism. The following sections will outline how these parameters are altered following NO₃⁻ supplementation and their potential with changes in exercise tolerance.
Chapter 8: General Discussion

**Exercise efficiency**

In 3 of the 4 experimental Chapters in this thesis the O\textsubscript{2} cost of moderate-intensity exercise was assessed during constant work rate exercise trials. Chapter 6 assessed treadmill walking exercise in an ageing population (60-70 years). Analysis revealed that no effects on the O\textsubscript{2} cost of walking were evident, which is in contrast to results previously reported in younger adults (Lansley *et al.*, 2011b) and to the body of literature, which indicates that NO\textsubscript{3}– supplementation improves exercise efficiency (Bailey *et al.*, 2009 Vanhatalo *et al.*, 2010 Larsen *et al.*, 2011). Whilst surprising, the lack of significant change in walking economy in Chapter 6 was consistent with the lack of change in muscle metabolic responses and functional capacity observed.

Chapters 5 and 7 assessed exercise efficiency during cycle ergometer exercise in a young healthy population in both normoxia (Chapters 5 & 7) and hypoxia (Chapter 5). These two studies demonstrated that during moderate-intensity cycle ergometry in normoxia, amongst young healthy subjects, NO\textsubscript{3} had no effect upon the O\textsubscript{2} cost of exercise. Again, these findings are in contrast to some (Bailey *et al.*, 2009 Vanhatalo *et al.*, 2010 Larsen *et al.*, 2011) but not all (Bescos *et al.*, 2012; Breese *et al.*, 2013; Wilkerson *et al.*, 2012) previous research.

However, in Chapter 5, NO\textsubscript{3}– supplementation was seen to reduce baseline (unloaded) cycling VO\textsubscript{2} by 10% and moderate-intensity exercise VO\textsubscript{2} by 7% in hypoxia, compared to PL. These findings are consistent with previous studies which have reported 4-8% reductions in steady state VO\textsubscript{2} during moderate-intensity cycle exercise in hypoxia, as a result of NO\textsubscript{3}– supplementation (Masschelein *et al.*, 2012; Muggeridge *et al.*, 2014). As outlined in the literature review, the mechanistic bases behind reductions in the O\textsubscript{2} cost of exercise may include improved mitochondrial efficiency (Larsen *et al.*, 2011) and/or reductions in the ATP cost of muscle force production (Bailey *et al.*, 2009), mediated by enhanced calcium-related muscle contractility (Hernandez *et al.*, 2012). NO is involved in the regulation of mitochondrial O\textsubscript{2} consumption and it is known to have a strong affinity for cytochrome-c oxidase (COX) (Brown, 2001). The competition for the COX binding site between NO and O\textsubscript{2} may be responsible, in part, for the reduced O\textsubscript{2} cost of exercise following NO\textsubscript{3}– supplementation (Larsen *et al.*, 2007; Bailey *et al.*, 2009). This may also initiate a signaling cascade resulting in mitochondrial protein changes which collectively enhance respiratory chain efficiency (Larsen *et al.*, 2011). Hypoxia, itself, may also result
in an acute, reversible inhibition of COX (Brown et al., 1999). The combined effects of hypoxia and NO\textsuperscript{3} supplementation may therefore make it more likely for these effects to occur in hypoxic conditions. While a significant improvement in exercise efficiency was only evident in one study of the current thesis, an intriguing relationship between submaximal exercise efficiency and severe-intensity exercise was revealed.

**Relationship between submaximal exercise efficiency and exercise tolerance**

An important and novel common theme that emerged from this thesis was a clear relationship between changes in the O\textsubscript{2} cost of submaximal exercise and changes in severe-intensity exercise tolerance following NO\textsuperscript{3} supplementation. Chapter 5 found a significant correlation between the reduction in steady-state V\textsubscript{O}_2 and the improvement in exercise tolerance following NO\textsuperscript{3} supplementation in hypoxia. Similarly, Chapter 7 demonstrated that the change in steady-state V\textsubscript{O}_2 during moderate-intensity exercise following NO\textsuperscript{3} supplementation was negatively correlated with the change in severe-intensity exercise tolerance. These data may suggest that NO\textsuperscript{3} supplementation is more effective in some individuals than others (discussed in a later section). Furthermore, why reductions in the O\textsubscript{2} cost of exercise or alterations in the V\textsubscript{O}_2 slow component were not evident during subsequent severe-intensity exercise bouts is not clear. It could be speculated that certain underlying mechanisms of NO\textsuperscript{3} supplementation become more prominent at different exercise intensities. For example, during moderate-intensity exercise the most prominent effect of NO\textsuperscript{3} supplementation is the reduction in the O\textsubscript{2} cost of exercise (evidenced by increased exercise efficiency), perhaps due to a combination of improved mitochondrial efficiency and a reduced ATP cost of force production. However, during severe-intensity exercise, mechanisms involved in vasodilatory-mediated increases in muscle blood flow and preferential distribution of blood to type II muscle fibers (evidenced by speeded V\textsubscript{O}_2 kinetics and improved exercise tolerance) may be relatively more important. Regardless of the underpinning explanation, these results do suggest that improved skeletal muscle efficiency during moderate-intensity exercise, consequent to greater NO bioavailability following NO\textsuperscript{3} supplementation, may promote improved severe-intensity exercise tolerance and performance.

**Power-duration relationship**

While accepting that improved exercise tolerance to any particular constant work rate is reflective of a physiological benefit of an intervention, it does not act as a sufficient
quantitative measure of the actual improvement in function as it provides data from just a single point of that relationship. Ideally, characterisation of the pre- and post-intervention power-duration relationship is necessary (Whipp & Ward, 2009). Chapter 4 examined the effects of NO₃⁻ supplementation upon four exercise intensities spanning the severe-intensity exercise domain. The rationale for this was two-fold: 1) to establish whether NO₃⁻ supplementation was as effective at higher severe-intensities (not previously investigated) as it was in the previously reported lower severe-intensities; and 2) to characterise the power-duration relationship, with and without NO₃⁻ supplementation. We therefore used the four constant power output exercise bouts in the BR and PL conditions to calculate the CP and W′, characterizing the power-duration relationship. NO₃⁻ supplementation resulted in a 1.4% (3 W) increase in CP and an 8.4% (1.5 kJ) increase in W′. While the modest improvements in CP and W′ did not appear to be substantial and were not statistically significant, when the two parameters were combined to predict performance, the time to complete a fixed amount of work was significantly less in BR compared to PL across the power-duration relationship. The potential benefits highlighted for performance (approximately 2-3%) were much greater than the 0.6% value suggested to be the smallest ‘worthwhile’ improvement for road TT cyclists (Paton & Hopkins, 2006). Interestingly, the differences between PL and BR in predicted performance were very similar to the beneficial effects of NO₃⁻ supplementation reported for cycling TT performance previously (4 km TT improved by 2.8% (Lansley et al., 2011); 10 km TT improved by 1.2% (Cermak et al., 2012); and 16.1 km TT improved by 2.7% (Lansley et al., 2011)). These data provide evidence to support that the beneficial effects of NO₃⁻ supplementation upon severe-intensity exercise tolerance can be explained by, and are coupled with, an up- and rightward shifting of the power-duration relationship.

The rate of adaptation of \(\dot{V}O_2\) at the onset of exercise (phase II time constant) and the trajectory of the \(\dot{V}O_2\) response towards its maximum (\(\dot{V}O_2\) slow component), are vitally important during constant work rate exercise. These responses, termed \(\dot{V}O_2\) kinetics, are associated with the CP and W′ and have been strongly linked with determining exercise tolerance during severe-intensity exercise. Slow \(\dot{V}O_2\) kinetics and the \(\dot{V}O_2\) slow component are associated with a greater depletion of intramuscular [PCr], greater utilization of intramuscular glycogen stores and the accumulation of fatiguing metabolites within the exercising muscle (Poole et al., 1991; Rossiter et al., 2002; Krustrup et al., 2004), all of which may lead to reduced exercise tolerance.
Chapter 8: General Discussion

Alterations in these responses following NO$_3^-$ supplementation may provide underlying mechanistic explanations behind the positive shifting of the power-duration relationship and improved exercise tolerance reported.

$\dot{V}O_2$ kinetics

Faster $\dot{V}O_2$ kinetics would be expected to reduce the reliance on non-oxidative metabolic processes across the transition from a lower to a higher metabolic rate and, therefore, to reduce muscle metabolic perturbation (Jones & Poole, 2005). In fact $\dot{V}O_2$ kinetics is considered as a key determinant of high-intensity exercise tolerance in humans (Murgatroyd et al., 2011). Specifically the phase II time constant is known to be closely related to CP; while the $\dot{V}O_2$ slow component amplitude is associated with the W’ (Murgatroyd et al., 2011). Therefore, any improvements in exercise tolerance may be explained, in part, by faster $\dot{V}O_2$ kinetics following NO$_3^-$ supplementation. This beneficial effect has emerged as a novel finding from this thesis and contributes significantly to our understanding of NO$_3^-$ supplementation as an ergogenic aid.

Chapter 6 revealed a small but significant speeding of $\dot{V}O_2$ kinetics following the onset of exercise as a result of NO$_3^-$ supplementation in older adults. As a function of this $\dot{V}O_2$ speeding, the O$_2$ deficit was reduced by 15%. Research prior to this thesis, in young adults had not demonstrated speeded $\dot{V}O_2$ kinetics during moderate-intensity exercise, following NO$_3^-$ supplementation. However, older adults typically have slower $\dot{V}O_2$ kinetics (Babcock et al., 1994; Chilibeck et al., 1996; Delorey et al., 2005) and are more likely to exhibit a speeding of $\dot{V}O_2$ kinetics following interventions designed to enhance muscle O$_2$ delivery (Scheuermann et al., 2002) than younger individuals. The faster $\dot{V}O_2$ kinetics observed in Chapter 6 could have been linked to NO-mediated enhanced muscle vasodilatation and blood flow (Ferguson et al., 2013), which may have offset a possible O$_2$ delivery limitation to $\dot{V}O_2$ kinetics in the older subjects. It would be prudent to note here that the $\dot{V}O_2$ mean response times evident in this cohort of older subjects were remarkably fast. This may suggest that this group of older individuals were not particularly representative of the aging population when compared to previous literature. Even so, the speeding of the $\dot{V}O_2$ MRT is feasibly explained by increased O$_2$ delivery to an environment that was relatively hypoxic.

Interestingly, the $\dot{V}O_2$ phase II time constant during moderate-intensity exercise was also reduced by NO$_3^-$ supplementation in hypoxia, in young healthy individuals, in Chapter 5. The hypoxic inspirate showed a trend toward slowing $\dot{V}O_2$ kinetics (increasing phase II
time constant) in the young healthy participants, as expected (Hughson & Kowalchuk., 1995, Springer et al., 1991). Remarkably, NO\textsubscript{3} supplementation speeded the phase II time constant in hypoxia toward values recorded in normoxia. NO\textsubscript{3} supplementation also tended to ameliorate the negative effects of hypoxia upon total oxygenation index (TOI) of the vastus lateralis muscle, as measured by NIRS, in a similar fashion to previous research (Masschelein et al., 2012). The improved TOI with NO\textsubscript{3} supplementation indicates better muscle oxygenation (Ferrari et al., 2004) which, as previously discussed, may be responsible for the speeding of the VO\textsubscript{2} phase II time constant. These findings suggest that NO\textsubscript{3} supplementation can help to reverse the detrimental effect of a reduced FIO\textsubscript{2} on VO\textsubscript{2} kinetics, during moderate-intensity exercise. In support of this, emerging data utilizing \textsuperscript{31}P-MRS techniques indicate that muscle PCr recovery kinetics (which reflect maximal rate of mitochondrial ATP resynthesis) appear to be unaffected by NO availability in normoxia (Fulford et al., 2012), but are speeded following NO\textsubscript{3} supplementation in hypoxia (Vanhatalo et al., 2011; Vanhatalo et al., 2014). The fact that PCr recovery kinetics are affected by NO availability in hypoxia but not normoxia may be a result of the vasodilatory effect of NO, which accelerates such recovery in an O\textsubscript{2}-limited condition, but has no influence under normoxic exercise and recovery. Collectively, these data support the findings in Chapter 6 where NO\textsubscript{3} supplementation reversed the potential O\textsubscript{2} delivery limitation on VO\textsubscript{2} kinetics in an older population.

Considering improvements in exercise efficiency following NO\textsubscript{3} supplementation were not evident during severe-intensity in the current thesis, it was interesting to note that the phase II time constant was also influenced during severe-intensity exercise. When considered in isolation, analyses in Chapter 4 revealed no significant changes in the VO\textsubscript{2} phase II time constant at power outputs representing 60% Δ, 70% Δ, 80% Δ or 100% peak, following NO\textsubscript{3} supplementation. However, the VO\textsubscript{2} phase II time constant was slightly and significantly faster in BR compared to PL when all data were considered together, irrespective of exercise intensity. One potential explanation of the speeding kinetics as a result of NO\textsubscript{3} supplementation during severe-intensity exercise is a preferential distribution of O\textsubscript{2} delivery to Type II fibres (Ferguson et al., 2012) and/or to muscle loci that may be relatively more hypoxic (Thomas et al., 2001; Hagen et al., 2003; Victor et al., 2009). As previously discussed, faster VO\textsubscript{2} kinetics may reduce the contribution of substrate-level phosphorylation to energy turnover in the first 1-2 min following the transition to high-
intensity exercise and may help to improve exercise tolerance (Burnley & Jones 2007; Murgatroyd et al., 2011).

Novel data from this thesis consistently demonstrate that NO\textsuperscript{3−} supplementation can positively modulate the VO\textsubscript{2} kinetic response, specifically speeding the phase II time constant. This occurred during both moderate- and severe-intensity exercise in situations where the intramuscular environment may have been more hypoxic. Research subsequent to the completion of the experimental chapters in question have corroborated these findings (Fulford et al., 2012; Vanhatalo et al., 2014). These data add a new mechanistic insight into the beneficial effects of NO\textsuperscript{3−} supplementation and may have important implications in utilising NO\textsuperscript{3−} supplementation to improve exercise tolerance in both athletic and ageing/clinical populations.

Muscle metabolism

Chapter 6 utilised \textsuperscript{31}P-MRS to assess the muscle metabolic response to low- and high-intensity knee-extensor exercise in a healthy older population with and without NO\textsuperscript{3−} supplementation. The findings indicated a 15% attenuation of muscle [PCr] degradation during low-intensity exercise following NO\textsuperscript{3−} supplementation, although this was not statistically significant. Previous research in younger adults has reported a reduction in the amplitude of [PCr] depletion during low-intensity (Bailey et al., 2010) and heavy-intensity exercise (Vanhatalo et al., 2014), following NO\textsuperscript{3−} supplementation. It is currently unclear why the fall in muscle [PCr] was not significantly spared in this older population, although inter-individual variability may have precluded the attainment of statistical significance. In addition to this, a lower ATP cost of muscle contraction in older adults (Tevald et al., 2010) may have served to reduce the impact of NO\textsuperscript{3−} supplementation on muscle contraction efficiency. Chapter 6 also assessed the kinetic recovery of [PCr] following high-intensity exercise. This rate of recovery is thought to reflect the maximal rate of oxidative synthesis of ATP (Kemp et al., 1993) via increased mitochondrial volume and/or oxidative enzyme activity or, in the event of hypoxia, increased O\textsubscript{2} supply. Consistent with previous research in younger adults in normoxia (Lansley et al., 2011; Fulford et al., 2013; Vanhatalo et al., 2014), NO\textsuperscript{3−} supplementation did not significantly alter muscle [PCr] recovery kinetics. This was surprising, given that we may expect the older individuals to be experiencing age-related tissue hypoxia and may have been sensitive to an increase in O\textsubscript{2} delivery as a result of NO\textsuperscript{3−} supplementation.
Chapter 8: General Discussion

Chapter 7 assessed the muscle metabolic response alongside the VO\textsubscript{2} response to moderate- and severe-intensity exercise following NO\textsubscript{3}\textsuperscript{−} supplementation. Data obtained from muscle biopsy samples indicated that NO\textsubscript{3}\textsuperscript{−} supplementation significantly elevated muscle [NO\textsubscript{3}\textsuperscript{−}]. However, analyses revealed that NO\textsubscript{3}\textsuperscript{−} supplementation had no effect upon [citrate synthase], [HAD] [ATP], [PCr], [lactate], [creatine] and pH. As would be expected, the overall lack of significant change in muscle [ATP], [PCr], [lactate], [creatine] and pH during exercise between the BR and PL conditions was associated with no significant changes in the group mean VO\textsubscript{2} response or exercise tolerance.

Data from this thesis demonstrated for the first time that human muscle [NO\textsubscript{3}\textsuperscript{−}] is significantly elevated following NO\textsubscript{3}\textsuperscript{−} supplementation. Assessment of key fatigue-related muscle metabolites was made during exercise in the current thesis using both \textsuperscript{31}P-MRS and muscle biopsy techniques. The presented data suggest that NO\textsubscript{3}\textsuperscript{−} supplementation had no effect upon muscle metabolism. These findings were accompanied by no changes in pulmonary VO\textsubscript{2} responses or exercise tolerance and were therefore not surprising. However, these findings contradict previous research (Bailey \textit{et al.}, 2010; Vanhatalo \textit{et al.}, 2014). When NO\textsubscript{3}\textsuperscript{−} supplementation-mediated alterations in exercise efficiency (assessed by pulmonary VO\textsubscript{2}) are evident, it is expected that changes in muscle metabolism would also be present.

\textbf{Therapeutic effects of NO\textsubscript{3}\textsuperscript{−} supplementation}

\textit{Blood pressure}

The reduction of blood pressure is arguably one the most important physiological benefits of NO\textsubscript{3}\textsuperscript{−} supplementation as it has potential implications for the prevention and/or treatment of hypertension. It is thought that increased plasma [NO\textsubscript{2}\textsuperscript{−}] augments the bioavailability of NO. Increased intracellular NO promotes smooth muscle relaxation via the synthesis of cyclic guanosine monophosphate and it is this NO-mediated smooth muscle relaxation that is considered to be responsible for the reduction in blood pressure reported (Archer \textit{et al.}, 1994). Findings from the current thesis present positive results with regard to the reduction of blood pressure following NO\textsubscript{3}\textsuperscript{−} supplementation. Chapter 4 assessed resting blood pressure in young, healthy individuals and consistent with previous research (Larsen \textit{et al.}, 2006; Webb \textit{et al.}, 2008; Kapil \textit{et al.}, 2010; Bailey \textit{et al.}, 2010; Kelly \textit{et al.}, 2013), demonstrated a significant 4 mmHg reduction in systolic blood pressure. Likewise, Chapter 6, which, for the first time, assessed the effects of NO\textsubscript{3}\textsuperscript{−} supplementation on blood pressure
Chapter 8: General Discussion

in a healthy, older population, demonstrated significant reductions in systolic (5 mm Hg),
diastolic (3 mm Hg) and mean arterial pressure (3 mm Hg).

Importantly, data from Chapters 4 and 6 provide positive and encouraging indications that
NO$_3^-$ supplementation could be used as a practical, relatively cheap, prophylactic and/or
therapeutic aid in the prevention or treatment of hypertension across the lifespan.

_Cerebral measures ($^1$H MRS, ADC) and cognitive function_

Chapter 6 considered whether NO$_3^-$ supplementation may provide beneficial effects upon
metabolic efficiency and blood flow within the brain, in a similar fashion to what had been
reported within skeletal muscle (Larsen et al., 2007; Vanhatalo et al., 2011). However, no
significant differences in [NAA] (an amino acid suggested to be a marker of neuronal
viability, as well as an intellectual and neuropsychological measure of cognition) was
evident following NO$_3^-$ supplementation. Similarly, [myo-inositol] (a carbohydrate found in
the brain that is elevated in patients with Alzheimer’s disease) was unaffected by NO$_3^-$
supplementation. Moreover, there were no changes in the concentrations of creatine or
choline in the brain, both of which are considered important in neurological health and
cognitive ability. There were also no changes to apparent diffusion coefficients in key
regions of the brain following NO$_3^-$ supplementation, a finding which was contrary to a
previous study (Pressley et al., 2010). As discussed in the experimental chapter, the lack of
findings may be due to the relatively young age and good health of the recruited
participants.

Chapter 6 also explored whether NO$_3^-$ supplementation could positively impact upon
cognitive function in an older population. Measures of attention, concentration, information
processing and working memory were completed using validated cognitive function tests.
No significant differences in cognitive function were evident following NO$_3^-$
supplementation, which may not be considered surprising given that there were no
significant changes in the NMR parameters of cerebral functionality or metabolism. The
absence of any effects here may be explained, in part, by the good general health of the
participants.

Whilst the findings from Chapter 6 indicate that there were no effects of NO$_3^-$
supplementation upon cerebral measures and cognitive function, the rationale and potential
Chapter 8: General Discussion

effects of this supplementation upon cerebral physiology and function needs to be further explored and provides an exciting avenue of further research.

*Arterial oxygen saturation and muscle oxygenation*

During Chapter 5, arterial oxygen saturation (SaO$_2$) was continuously assessed during the testing protocol, in order to examine the effects of FIO$_2$, NO$_3^-$ supplementation and as a safety requirement. The study identified that hypoxia significantly reduced SaO$_2$ during rest, moderate and severe-intensity exercise. Further analyses revealed that at rest, NO$_3^-$ supplementation tended to blunt the reductions in SaO$_2$ caused by hypoxia, which may indicate improved oxygenation. Previous research has reported that dietary NO$_3^-$ supplementation results in small increases in arterial oxygen saturation during exercise in hypoxia (Masschelein et al., 2012; Schiffer et al., 2013; Muggeridge et al., 2014) although this was not reflected in Chapter 5 of this thesis. Indices of muscle oxygenation were measured using NIRS during exercise and recovery in hypoxia and normoxia and following NO$_3^-$ supplementation in Chapter 5. During moderate-and severe-intensity exercise, the manipulated FIO$_2$ altered muscle oxygenation while BR supplementation had no significant influence upon the response. Specifically, [HHb] was greater in hypoxia, indicating that muscle fractional O$_2$ extraction was increased, while [HbO$_2$] and TOI were significantly reduced in hypoxia compared to normoxia, findings which were all consistent with previous research (Masschelein et al., 2012). Interestingly, during moderate-intensity exercise NO$_3^-$ supplementation tended to ameliorate the negative effects of hypoxia upon TOI, indicating improved muscle oxygenation (Ferrari et al., 2004). Furthermore and consistent with a possible improvement in oxygenation status, the typical compensatory rise in heart rate in hypoxia was attenuated during moderate-intensity exercise, following NO$_3^-$ supplementation.

Collectively, the blunting of reductions in SaO$_2$ at rest, improved TOI and attenuation of heart rate during moderate-intensity exercise in hypoxia could have important implications for individuals suffering from tissue hypoxia inducing pathologies (anaemia, COPD, diabetes) and suggests an additional therapeutic mechanism of NO$_3^-$ supplementation.

To summarise and in line with the aims outlined at the end of the literature review, this thesis has examined the potential ergogenic and therapeutic capabilities of dietary NO$_3^-$ supplementation, in the form of NO$_3^-$ rich beetroot juice. The findings of this thesis demonstrate that indeed NO$_3^-$ supplementation may be considered as an ergogenic aid for
severe-intensity cycle exercise and may elicit therapeutic effects in hypoxic environments as well as upon cardiovascular health across both young and old populations. However, data from this thesis also clearly outlines that NO₃⁻ supplementation may not always be effective.

**Effectiveness of NO₃⁻ supplementation**

Chapters 5 and 7 indicate that NO₃⁻ supplementation elicits beneficial effects in some individuals but not others. Chapter 5 found a significant correlation between the reduction in steady-state VO₂ and the improvement in exercise tolerance following NO₃⁻ supplementation in hypoxia. Similarly, Chapter 7 demonstrated that the change in steady-state VO₂ during moderate-intensity exercise following BR supplementation was negatively correlated with the change in severe-intensity exercise tolerance. In Chapter 7, the change in muscle [NO₃⁻] following BR supplementation was positively and significantly correlated with the change in severe-intensity exercise tolerance. It was evident in Chapter 7 that 4 subjects responded to the supplementation and 4 subjects did not. The ‘responders’ had a significantly greater increase in plasma [NO₂⁻]; a significantly reduced steady-state VO₂ during moderate-intensity exercise; and a significantly improved severe-intensity exercise tolerance, compared to the ‘non responders’. In addition, although not statistically significant, the ‘responders’ had more than twice the muscle [NO₃⁻] compared to the ‘non-responders’. The mechanistic bases behind differences in the effectiveness of NO₃⁻ supplementation, or responsiveness of the participant are not fully understood and obligate further research. Previous research (Wilkerson et al., 2012) has also outlined the potential for ‘responders’ and ‘non-responders’ to NO₃⁻ supplementation and suggest a number of explanations for this. Some studies indicate that NO₃⁻ supplementation may be less effective as an ergogenic aid in highly-trained endurance athletes, at least when NO₃⁻ is ingested acutely and/or longer duration, lower-intensity endurance performance is assessed (Bescos et al., 2012, Cermak et al., 2012, Wilkerson et al., 2012; Christensen et al., 2013). Compared to less well-trained subjects, endurance athletes have higher baseline plasma [NO₂⁻], greater training-related NOS activity, a higher proportion of type I fibres, and greater mitochondrial and capillary density, all of which may reduce the potential benefits of NO₃⁻ supplementation (Wilkerson et al., 2012).

Each of the experimental chapters in this thesis have utilised chronic supplementation periods (2-3 days), moderate- and severe-intensity exercise and have purposefully recruited
recreationally active individuals. Therefore, the evidence of ‘responders’ and ‘non-responders’ in the current thesis, is particularly interesting. An additional potential explanation behind non response to supplementation could be that of oral bacteria. The oral microbiome is vital in the reduction of NO$_3^-$ to NO$_2^-$, a process that occurs on the surface of the tongue. If particular nitrate-reducing bacteria are not present in certain individuals, this may result in a lack of any beneficial effects. The oral microbiome is known to alter over the lifespan and should be further explored with regard to the effects of increased NO$_3^-$. This would provide valuable information about the effectiveness of NO$_3^-$ supplementation over the lifespan, but may also provide vital information for public health policy makers in charge of formulating nutritional intake recommendations. The current thesis did put particular controls in place to reduce the likelihood of variability in oral bacteria (restricted age range for individual studies, subjects were asked to refrain from using anti-bacterial mouthwash throughout the testing period and recruitment of non-smokers). Furthermore, there may also be some suggestion (Porcelli et al., 2014) that certain individuals require larger doses in order to benefit from NO$_3^-$ supplementation, which may be based upon body mass and/or training status.

It is clear from this thesis and from existing literature that NO$_3^-$ supplementation can elicit beneficial effects in some individuals but not others. It is likely that a combination of the oral microbiome, training status and dosing have an influence upon an individual’s responsiveness to the supplementation. Athletes, coaches, clinicians and the general public should be aware of these potential issues and should tailor the use of NO$_3^-$ supplementation accordingly.

Ergogenic applications

Importantly, this thesis provides data to suggest that NO$_3^-$ supplementation can have some direct practical applications. The improvements in exercise tolerance in shorter-duration, higher-intensity cycle exercise evidenced in Chapter 4 may be of interest to athletes and coaches involved in sports performance of > 3 but < 20 minutes. These data suggest that developing and utilizing specific pre-race nutritional plans targeted at increasing NO$_3^-$ intake may improve sports performance.

Data from this thesis also demonstrate that NO$_3^-$ supplementation caused a speeding of the $\dot{V}O_2$ phase II time constant during severe-intensity exercise in normoxia (Chapter 4) and moderate-intensity exercise in hypoxia (Chapter 5) in a young healthy population. A
speeding of the $\dot{V}O_2$ mean response time was also evident during moderate –intensity walking in a healthy older population (Chapter 6). These modulations to the $\dot{V}O_2$ response may have important implications to improving exercise tolerance and performance in a young population as well enhanced functional capacity in an older population.

Furthermore, data from Chapter 5 indicate that the oxygen cost of exercise is reduced during moderate-intensity exercise in hypoxia, with subsequent improved tolerance to severe-intensity exercise. Chapter 5 also revealed that during rest and moderate-intensity exercise NO$_3^-$ supplementation offset the typical hypoxia-induced increase in heart rate. These findings may provide valuable information for athletes and/or explorers exercising at altitude, where the oxygen content of atmospheric air is reduced.

**Therapeutic applications**

The findings of this thesis may also have a number of important therapeutic implications and ‘real life’ applications to a range of populations. The reductions in resting blood pressure as a result of increased NO bioavailability following NO$_3^-$ supplementation, reported in Chapters 4 and 6, may have important public health implications. Reducing resting systolic blood pressure in young and old normotensive individuals by 4 mmHg (Chapter 4) and 5 mmHg (Chapter 6) respectively, suggests that NO$_3^-$ supplementation may prove to be beneficial in the prevention and/or treatment of hypertension. In fact, a reduction in systolic blood pressure of just 2mmHg in individuals aged 40-69 could reduce CVD related mortality by 10% (Prospective Studies Collaboration, 2002). Moreover, it has been estimated that a reduction of 2 mmHg in the average adult’s systolic blood pressure could save more than 14,000 UK lives per year (Critchley & Capewell, 2003). This relatively low-cost, naturally-occurring, therapeutic aid may provide a cost effective method for off-setting the leading cause of cardiovascular disease (Hajjar *et al.*, 2006) and improving cardiovascular health worldwide.

In addition, the reduction in the oxygen cost of exercise during moderate-intensity exercise in hypoxia, and subsequent improvement in tolerance to severe-intensity exercise suggest that NO$_3^-$ supplementation may have therapeutic benefits for individuals suffering from pathological conditions, such as such as chronic obstructive pulmonary disease, diabetes, anaemia and peripheral arterial diseases, which induce tissue hypoxia, reduce functional capacity and ultimately compromise quality of life.
Chapter 8: General Discussion

These data could be informative to clinicians and policy makers in formulating public health nutritional recommendations. It provides additional evidence that increasing NO$_3^-$ intake can provide therapeutic health effects which may contribute to improving individual’s quality of life, decreasing disease incidence/prevalence and lowering the cost of care.

**Limitations**

*Nitrate dose*

In each experimental chapter of this thesis, all subjects were administered a fixed dose of NO$_3^-$. The administration of NO$_3^-$ dose relative to body mass may be a future avenue of investigation, to elucidate whether larger (or highly trained) individuals would require larger quantities of NO$_3^-$ in order to elicit beneficial effects. However, the doses (8-9 mmol of NO$_3^-$ per day) and supplementation periods (2-3 days) utilized were employed with knowledge of previous research which had demonstrated beneficial effects (significant increase in plasma NO$_2^-$, reductions in blood pressure and oxygen cost of exercise and improvements in exercise tolerance) following acute ingestion of nitrate supplementation (~5.2 mmol NO$_3^-$, 2.5 hours prior to testing) (Vanhatalo *et al.*, 2010).

*Dietary control*

Throughout all experimental chapters, subjects were instructed to maintain their normal daily food intake. This is in contrast to some early studies (Bailey *et al.*, 2009, Bailey *et al.*, 2010, Larsen *et al.*, 2007, Larsen *et al.*, 2010), in which subjects were instructed to exclude NO$_3^-$-rich foods (such as certain vegetables and cured meats) from their diet. Whilst the unrestricted dietary approach employed may have increased variability in NO$_3^-$ intake, this thesis aimed to investigate whether the positive effects of NO$_3^-$ supplementation were present when habitual NO$_3^-$ intake was not restricted. This provides ecological validity to using the supplementation and allows conclusions and implications to be made applicable to the wider public, who would not typically restrict their diet. Subjects were, however, asked to maintain a food diary throughout the supplementation periods. These were monitored for any individuals who had particularly high or low habitual NO$_3^-$ intake and whether this affected the efficacy of the supplementation.
Chapter 8: General Discussion

**Measurement of NO markers restricted to NO\textsubscript{2} and NO\textsubscript{3}**

The main aim of administering NO\textsubscript{3} supplementation was to promote the production and bioavailability of NO. The direct measurement of NO is extremely difficult due its very short half-life in vivo, of less than 0.1s (Kelm et al., 1990). In contrast, NO\textsubscript{2} and NO\textsubscript{3} are both stable metabolites of NO. They are present in blood and urine and are easily accessible to quantitative analysis. Therefore, measurement of NO\textsubscript{2} and NO\textsubscript{3} in various biological fluids (predominantly plasma in this thesis) has been shown to provide the most suitable and practical method to assess NO synthesis in vivo. In fact, research suggests that in particular, short-term changes in NO synthesis are best assessed by measuring plasma NO\textsubscript{2} concentration (Lauer et al., 2001), and so this measurement was employed throughout. It is appreciated that additional measures could have been made to provide a more comprehensive assessment of NO bioavailability, including concentrations of the cyclic nucleotide cGMP. Furthermore, we could have measured the concentrations of NO\textsubscript{2} and NO\textsubscript{3} in saliva and urine as well as exhaled NO in addition to our plasma and muscle analyses. This would have allowed the tracking of these metabolites from consumption to excretion/exhalation. These measures and approaches may be utilized in future research but fell outside of the scope of this thesis. The assessment of plasma NO\textsubscript{2} and NO\textsubscript{3} and muscle NO\textsubscript{3} provided the most suitable, appropriate and practical characterization of NO bioavailability following NO\textsubscript{3} supplementation.

**Constant work rate tests to exhaustion**

The aim of this thesis was to assess the ergogenic and therapeutic qualities of dietary NO\textsubscript{3} supplementation. A key aspect of each study was to assess the effects of NO\textsubscript{3} supplementation on the VO\textsubscript{2} kinetic response to exercise and whether these changes may influence exercise tolerance. In order to achieve this, it was necessary to assess these parameters in the same exercise test. Accurate assessment of pulmonary VO\textsubscript{2} kinetics requires the completion of a ‘step’ exercise test, in which the work rate is abruptly increased from an ‘unloaded’ baseline to a target work rate. This approach enhances the validity of the investigation into the influence of VO\textsubscript{2} kinetics on exercise tolerance, but limits the generalizability of our data to ‘real life’ sporting performance where success is determined by completing a given distance in the fastest time, not by sustaining a given power output for the longest possible time. It is known that constant work rate tests to exhaustion have lower ecological validity and are less reliable compared to time-trial
Chapter 8: General Discussion

performance tests (Laursen et al., 2007). However, time-to-exhaustion and time-trial tests have been shown to have a similar level of sensitivity in detecting changes in exercise performance in response to an experimental intervention (Amann et al., 2008). The authors (Amann et al., 2008) discussed that while the error of measurement is higher with time-to-exhaustion trials, the magnitude of change is also far greater such that the sensitivity of these performance tests is similar in detecting changes in exercise performance with an intervention. Along with the additional benefits of accurately assessing VO₂ kinetics, time-to-exhaustion tests were considered most appropriate to use in 3 of the 4 experimental chapters in the current thesis.

Is the 6-minute walk test a reliable and valid measure of functional capacity?

Research using older individuals (65-89 years) demonstrated a one-week test-retest reliability coefficient of r = 0.95 (Harada et al., 1999), while a correlation was evident between the distance covered in the 6-minute walk test and VO₂peak (r = 0.70; Nixon et al., 1996). The physiological demand of the walk test appears to be distinct from that of cycle ergometer tests and, therefore, may be a better indicator of function in normal daily life. The 6-minute walk test provides a simple, safe and cost effective measurement of functional movement considered essential in all daily activities in older and clinical populations.

Does pulmonary VO₂ accurately represent muscle VO₂?

Whilst assessing the effectiveness of NO₃⁻ supplementation as a therapeutic and ergogenic aid, it is important that the pulmonary VO₂ signal measured is reflective of muscle VO₂. A number of techniques have been employed in the present thesis to minimize the breath-by-breath variability which is inherent with measuring VO₂ during exercise and to enhance the signal-to-noise ratio, which enhances confidence in the parameters assessed. These techniques have been discussed in the general methodology chapter and include appropriate cleaning of data and the completion of repeat transitions where appropriate. When these procedures have been employed it has been shown that pulmonary VO₂ kinetics does indeed accurately represent muscle VO₂ kinetics (Barstow et al., 1990; Poole et al., 1991; Grassi et al., 1996; Krstrup et al., 2009).
Chapter 8: General Discussion

**Future research questions**

**Oral microbiome**

The requisite role of the oral microbiome for the NO$_3^-$ -NO$_2^-$ -NO pathway highlights an area that may prove important in the effectiveness of NO$_3^-$ supplementation. The oral microbiome plays a key role in systemic NO homeostasis and the modulation of cardiovascular and metabolic functions. However, its interactive role with NO$_3^-$ supplementation is not yet well understood. Uptake mechanisms and excretion of NO$_3^-$ in the salivary glands, along with inter-individual differences in oral microbiome, needs to be explored, specifically the potential effects of age.

**NO$_3^-$ supplementation effectiveness**

Whilst oral microbiome may be a major factor in determining the effectiveness of NO$_3^-$ supplementation, the notion of ‘responders’ and ‘non-responders’ needs to be more fully understood. Most explanations of non-responders currently refer to the training status of the individual or the amount and timing of juice consumption and exercise testing. Research which strives to understand the underlying mechanisms or explanations behind responding or not-responding to the supplementation, would add substantial knowledge to the developing topic of dietary NO$_3^-$ supplementation.

**Clinical populations**

Chapter 5 aimed to identify the effects of NO$_3^-$ supplementation in a hypoxic environment (which acted to mimic some disease states), while Chapter 6 assessed the effect of NO$_3^-$ supplementation in a healthy older population (60-70 years). In addition to this, existing literature has assessed the effects of NO$_3^-$ in diabetic (Gilchrist et al., 2013), PAD (Kenjale et al., 2011) and COPD (Berry et al., 2014) populations, with mixed findings. Future research should focus on additional specific diseased populations where the effects of NO$_3^-$ supplementation may have particular benefits (i.e. anaemia). Furthermore, an even older population than that seen in Chapter 6 should also be targeted as the decline in physical and cognitive capacity has been shown to accelerate from 70 years onwards. The translation of findings from young, healthy- to elderly and pathological populations may have much larger public health implications and may contribute to further improvements in quality of life.
Chapter 8: General Discussion

_Cerebral physiology & cognitive function_

Recent research provided promising potential with regard to the role of NO\textsuperscript{3−} supplementation in improving cerebral physiology and function, via increasing cerebral blood flow to areas important in executive functioning (Presley et al., 2011). While the findings from Chapter 6 indicated that there were no effects of NO\textsuperscript{3−} supplementation upon cerebral measures and cognitive function in healthy older individuals, the potential implication of NO\textsuperscript{3−} supplementation in improving cerebral physiology and function needs to be explored further. It provides an exciting avenue of further research and may provide important insight into preventing or offsetting age-related declines in cognitive function.

_Conclusions_

Investigating the physiological effects of dietary NO\textsuperscript{3−} supplementation is a current and relevant topic in exercise and health physiology. From elucidating the mechanisms behind alterations to the V\textsubscript{O2} kinetic response to exercise and subsequent improved athletic performance, to quantifying the extent to which NO\textsuperscript{3−} supplementation may be used as a therapeutic aid in the prevention and treatment of pathological conditions, ongoing scientific research aims to understand the potential of this dietary supplement. The current thesis has contributed to this understanding and has also presented findings which highlight unanswered questions obligating further research. This thesis has provided data with regard to the potential ergogenic and therapeutic qualities of NO\textsuperscript{3−} supplementation. These data demonstrate that dietary NO\textsuperscript{3−} supplementation may be considered as an ergogenic aid for severe-intensity cycle exercise and may elicit therapeutic effects in hypoxic environments as well as upon cardiovascular health across both young and old populations. However, the presented data also outline that supplementation may not always be effective. The underlying mechanisms and parameters which may influence its effectiveness are not yet fully understood and so supplementation should be carefully considered, monitored and tailored specifically for individuals and their particular requirements.

Based on the findings presented in this thesis, it can be concluded that while NO\textsuperscript{3−} supplementation may not _always_ be effective, it _can_ modulate the V\textsubscript{O2} response to exercise, improve athletic performance and reduce blood pressure. As such it should be considered to have, and be utilized for, its ergogenic and therapeutic qualities.
References


References


Barstow TJ, Scheuermann BW. Effects of maturation and ageing on VO₂ kinetics. In AM Jones, DC Poole (Eds.), *Oxygen uptake kinetics in sport, exercise and medicine*: 331-352, 2005.


Bell C, Paterson DH, Kowalchuk JM, Cunningham DA. Oxygen uptake kinetics of older humans are slowed with age but are unaffected by hyperoxia. *Experimental Physiology* 84: 747-759, 1999.


References


References


References


Jones AM, Poole DC. Introduction to oxygen uptake kinetics. In AM Jones, DC Poole (Eds.), *Oxygen uptake kinetics in sport, exercise and medicine*: 3-35, 2005.


References


References


References


References


References


References


References


References


References


References


