DIETARY NITRATE SUPPLEMENTATION AS AN ERGOGENIC AND THERAPEUTIC AID

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

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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.

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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 4mmHg (BR: 118 ± 5 vs. PL: 122 ± 5mmHg) 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 $L \cdot min^{-1}$) and Normoxia-PL (N-PL) (1.97 ± 0.25 $L \cdot min^{-1}$). $\dot{V}O_2$ kinetics were faster in H-BR compared to H-PL (phase II τ, 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 O_2 cost of exercise remained unchanged. Functional capacity (6-minute walk test), the muscle metabolic response to low-intensity exercise, brain metabolite

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Symbols and abbreviations

[ ]	concentration
Δ	difference
^{1}H-MRS	^{1}proton nuclear magnetic resonance spectroscopy
6MWT	six-minute walk test
^{31}P-MRS	^{31}Phosphorous nuclear magnetic resonance spectroscopy
A	exponential response amplitude
ADC	apparent diffusion coefficient
ADP	adenosine diphosphate
AMARES	advanced method for accurate, robust and efficient spectral fitting
ANOVA	analysis of variance
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
BP	blood pressure
BR	beetroot
Ca^{2+}	calcium
Ch	choline
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
CON	control
CP	critical power
Cr	creatine
CV	coefficient of variation
eNOS	endothelial nitric oxide synthase
GET	gas exchange threshold
H^+	hydrogen ion, proton
Hb	haemoglobin
HbO_2	oxygenated haemoglobin
Hb_{tot}	total haemoglobin

HHb	deoxygenated haemoglobin
HR	heart rate
iNOS	inducible nitric oxide synthase
jMRUI	java-based magnetic resonance user interface
K^+	potassium ion
kJ	kilojoules
MAP	mean arterial pressure
MR	magnetic resonance
MRS	magnetic resonance spectroscopy
MRT	mean response time
mI	myo-inositol
NAA	*N*-acetyl aspartate
$NaNO_3^-$	sodium nitrate
NIRS	near-infrared spectroscopy
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
O_2	oxygen
O_2^-	superoxide
P	power output
PCr	phosphocreatine
P_i	inorganic phosphate
PL	placebo
P/O	oxygen cost of ATP resynthesis
PRESS	point resolved spectroscopy
RER	respiratory exchange ratio
RMR	resting metabolic rate

ROS	reactive oxygen species
SD	standard deviation
SE	standard error
SENSE	sensitivity encoding
τ	time constant
TD	exponential response time delay
T_{lim}	limit of tolerance
TT	time trial
VCl_3	vanadium chloride
$\dot{V}CO_2$	pulmonary carbon dioxide output
$\dot{V}_E$	minute ventilation (expired)
$\dot{V}O_2$	pulmonary oxygen uptake
$\dot{V}O_{2max}$	maximal oxygen uptake
$\dot{V}O_{2peak}$	peak oxygen uptake
W	Watt
W′	curvature constant of the power-duration relationship

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

Kelly J, Vanhatalo A, Wilkerson, DP, Wylie LJ, Jones AM. Effects of nitrate on the power-duration relationship for severe-intensity exercise, *Medicine & Science in Sports & Exercise*, 45 (9): 1798-1806, 2013.

Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, Gilchrist M, Winyard PG, Jones AM. Effects of short-term dietary nitrate supplementation on blood pressure, O_2 uptake kinetics, and muscle and cognitive function in older adults. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, 304 (2): R73-83, 2013.

Kelly J, Wylie, LJ, Black MI, McDonagh S, Jackman S, List S, Tucker C, Winyard P, Blackwell J, Vanhatalo A, Jones AM. Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, 307: R920-R930, 2014.

Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. Beetroot juice and exercise: the pharmacokinetic-pharmacodynamic and dose-response relationships. *Journal of Applied Physiology*, 115 (3): 325-336, 2013.

Wylie LJ, Mohr M, Krustrup P, Jackman SR, Ermidis G, Kelly J, Black MI, Bailey SJ, Vanhatalo A, Jones AM. Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. *European Journal of Applied Physiology*, 113 (7): 1673-1684, 2013.

Shepherd A, Wilkerson D, Dobson L, Kelly J, Winyard P, Jones AM, Benjamin N, Shore A, Gilchrist M. Dietary nitrate's effect on the oxygen cost of cycling, walking performance and BP in COPD. *Nitric Oxide*, 2015.

Other publications

Jones AM, Kelly J, McDonagh S, Wylie LJ. Dietary nitrate and exercise. *Professionals in Nutrition for Exercise and Sports Newsletter*, 2013.

Works in review

Kelly J, Vanhatalo A, Mohr M, Krustrup P, Wylie LJ, Jackman SR, Black MI, Blackwell JR, Winyard PG, Bangsbo J, Jones AM. Dietary nitrate supplementation: effects on muscle nitrate, muscle metabolism and pulmonary O_2 uptake kinetics during moderate- and high-intensity cycle exercise. *Journal of Applied Physiology* (In Review).

Conference Activity

Oral Presentation: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. 19th annual Congress of the European College of Sports Science, VU University, Amsterdam, July 2014.

Poster Presentation: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. University of Exeter Postgraduate Research Showcase, April 2014 and University of Exeter Health Showcase Event, June 2014.

Oral Presentation: Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. BASES Student Conference, University of Portsmouth, Portsmouth, April 2014.

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

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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_2O 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.

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 ($\dot{V}O_2$) 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ (Phase I) is followed by a rapid exponential increase in $\dot{V}O_2$ (Phase II) which has a time constant (time taken to achieve 63% of the change in $\dot{V}O_2$) of 25-40 s in healthy individuals. This rapid exponential increase drives $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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.

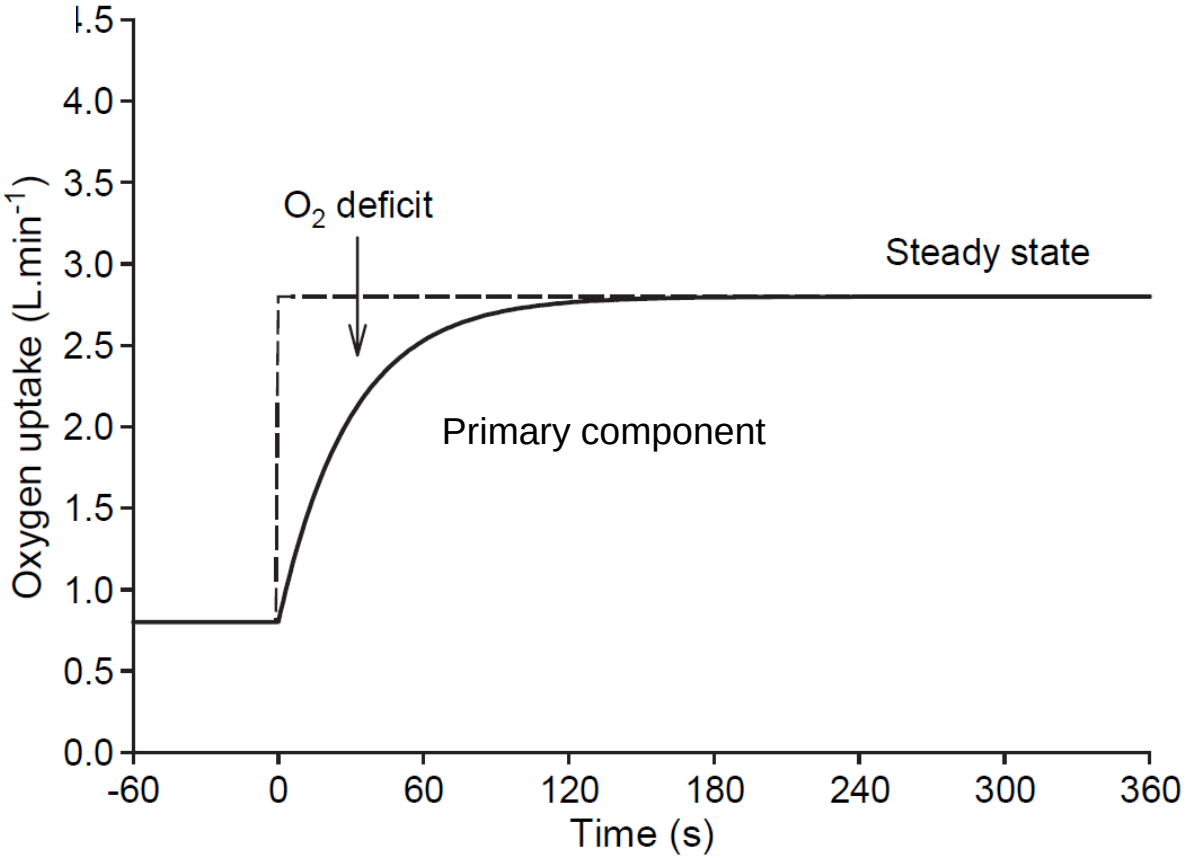

Fig 1.1: Schematic illustration of the $\dot{V}O_2$ response to cycle exercise. At the onset of exercise, an oxygen deficit is incurred due to the $\dot{V}O_2$ response lagging behind the energy requirements of the task. The primary component of the $\dot{V}O_2$ response increases in an exponential fashion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

During exercise completed above the GET, a rise in the $\dot{V}O_2$ response in addition to the primary component is evident. This additional superimposed elevation in $\dot{V}O_2$ is termed the 'slow component' and can be stabilised during heavy-intensity exercise (below the critical power (CP)), but continues to drive the $\dot{V}O_2$ to maximum during severe-intensity exercise (above CP). Importantly, the $\dot{V}O_2$ 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; Krustrup *et al*., 2004).

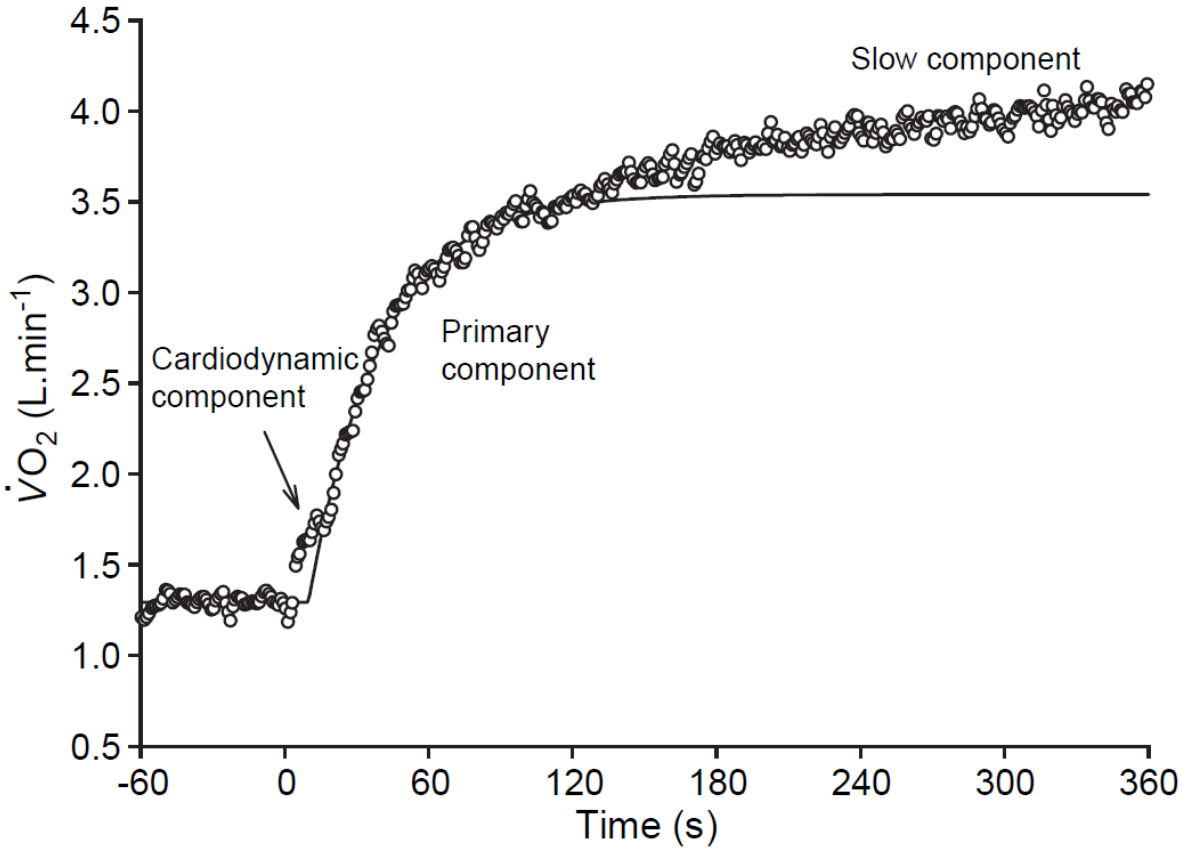

Figure 1.2: The $\dot{V}O_2$ response to exercise above the CP in a healthy individual. Note that $\dot{V}O_2$ continues to increase beyond the primary component, leading to an end-exercise $\dot{V}O_2$ that is ~500 ml·min^{-1} higher than expected. This additional rise in $\dot{V}O_2$ is termed the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

With this in mind, reducing the $\dot{V}O_2$ steady-state, speeding the kinetic response and/or reducing the magnitude of the $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 ~ 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.

Chapter 2: Literature Review

Nitric oxide

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 (BH_4), 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 (Förstermann, 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 (NO_3^-) to nitrite (NO_2^-) 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.

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 ~5 h 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^-.

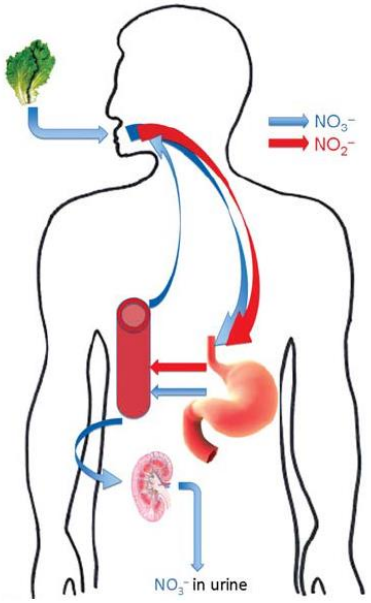

Figure 2.1: Schematic diagram of the enterosalivary circulation of nitrate in the human body. Nitrate is represented by the blue arrows and nitrite represented by the red arrows. (From Gilchrist *et al*., 2010)

Finally, NO_2^- is reduced into bioactive NO. This reduction is actuated by a number of catalysts including deoxyhaemoglobin (Cosby *et al*., 2003), deoxymyoglobin (Shiva *et al*., 2007), xanthine oxidase (Zhang *et al*., 1998), aldehyde oxidase (Li *et al*., 2008), eNOS (Vanin *et al*., 2007), and the mitochondrial electron transfer complexes (Kozlov *et al*., 1999). This reduction reaction 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 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 *et al*., 2008) and NO_3^- (Carlström *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.

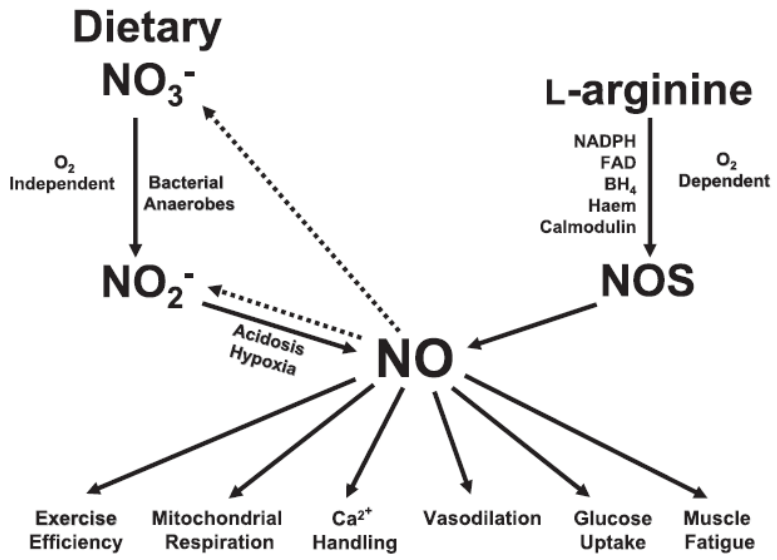

Fig 2.2: A schematic diagram outlining the pathways of NO generation and the roles it can have within the human body. (From Bailey *et al*., 2012).

Beetroot and typical NO_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_3^-. NO_3^- is naturally ingested as part of a healthy diet, with 60-80% of daily NO_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_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_3^- intake of adults in the US and Europe is 1-2 mmol/d. Vegetarians are likely to consume more NO_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_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.

Toxicity

Concerns related to adverse effects of inorganic NO_2^- and NO_3^- have previously been proposed in the literature including the development of methaemoglobinaemia (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.

Methaemoglobinaemia

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 methaemoglobinaemia which can cause cellular hypoxia. Concern about NO_3^- and methaemoglobinaemia 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 methaemoglobinaemia.

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

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 NO_3^- supplementation (Bescos *et al*., 2011). During incremental exercise protocols, NO_3^- supplementation has been reported to both increase (Vanhatalo *et al*., 2010) and decrease (Larsen *et al*., 2010; Bescos *et al*., 2011) the $\dot{V}O_{2max}$. Explanations of the increase in $\dot{V}O_{2max}$ 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 $\dot{V}O_{2max}$ 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 NO_3^- 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 ($\dot{V}O_2$), 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:

$$T_{lim} = W'/ (P-CP) \quad \text{(Eqn. 1)}$$

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:

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

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

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

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:

$$T_{lim} = (W-W')/CP \quad \text{(Eqn. 4)}$$

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.

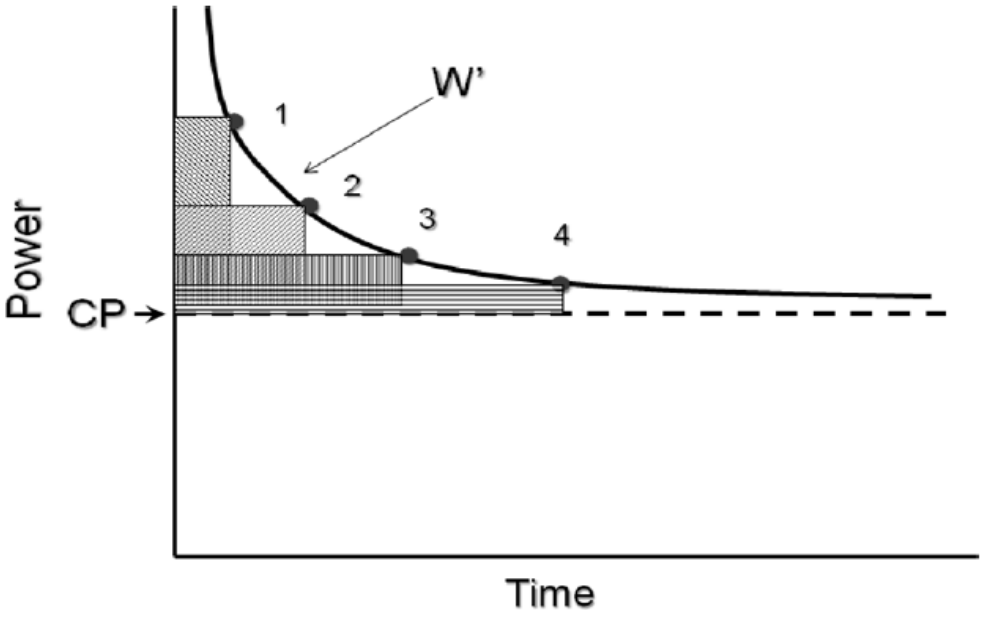

Figure 2.3: Illustration of the hyperbolic power-duration relationship. When time-to exhaustion, during exercise at four different intensities above the critical power (severe-intensity), is plotted against the power output, time to exhaustion increases hyperbolically. The filled circles represent the time to exhaustion at a given power output. The solid line hyperbola characterises the power-duration realtionship 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 $\dot{V}O_2$

kinetics and $\dot{V}O_{2max}$ (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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 NO_3^- 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 NO_3^- 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 NO_3^- 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 $[NO_2^-]$, 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 NO_3^- -mediated alterations in the $\dot{V}O_2$ response and improved exercise performance include that of changes to intracellular Ca^{2+} handling. In 2012, Hernandez and colleagues reported that fast-twitch skeletal muscle harvested from mice supplemented with NO_3^- in drinking water displayed increased tetanic $[Ca^{2+}]$, 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 Ca^{2+} handling) being increased. These findings show that fast twitch muscles of NO_3^- 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, NO_3^- 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_2 delivery to Type II fibers offers important information regarding the NO_3^- -mediated vascular and metabolic control that has previously been reported in humans during exercise (Larsen *et al*., 2007; Bailey *et al*., 2009, Bailey *et al*., 2010). In order to add to the existing proposed mechanisms behind the beneficial effects of NO_3^- supplementation, direct assessment of human skeletal muscle metabolism during exercise must be investigated.

Muscle metabolism

Following the onset of exercise, an immediate increase in ATP turnover and an exponential rise in oxygen (O_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 *et al*., 2008). As previously discussed, pulmonary $\dot{V}O_2$, which provides a close representation of muscle $\dot{V}O_2$ (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 $\dot{V}O_2$ response, the $\dot{V}O_2$ 'slow component', is evident which delays and/or prevents the attainment of a steady state. The development of the $\dot{V}O_2$ 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^+, P_i, ADP). Interventions that alter the $\dot{V}O_2$ 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 *et al*., 2009).

The effects of NO_3^- supplementation upon the $\dot{V}O_2$ response to exercise are well documented. While non-invasive measures (^{31}P-MRS) of muscle metabolism during exercise, following NO_3^- supplementation, have been recorded (Bailey *et al*., 2010), the effects of NO_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_3^- supplementation can affect the muscle metabolic response to exercise in humans.

It is also currently unknown whether NO_3^- supplementation can increase skeletal muscle [NO_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_3^-] of sedentary, resting rats have been reported as ~46 nM g^{-1} (Perez *et al*., 2002). Assessing the effects of NO_3^- supplementation upon skeletal muscle [NO_3^-] in humans is important. This may elucidate whether skeletal muscle [NO_3^-] is influenced by NO_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_2^- to bioactive NO in the NO_3^- - NO_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_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. 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_2 delivery versus O_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_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_2 in inspired air results in a reduction in arterial oxygen concentration and a decrease in intracellular partial pressure of O_2 (PO_2) (Richardson *et al*., 1995). In order to restore sufficient O_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 $\dot{V}O_2$ but greater muscle metabolic perturbation (Wilkins *et al*., 2006). A reduction in

intracellular 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, 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, P_i, 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). 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 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 $\dot{V}O_{2max}$ 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 heamodynamics (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_{2max} (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 $\dot{V}O_2$ kinetics. In support of this notion, a bout of heavy priming exercise speeded $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics in older people. However, conflicting research suggests that the slow $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics in an older population.

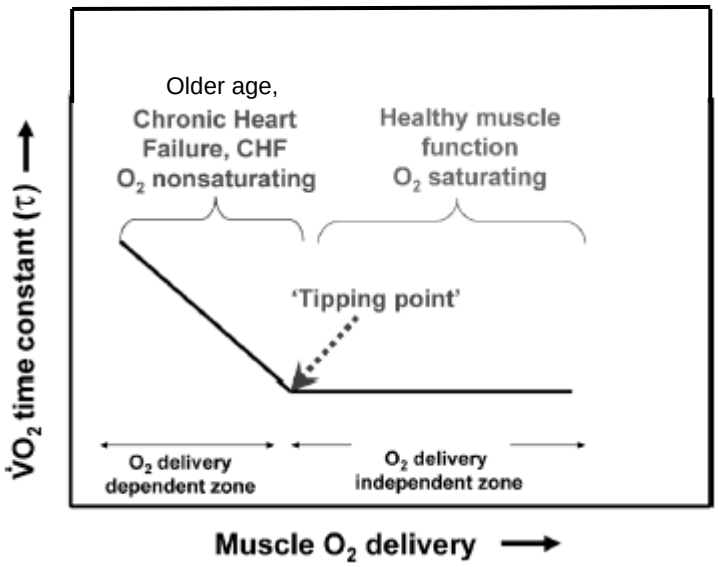

Fig 2.4: A schematic diagram outlining the the dependence of $\dot{V}O_2$ kinetics on muscle O_2 delivery in various populations (From Poole & Jones. 2005).

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 peryoxynitrite and oxidises BH_4, 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 O_2 delivery, speed $\dot{V}O_2$ kinetics, improve mitochondrial efficiency, that possesses antioxidant properties and which may help to increase the bioavailability of NO, such as dietary NO_3^- 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 NO_2^- infusion led to increases in cerebral blood flow in rats measured using laser Doppler flowmetery (Rifkind *et al*., 2007). Subsequent research demonstrated that a high-NO_3^- diet elevated plasma NO_2^- 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 NO_3^- 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 NO_3^- 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 $\dot{V}O_2$ 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 a*l., 2012). NO_3^- 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 NO_3^- supplementation. A reduced ATP cost of muscle power output (Bailey *et al*., 2010), increased mitochondrial efficiency (Larsen *et al*., 2011), improved intracellular Ca^{2+} 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 $\dot{V}O_2$ response and improved exercise capacity.

Despite the publication of many dietary NO_3^- supplementation research papers in recent years, a number of outstanding questions remain to be answered regarding the ergogenic and therapeutic capabilities of dietary NO_3^- supplementation. Among others, these include investigating the effects of NO_3^- supplementation upon the power-duration relationship; NO metabolism in differing environmental conditions; and effectiveness of NO_3^- supplementation in an older population. Furthermore, a deeper understanding of the effects that NO_3^- 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 NO_3^- supplementation modulate the power-duration relationship for severe-intensity exercise in young, healthy, recreationally active males?
    - Does dietary NO_3^- improve exercise tolerance at intensities spanning the severe domain?
    - Does dietary NO_3^- increase critical power and/or W′?
    - Does dietary NO_3^- decrease resting metabolic rate?

2) How does dietary NO_3^- supplementation affect NO metabolism and does it have beneficial effects on exercise tolerance in hypoxic conditions?
    - What is the kinetic response of plasma [NO_3^-] and [NO_2^-] 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 NO_3^- supplementation elicited in young, healthy participants also evident in a healthy, older population?
    - Can NO_3^- supplementation reduce resting blood pressure in older individuals?
    - Does dietary NO_3^- modulate intramuscular metabolite responses in response to low- and high- intensity exercise?
    - Can dietary NO_3^- improve functional capacity in older adults?
    - Does dietary NO_3^- alter cerebral blood flow or the concentrations of important cerebral metabolites?
    - Can dietary NO_3^- be beneficial to cerebral health and cognitive function in an older population?
4) How does dietary NO_3^- supplementation influence skeletal muscle [NO_3^-], pulmonary $\dot{V}O_2$ and muscle metabolic responses to constant-work-rate moderate- and severe-intensity exercise.
    - Are NO_3^- levels increased within skeletal muscle tissue as a result of dietary NO_3^- supplementation?
    - Is the muscle metabolic response to exercise altered as a result of dietary NO_3^- 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) NO_3^- supplementation will 1) improve exercise tolerance across a range of severe-intensity exercise bouts by increasing the CP and/or W′ and 2) NO_3^- supplementation will reduce the resting metabolic rate.

2) A reduced fraction of inspired O_2 (FIO_2) will accentuate the depletion of NO_2^- during exercise compared to normoxia. NO_3^- supplementation will improve tolerance to severe-intensity, constant-work-rate cycle exercise in hypoxia, and this improvement will be greater than the effect of NO_3^- on exercise tolerance in normoxia.

3) Dietary supplementation with NO_3^- -rich beetroot juice will reduce resting blood pressure, speed the $\dot{V}O_2$ kinetics and lower the O_2 cost of treadmill walking, and improve functional capacity and cognitive function in healthy older adults.

4) Muscle [NO_3^-] will be elevated as a result of NO_3^- supplementation. The magnitude of muscle PCr degradation and fatiguing metabolite accumulation will be attenuated and exercise tolerance will be improved as a result of NO_3- 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 ^{TM} 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 $ml \cdot h^{-1}$ 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 [NO_2^-] and [NO_3^-] 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 [NO_2^-] and [NO_3^-] via chemiluminescence.

Prior to, and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO_2^-. Plasma NO_2^- and NO_3^- were analysed by gas phase chemiluminescence analysis. This initially required NO_2^- and NO_3^- to be reduced to NO gas. For reduction of NO_2^-, undiluted plasma was injected into a glass purge vessel containing 5 ml glacial acetic acid and 1 ml NaI solution. For NO_3^- 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 NO_2^- and NO_3^- 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 (O_2) and infrared absorption (CO_2) (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 $\dot{V}O_2$, $\dot{V}CO_2$ 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 $W \cdot min^{-1}$ 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), $\dot{V}O_{2peak}$ 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 $\dot{V}O_{2peak}$ 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 *et al*., 1981). Therefore, the work rates associated with the GET and $\dot{V}O_{2peak}$ 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 $\dot{V}O_{2peak}$ 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% Δ (a power output representing the power output at GET plus 75% of the difference between the power outputs at $\dot{V}O_{2peak}$ and GET). In Chapter 4, four different severe-intensity work rates were imposed and these were 60%Δ, 70%Δ, 80%Δ and 100% $\dot{V}O_{2peak}$.

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; Krustrup *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:

$$\dot{V}O_2(t) = \dot{V}O_{2baseline} + A_p [1 - e^{-(t-TDp/\tau p)}] \quad \text{(Eqn. 5)}$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time *t*; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ in the baseline period; A_p, TD_p, and τ_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:

$$\dot{V}O_2(t) = \dot{V}O_{2baseline} + A_p [1 - e^{-(t-TDp/\tau p)}] + A_s [1 - e^{-(t-TDs/\tau s)}] \quad \text{(Eqn. 6)}$$

Where $\dot{V}O_2(t)$ represents the absolute $\dot{V}o_2$ at a given time *t*; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ during the final 90 s of the baseline period; A_p, TD_p and τ_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 τ_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.

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

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ABSTRACT

KELLY, J., A. VANHATALO, D. P. WILKERSON, 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}, ~0.006 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% Δ), 70% Δ, 80% Δ, and 100% peak power, and the results were used to establish CP and W'. **Results**: Nitrate supplementation improved exercise tolerance during exercise at 60% Δ (BR, 696 ± 120 vs PL, 593 ± 68 s; $P < 0.05$), 70% Δ (BR, 452 ± 106 vs PL, 390 ± 86 s; $P < 0.05$), and 80% Δ (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 heavy-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 ($\dot{V}O_2$), 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).

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The hyperbolic power–duration relationship is given as follows:

$$T_{lim} = W'/(P-CP) \quad [1]$$

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

$$P = W'(1/T_{lim}) + CP \quad [2]$$

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

$$W = CPT_{lim} + W' \quad [3]$$

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):

$$T_{lim} = (W-W')/CP \quad [4]$$

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

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_2^- 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 salts or 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 $\dot{V}O_2$ 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; $\dot{V}O_{2peak}$ = 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 preexercise 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 $\dot{V}O_{2peak}$ 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.

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 $W \cdot min^{-1}$ 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. $\dot{V}O_{2peak}$ was determined as the highest mean $\dot{V}O_2$ during any 30-s period. The GET was determined from a cluster of measurements, including 1) the first disproportionate increase in CO_2 production ($\dot{V}CO_2$) from visual inspection of individual plots of $\dot{V}CO_2$ vs $\dot{V}O_2$, 2) an increase in expired ventilation ($\dot{V}_E$)/$\dot{V}O_2$ with no increase in $\dot{V}_E/\dot{V}CO_2$, and 3) an increase in end-tidal O_2 tension with no fall in end-tidal CO_2 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 (2×250 $mL \cdot d^{-1}$ of BR providing a total of 8.2 mmol nitrate per day; Beet it, James White Drinks, Ipswich, UK) or nitrate-depleted PL (2×250 $mL \cdot d^{-1}$ 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 nitrate ions (23). 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 $\dot{V}O_{2peak}$), 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 × 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 [NO_2^-]. 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 [NO_2^-]. Before and regularly during

analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO_2^-. After plasma samples were thawed at room temperature, the $[NO_2^-]$ 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_2) and infrared (CO_2) analyzers (Oxycon 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 (Oxycon beta; Mijnhardt) placed over their head instead of the aforementioned mouthpiece and nose clip. HR was measured using short-range radiotelemetry (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 $\dot{V}O^2$ 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 first 20 s of data after the onset of exercise (the phase I response) were deleted and a nonlinear least-square algorithm was used to fit the data. A biexponential model was used to characterize the $\dot{V}O_2$ responses to the severe-intensity exercise bouts as described in the following equation:

$$\dot{V}O_2(t) = \dot{V}O_{2baseline} + A_p\left[1-e^{-(t-TDp/\tau p)}\right] + A_s\left[1-e^{-(t-TDs/\tau s)}\right] \quad [5]$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time t; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ during the final 90 s of the baseline period; A_p, TD_p, and τ_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 τ_s represent the amplitude of, time delay before the onset of, and time constant describing the development of the $\dot{V}O_2$ 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 $\dot{V}O_2$ for all four work rates was defined as the mean $\dot{V}O_2$ measured during the final 15 s of exercise. The same "filtering" technique was used for the breath-by-breath $\dot{V}O_2$ data collected during the RMR tests. A mean value during the 10-min collection period was calculated.

Statistical analyses. Differences in plasma $[NO_2^-]$, 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 $\dot{V}O_2$ 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 $[NO_2^-]$ 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 $\dot{V}O_2$ 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 $[NO_2^-]$ and BP. ANOVA revealed that plasma $[NO_2^-]$ 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 $[NO_2^-]$ was 98 ± 41, 96 ± 29, 75 ± 16, 86 ± 26, and 73 ± 31 nM, respectively; whereas for BR visits 1–5, the plasma $[NO_2^-]$ was 297 ± 98, 262 ± 107, 295 ± 108, 209 ± 86, and 228 ± 135 nM, respectively. The plasma $[NO_2^-]$ 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 $[NO_2^-]$ by 197% when compared with PL (BR, 255 ± 70 vs PL 86 ± 21 nM; $P < 0.01$). BR also reduced systolic BP compared with PL (BR, 118 ± 5 vs 122 ± 5 mm Hg; $P < 0.01$), 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.

Ramp incremental test. The main experiment resulted in no training effect ($P > 0.05$) upon $\dot{V}O_{2peak}$ (initial test, 4.13 ± 0.44 vs final test, 4.09 ± 0.56 mL·min^{-1}), peak power output (initial test, 344 ± 34 vs final test, 338 ± 32 W), or power output at GET (initial test, 129 ± 30 vs final test, 125 ± 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% Δ (BR, 696 ± 120 vs PL, 593 ± 68 s; $P < 0.05$), 70% Δ (BR, 452 ± 106 vs PL, 390 ± 86 s; $P < 0.05$), and 80% Δ (BR, 294 ± 50 vs PL, 263 ± 50 s; $P < 0.05$) but not at 100% peak (BR, 182 ± 37 vs PL, 166 ± 26 s; $P = 0.10$). 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 ± 26 and 221 ± 27 W for PL and BR, respectively, and the values for *W′* were 17.8 ± 3.0 and 19.3 ± 4.6 kJ for PL and BR, respectively. For the work–time model, the values for CP were 218 ± 26 and 217 ± 28 W for PL and BR, respectively, and the values for *W′* were 17.7 ± 3.0 and 19.7 ± 4.1 kJ for PL and BR, respectively. For the power–time model, the values for CP were 216 ± 26 and 214 ± 27 W for PL and BR, respectively, and the values for *W′* were 18.5 ± 3.3 and 21.2 ± 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.

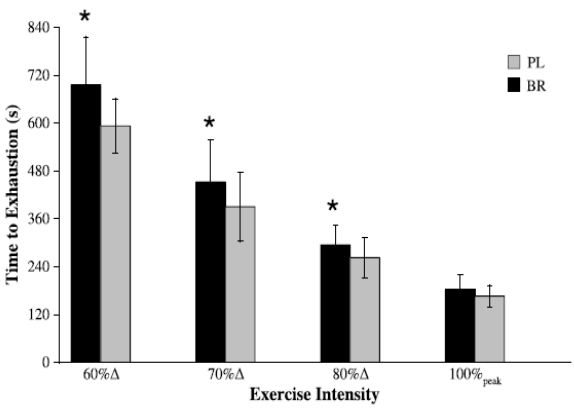

FIGURE 1—Group mean ± SD times to exhaustion across four severe-intensity power outputs, after PL (*gray bars*) and BR (*black bars*) supplementation. *$P < 0.05$.

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

	PL	BR
60% Δ		
Baseline heart rate, bpm	101 ± 11	101 ± 7
End exercise heart rate, bpm	188 ± 8	189 ± 10
Baseline blood [lactate], mM	0.9 ± 0.2	0.8 ± 0.1
End exercise blood [lactate], mM	8.1 ± 2.3	7.9 ± 1.9
70% Δ		
Baseline heart rate, bpm	100 ± 9	97 ± 9
End exercise heart rate, bpm	189 ± 9	190 ± 9
Baseline blood [lactate], mM	0.9 ± 0.2	0.8 ± 0.3
End exercise blood [lactate], mM	8.7 ± 1.6	8.4 ± 1.8
80% Δ		
Baseline heart rate, bpm	97 ± 12	98 ± 11
End exercise heart rate, bpm	184 ± 9	184 ± 9
Baseline blood [lactate], mM	0.9 ± 0.2	0.9 ± 0.2
End exercise blood [lactate], mM	8 ± 1.6	8.6 ± 1.2
100% peak		
Baseline heart rate, bpm	99 ± 11	101 ± 10
End exercise heart rate, bpm	180 ± 9	182 ± 9
Baseline blood [lactate], mM	1 ± 0.1	0.9 ± 0.2
End exercise blood [lactate], mM	8.4 ± 1.1	8.8 ± 1.4

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 ± 27 vs PL, 218 ± 26 W) and *W′* (BR, 19.3 ± 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 $\dot{V}O_2$ parameters derived from the model fit, during each of the prediction trials, are presented in Table 2. $\dot{V}O_2$ 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

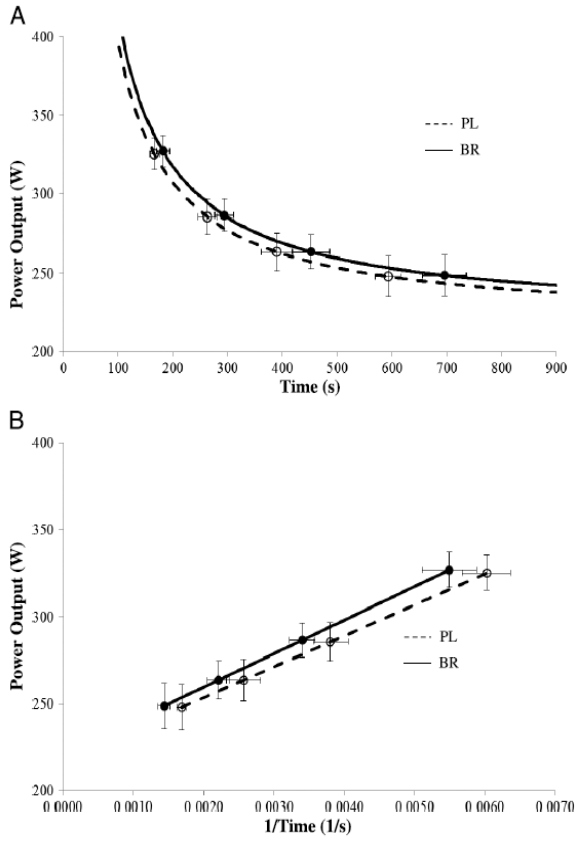

FIGURE 2—Effects of BR on the power–duration relationship established from four severe-intensity prediction trials. The group mean ± SE power–duration profiles are shown in panel (A), and the group mean ± SE power–1/time relationships are shown in panel (B). Responses after PL are represented by the 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 $\dot{V}O_{2peak}$ attained during the ramp incremental tests.

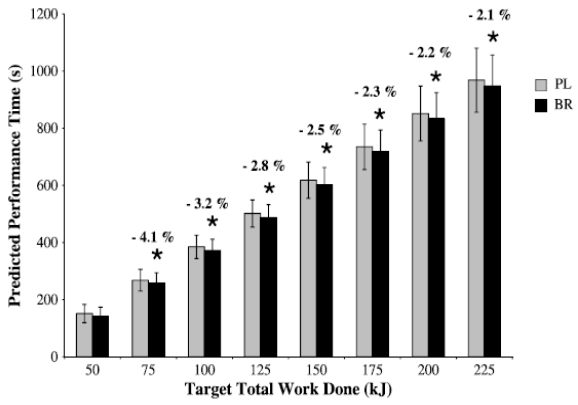

FIGURE 3—Mean ± SD predicted time to complete given amounts of work as calculated using the CP and W' estimates from the power–1/time model after PL (*gray bars*) and BR (*black bars*) supplementation. BR is expected to improve performance compared with PL by 2.1%–4.1% for target work done trials between 75 and 225 kJ. *$P < 0.05$.

TABLE 2. Mean ± SD oxygen uptake dynamics during four different severe-intensity exercise bouts.

	PL	BR
60% Δ ($\dot{V}O_2$)		
Baseline, mL·min^{-1}	1125 ± 158	1072 ± 85
End exercise, mL·min^{-1}	4382 ± 270	4369 ± 329
Phase II time constant, s	23 ± 5	20 ± 4
Primary amplitude, mL·min^{-1}	2081 ± 381	2060 ± 313
Slow component amplitude, mL·min^{-1}	1180 ± 246	1208 ± 52
70% Δ ($\dot{V}O_2$)		
Baseline, mL·min^{-1}	1151 ± 183	1142 ± 118
End exercise, mL·min^{-1}	4405 ± 476	4499 ± 371
Phase II time constant, s	30 ± 8	28 ± 8
Primary amplitude, mL·min^{-1}	2357 ± 458	2345 ± 329
Slow component amplitude, mL·min^{-1}	896 ± 217	1027 ± 116
80% Δ ($\dot{V}O_2$)		
Baseline, mL·min^{-1}	1080 ± 144	1063 ± 106
End exercise, mL·min^{-1}	4115 ± 425	4222 ± 320
Phase II time constant, s	25 ± 5	22 ± 10
Primary amplitude, mL·min^{-1}	2272 ± 252	2261 ± 252
Slow component amplitude, mL·min^{-1}	721 ± 139	856 ± 61
100% Peak ($\dot{V}O_2$)		
Baseline, mL·min^{-1}	1151 ± 172	1125 ± 118
End exercise, mL·min^{-1}	4119 ± 420	4180 ± 390
Phase II time constant, s	24 ± 7	21 ± 5
Primary amplitude, mL·min^{-1}	2232 ± 397	2371 ± 330
Slow component amplitude, mL·min^{-1}	695 ± 215	686 ± 219

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 (1,14,25,36). 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

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–1/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 T_{lim} 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics. For example, Bailey et al. (1) reported that BR resulted in a 23% reduction in the $\dot{V}O_2$ 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 $\dot{V}O_2$ slow component would delay the time before $\dot{V}O_{2max}$ is attained and could therefore contribute to enhanced exercise tolerance. Also, Lansley et al. (22) found that BR increased power output for the same $\dot{V}O_2$ during cycling TT performances, implying improved muscle efficiency. An improved muscle efficiency after nitrate supplementation may be observed either as a lower $\dot{V}O_2$ for the same power output or, conversely, a higher power output for the same $\dot{V}O_2$. In the present study, the amplitudes of the $\dot{V}O_2$ primary and slow components were not different between BR and PL. Moreover, ANOVA revealed that the end-exercise $\dot{V}O_2$ was neither different between conditions nor different from the $\dot{V}O_{2peak}$ measured during ramp incremental exercise. This latter result indicates that $\dot{V}O_{2peak}$ is not reduced by nitrate supplementation, which is consistent with some (1,36), but not all (4,27), previous studies.

Interestingly, the $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics would reduce the contribution of substrate-level phosphorylation to 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 $\dot{V}O_2$ kinetics (~2.6 s) might have contributed to the slightly higher CP (~3 W) observed with BR compared with PL in the present study.

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 the present study can be applied to highly trained endurance athletes. Compared with less well-trained subjects, endurance athletes have higher baseline plasma [NO_2^-], 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 $\dot{V}O_2$ suggests either 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.

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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.

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

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Kelly J, Vanhatalo A, Bailey SJ, Wylie LJ, Tucker C, List S, Winyard PG, Jones AM. Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. *Am J Physiol Regul Integr Comp Physiol* 307: R920–R930, 2014. First published July 9, 2014; doi:10.1152/ajpregu.00068.2014.—We investigated the effects of dietary nitrate (NO_3^-) supplementation on the concentration of plasma nitrite ([NO_2^-]), oxygen uptake ($\dot{V}O_2$) kinetics, and exercise tolerance in normoxia (N) and hypoxia (H). In a double-blind, crossover study, 12 healthy subjects completed cycle exercise tests, twice in N (20.9% O_2) and twice in H (13.1% O_2). Subjects ingested either 140 ml/day of NO_3^--rich beetroot juice (8.4 mmol NO_3^-; BR) or NO_3^--depleted beetroot juice (PL) for 3 days prior to moderate-intensity and severe-intensity exercise tests in H and N. Preexercise plasma [NO_2^-] was significantly elevated in H-BR and N-BR compared with H-PL ($P < 0.01$) and N-PL ($P < 0.01$). The rate of decline in plasma [NO_2^-] was greater during severe-intensity exercise in H-BR [−30 ± 22 nM/min, 95% confidence interval (CI); −44, −16] compared with H-PL (−7 ± 10 nM/min, 95% CI; −13, −1; $P < 0.01$) and in N-BR (−26 ± 19 nM/min, 95% CI; −38, −14) compared with N-PL (−1 ± 6 nM/min, 95% CI; −5, 2; $P < 0.01$). During moderate-intensity exercise, steady-state pulmonary $\dot{V}O_2$ was lower in H-BR (1.91 ± 0.28 l/min, 95% CI; 1.77, 2.13) compared with H-PL (2.05 ± 0.25 l/min, 95% CI; 1.93, 2.26; $P = 0.02$), and $\dot{V}O_2$ kinetics was faster in H-BR (τ: 24 ± 13 s, 95% CI; 15, 32) compared with H-PL (31 ± 11 s, 95% CI; 23, 38; $P = 0.04$). NO_3^- supplementation had no significant effect on $\dot{V}O_2$ kinetics during severe-intensity exercise in hypoxia, or during moderate-intensity or severe-intensity exercise in normoxia. Tolerance to severe-intensity exercise was improved by NO_3^- in hypoxia (H-PL: 197 ± 28; 95% CI; 173, 220 vs. H-BR: 214 ± 43 s, 95% CI; 177, 249; $P = 0.04$) but not normoxia. The metabolism of NO_2^- during exercise is altered by NO_3^- supplementation, exercise, and to a lesser extent, hypoxia. In hypoxia, NO_3^- supplementation enhances $\dot{V}O_2$ kinetics during moderate-intensity exercise and improves severe-intensity exercise tolerance. These findings may have important implications for individuals exercising at altitude.

hypoxia; beetroot juice; nitric oxide; efficiency; performance

NITRIC OXIDE (NO) IS A UBIQUITOUS, water-soluble, free radical gas that plays a crucial role in many biological processes. Effective NO production is important in normal physiological functioning, from the regulation of blood flow, muscle contractility, and mitochondrial respiration to host defense, neurotransmission, and glucose and calcium homeostasis (11, 17, 60). NO production via the oxidation of L-arginine, in a process catalyzed by nitric oxide synthase (NOS), may be blunted in conditions of reduced O_2 availability (52). It is now widely accepted that NO can also be generated via an alternative pathway, whereby inorganic nitrate (NO_3^-) is reduced to nitrite (NO_2^-) and further to NO. This NOS- and O_2-independent NO_3^--NO_2^--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_3^- stores can be increased via the diet, with green leafy vegetables and beetroot being particularly rich in NO_3^-. Upon ingestion, inorganic NO_3^- is absorbed from 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 the NO_3^- to NO_2^- (21). Some salivary NO_2^- is converted into NO when swallowed into the acidic environment of the stomach (7), while the remainder is absorbed, increasing circulating plasma NO_2^- concentration [NO_2^-]. This NO_2^- may be reduced to NO via a number of enzymatic and nonenzymatic pathways (e.g., xanthine oxidoreductase and deoxyhemoglobin), 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. A reduced fraction of O_2 in inspired air results in reductions in arterial O_2 concentration and intracellular partial pressure of O_2 (P_{O_2}). 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_2 supply, local blood flow is increased via hypoxia-induced vasodilatation with NO being implicated as a major mediator of this process (12). NO_2^- may also promote hypoxic vasodilatation in an NO-independent manner (16).

Dietary NO_3^- supplementation, in the form of nitrate salts and nitrate-rich beetroot juice (BR), represents a practical method of increasing circulating plasma [NO_3^-] (31, 42, 67) and [NO_2^-] (4, 33, 62). NO_3^- supplementation has been shown to reduce resting blood pressure (3, 33, 42) and oxygen uptake ($\dot{V}O_2$) during submaximal exercise (4, 39, 40, 41, 62, 67) and to improve exercise performance in young, healthy individuals exercising in normoxic conditions (14, 38), but not necessarily in well-trained athletes (5, 6, 66). These changes may be related to NO-mediated alterations in mitochondrial efficiency (39), 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_2 availability (~21%) is reduced. Indeed, NO_3^- supplementation in the form of BR has recently been shown to

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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 [NO_2^-] values. However, the kinetics of plasma [NO_2^-] 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 [NO_2^-] 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 [NO_2^-] during exercise. In addition to exercise, the metabolism of NO and its derivatives are known to be influenced by intracellular P_{O_2} and the fraction of inspired oxygen ($F_{I_{O2}}$). 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 [NO_2^-] during exercise and recovery at different $F_{I_{O2}}$ 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 NO_3^--NO_2^--NO pathway is facilitated in hypoxic conditions (48), we reasoned that BR supplementation may modulate the changes in [NO_2^-] 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 [NO_2^-] dynamics, pulmonary $\dot{V}O_2$, and muscle oxygenation) and exercise tolerance, in both normoxia and hypoxia. We hypothesized that the reduction of [NO_2^-] during exercise would be greater in hypoxia compared with normoxia but that [NO_2^-] 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; $\dot{V}O_{2\,peak}$ = 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 NO_3^- to NO_2^- in the oral cavity (27). Subjects were also instructed to maintain their normal dietary intake for 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 a ramp incremental test to exhaustion for the determination of the maximal O_2 uptake ($\dot{V}O_2$) and the gas exchange threshold (GET). Subjects performed 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 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. $\dot{V}O_2$ was determined as the highest mean $\dot{V}O_2$ during any 30-s period. The GET was determined from a number of measurements, including *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 $\dot{V}E/\dot{V}CO_2$. Power outputs representing moderate- and severe-intensity exercise for each individual were calculated, taking into account the mean response time for $\dot{V}O_2$ 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 ml/day of NO_3^--rich BR or 140 ml/day of NO_3^--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 $\dot{V}O_2$ and plasma [NO_2^-] kinetics. In total, there were four different experimental conditions: *1)* hypoxia-BR (H-BR); *2)* hypoxia-PL (H-PL); *3)* normoxia-BR (N-BR); and *4)* normoxia-PL (N-PL). Trial order was randomly assigned in a balanced fashion, such that three subjects started on H-BR, three started on H-PL, three started on N-BR, and three started on the N-PL condition.

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. Prior to the exercise protocol, subjects lay in a supine position for 10 min breathing normoxic inspirate. A further 10-min period elapsed with subjects breathing either the hypoxic or normoxic inspirate. The exercise protocol involved two 5-min bouts 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 $\dot{V}O_{2\,peak}$) (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 $\dot{V}O_2$ 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 (36). 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 (ModBL) 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 Hypoxico HYP 100 filtration system (Sporting Edge UK, Basingstoke, UK), with the generator supplying the inspirate via an extension conduit to a 150-liter Douglas Bag (Cranlea, 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, Cranlea). 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 O_2 and CO_2 concentration of the inspirate was monitored during each test using a Servomex 5200 High Accuracy Paramagnetic O_2 and CO_2 Analyzer (Servomex, Crowborough, UK). The gas analyzer was calibrated prior to each test with a 16.0% O_2, 8.0% CO_2, and 76.0% N_2 gas mix (BOC Special Gases, Guildford, UK). For the N-PL and N-BR trials, the Hypoxico HYP-100 generator was switched to normoxic mode (i.e., all O_2 filters were turned off so that no O_2 was removed from the ambient air). However, during the H-PL and H-BR trials, the generator was set to maximum O_2 filtration, which supplied an F_{IO2} of 0.131 ± 0.02, and an F_{ICO_2} of 0.004 ± 0.00.

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 NO_3^- placebo supplementation before *visits 3–6*. The supplements were either concentrated, NO_3^--rich BR (2 × 70 ml/day of BR providing ~8.4 mmol NO_3^- per day; Beet it, James White Drinks, Ipswich, UK) or concentrated, NO_3^--depleted PL (2 × 70 ml/day of PL providing ~0.006 mmol NO_3^- 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 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 beeturia (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, Salon, OH) and analyzed to determine blood [lactate] and [glucose] (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). Blood samples for the determination of plasma [NO_2^-] and [NO_3^-] were collected into lithium-heparin tubes and immediately centrifuged at 4,000 rpm and 4°C for 8 min. Plasma was extracted and immediately frozen at −80°C for later analysis of [NO_2^-] and [NO_3^-].

Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO_2^-. Plasma [NO_2^-] and [NO_3^-] were analyzed using gas phase chemiluminescence. This initially required NO_2^- and NO_3^- to be reduced to NO gas. For reduction of NO_2^-, undiluted plasma was injected into a glass purge vessel containing 5 ml of glacial acetic acid and 1 ml NaI solution. For NO_3^- reduction, plasma samples were deproteinized in an aqueous solution of zinc sulfate (10% wt/vol) and 1 M sodium hydroxide, prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% wt/vol). 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 analyzer (Sievers NOA 280i; Analytix, Durham, UK). The concentrations of NO_2^- and NO_3^- 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 rate of change in plasma [NO_2^-] during the severe exercise bout was calculated as the difference between preexercise baseline and exercise [NO_2^-] values relative to exercise duration.

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 (O_2) and infrared (CO_2) analyzers (Oxycon Pro, Jaeger, Hoechburg, Germany) via a capillary line connected to the mouthpiece. Pulmonary gas exchange variables were calculated and displayed breath-by-breath. Heart rate (HR) and arterial oxygen saturation (Sa_{O2}) 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 musculus vastus lateralis of the right leg was monitored via near-infrared spectroscopy (NIRS) (NIRO 200; Hamamatsu Photonics KK, Hamamatsu City, Japan) during the exercise protocol, as described previously (4). Deoxyhemoglobin concentration ([HHb]), oxyhemoglobin concentration ([HbO_2]), total hemoglobin concentration ([Hb_{tot}]), and tissue oxygenation index (TOI) were measured.

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, 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 I 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 $\dot{V}O_2$

responses to both moderate- and severe- intensity exercise, as described in the following equation:

$$\dot{V}o_2(t) = \dot{V}o_{2\ baseline} + A_p\left[1 - e^{-(t-TD_p/\tau_p)}\right] \quad (1)$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time t; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ over the final 60 s of baseline cycling; A_p, TD_p, and τ_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. 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 [HHb] response to exercise was also modeled, as described previously (4). The [HHb] 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 [HHb] 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 [HHb], as determined by visual inspection of the residual plots. For severe-intensity exercise, the [HHb] fast and slow-phase responses were determined as described above for the $\dot{V}O_2$. The [HbO_2], [Hb_{tot}], and TOI responses were not modeled as they do not approximate an exponential. Rather, the changes in these variables were assessed by determining the [HbO_2], [Hb_{tot}], and TOI at baseline (60 s preceding step 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 × F_{IO2}) 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 [NO_2^-] and [NO_3^-]

Preexercise, plasma [NO_2^-] was significantly elevated in H-BR compared with H-PL (H-BR: 301 ± 89 vs. H-PL: 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 [NO_2^-] during moderate-intensity and severe-intensity exercise and subsequent recovery are presented in Fig. 1.

Plasma [NO_3^-] 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).

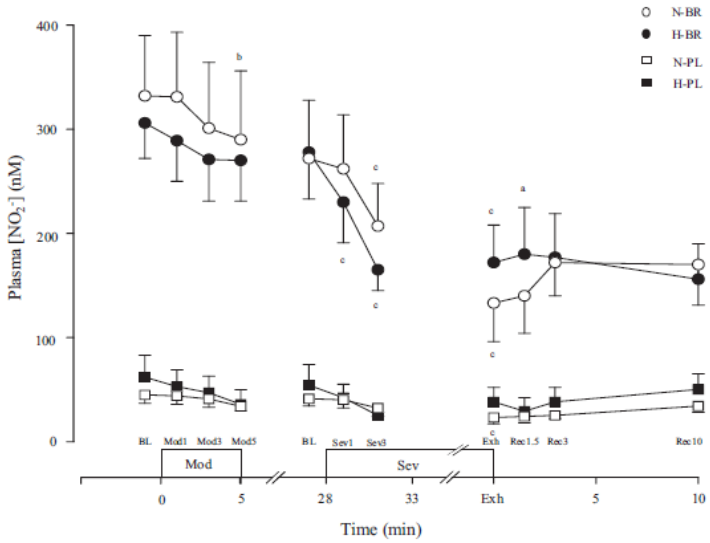

Fig. 1. Plasma [NO_2^-] response during moderate-intensity and severe-intensity exercise and recovery following beetroot juice (BR) and placebo (PL) supplementation in normoxia (N) and hypoxia (H). Group means ± SE plasma. H-BR was greater than H-PL at each time point, and N-BR was greater than N-PL at each time point. $^aP < 0.05$ for N-BR compared with H-BR. $^bP < 0.05$ compared with moderate baseline. $^cP < 0.05$ compared with severe baseline. Where error bars are not visible, the size of the data point exceeds the error.

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 (all $P < 0.05$). 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: 290 ± 207 nM; $P = 0.04$). However, the decrease in plasma [NO_2^-] in H-BR only showed a trend toward a reduction (ModBL: 306 ± 109 vs. Mod5: 270 ± 125 nM; $P = 0.10$). The rate of 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 (all $P < 0.05$). 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: 206 ± 129; $P = 0.01$; exhaustion: 132 ± 117 nM; $P < 0.01$). In H-BR, plasma [NO_2^-] decreased from SevBL (277 ± 142 nM) to Sev1 (229 ± 123 nM; $P = 0.01$), Sev3 ($n = 10$; 164 ± 64 nM; $P = 0.03$) and exhaustion (171 ± 115 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 ± 60 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 (22 ± 19 nM; $P = 0.02$). This decrease was not significant in H-PL (SevBL: 53 ± 65 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: −30 ± 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 ($P = 0.66$) 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^-] (Fig. 1). BR supplementation significantly elevated plasma [NO_2^-] across all time points compared with PL in both hypoxic and normoxic conditions (all $P < 0.05$). 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 Sa_{O2} data at rest and during moderate-intensity and severe-intensity exercise are reported in Table 1. Resting Sa_{O2} and HR prior to the administration of inspirate were not significantly different between conditions. However, ANOVA revealed a significant main effect by $F_{I_{O2}}$ following 10 min of breathing the hypoxic or normoxic inspirate, with Sa_{O2} being significantly reduced in H-PL compared with N-PL ($P < 0.01$) and H-BR compared with N-BR ($P < 0.01$). HR was significantly elevated in H-PL compared with N-PL ($P < 0.01$) and H-BR compared with N-BR ($P = 0.02$) in the final 30 s of gas inspiration.

Moderate exercise. During moderate-intensity exercise, Sa_{O2} 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. Sa_{O2} 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 Sa_{O2} between BR and

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

	N-PL	N-BR	H-PL	H-BR
Resting without inspirate				
Sa_{O2}, %				
10-min period	99 ± 1	99 ± 1	99 ± 1	99 ± 1
End	99 ± 1	99 ± 1	99 ± 1	99 ± 1
HR, beats/min				
10-min period	59 ± 9	61 ± 10	61 ± 10	61 ± 9
End	60 ± 9	61 ± 9	61 ± 10	61 ± 9
Resting with inspirate				
Sa_{O2}, %				
10-min period	99 ± 1	99 ± 1	93 ± 2†	93 ± 2*
End	99 ± 1	99 ± 1	90 ± 3†	91 ± 1*
HR, beats/min				
10-min period	58 ± 9	60 ± 11	68 ± 11	66 ± 10#
End	60 ± 8	60 ± 11	68 ± 11†	66 ± 10*
Moderate-intensity exercise				
Sa_{O2}, %				
Baseline	97 ± 3	98 ± 2	87 ± 4	85 ± 4
6-min period	97 ± 3	98 ± 2	83 ± 3†	84 ± 4*
End	97 ± 3	97 ± 3	81 ± 4†	82 ± 5*
HR, beats/min				
Baseline	82 ± 10	86 ± 12	101 ± 16	94 ± 13
6 min period	102 ± 15	107 ± 15	122 ± 15	117 ± 19#
End	105 ± 16	111 ± 17	130 ± 15†	124 ± 19*
Severe-intensity exercise				
Sa_{O2}, %				
Baseline	98 ± 2	97 ± 3	86 ± 4	87 ± 4
Exhaustion	94 ± 4	94 ± 4	80 ± 3†	80 ± 4*
HR, beats/min				
Baseline	97 ± 9	103 ± 12	113 ± 9	114 ± 12
Exhaustion	179 ± 4	180 ± 5	172 ± 6	171 ± 6

Data are presented as means ± SD. HR, heart rate; N-PL, normoxia placebo; N-BR, normoxia beetroot juice; H-PL, hypoxia placebo (NO_3^--depleted beetroot juice); H-BR, hypoxia beetroot juice. #$P < 0.05$ compared to H-PL. *$P < 0.05$ compared to N-BR. †$P < 0.05$ compared to N-PL.

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

$\dot{V}O_2$ Kinetics

Pulmonary $\dot{V}O_2$ 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 $\dot{V}O_2$ response to moderate-intensity exercise. The $\dot{V}O_2$ 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 $\dot{V}O_2$ during baseline (20 W) exercise in hypoxia compared with PL ($P = 0.02$). The $\dot{V}O_2$ phase II τ tended to be increased (i.e., slower kinetics) in hypoxia compared with normoxia ($P = 0.07$). Post hoc analyses revealed that the $\dot{V}O_2$ phase II τ was smaller (i.e., faster kinetics) in H-BR compared with H-PL ($P = 0.04$).

Severe exercise. During severe-intensity exercise, the $\dot{V}O_2$ slow-component amplitude ($P < 0.01$) and $\dot{V}O_2$ at exhaustion ($P < 0.01$) were significantly reduced as a result of the hypoxic inspirate in both PL and BR (Table 2). In hypoxia, BR tended to further reduce the end-exercise $\dot{V}O_2$ compared with H-PL ($P = 0.07$), while BR had no effect upon end-exercise $\dot{V}O_2$ in normoxia.

NIRS

The [HHb], [HbO_2], [Hb_{tot}], and TOI values measured during moderate- and severe-intensity exercise are shown in Table 3.

Moderate exercise. During moderate-intensity exercise, ANOVA revealed a significant main effect by F_{IO2}. The modeled [HHb] amplitude was significantly greater in hypoxia compared with normoxia in both supplemented conditions across all time points (all $P < 0.05$). The end-exercise [HbO_2] was lower in H-BR compared with N-BR ($P = 0.02$) and H-PL compared with N-PL ($P = 0.01$). TOI at baseline and throughout exercise was also significantly reduced in hypoxia compared with normoxia ($P < 0.05$). Post hoc analyses revealed that BR tended to offset the negative effects of hypoxia on TOI when compared with PL ($P = 0.08$).

Severe exercise. During severe-intensity exercise, ANOVA revealed a significant main effect by F_{IO2}. [HHb] was significantly increased in H-BR and H-PL compared with N-BR and N-PL ($P < 0.05$), whereas the [HHb] slow phase amplitude was larger in normoxia compared with hypoxia ($P < 0.05$). [HbO_2] was reduced in hypoxia compared with normoxia ($P < 0.05$), and TOI was lower as a result of hypoxia throughout exercise ($P < 0.05$). No differences in NIRS data between BR and PL were evident during severe-intensity exercise.

Exercise tolerance. ANOVA revealed that hypoxia resulted in a significant reduction in exercise tolerance compared with normoxia in both PL (H-PL: 197 ± 28 vs. N-PL: 431 ± 124 s, $P < 0.01$) and BR conditions (H-BR: 214 ± 43 vs. N-BR 412 ± 139 s, $P < 0.01$). Although the unspecific F-test for interaction effect across all four conditions did not attain significance at the 95% level, it should be noted that the specific test for a difference between exercise tolerance in H-BR and H-PL was significant (H-BR: 214 ± 43 vs. H-PL: 197 ± 28 s; $P = 0.04$), whereas the comparison between N-BR and N-PL was not (N-BR: 412 ± 139 vs. N-PL: 431 ± 124 s;

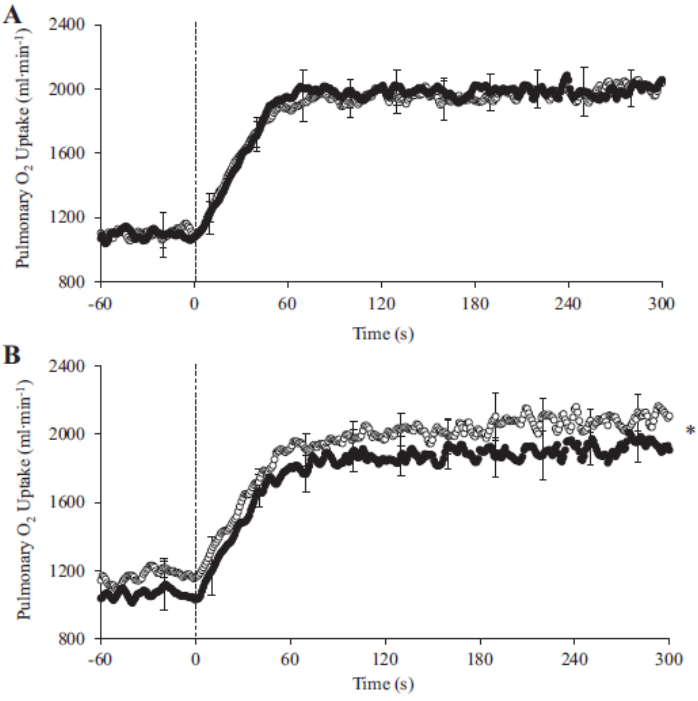

Fig. 2. Pulmonary O_2 uptake ($\dot{V}O_2$) responses during a step increment to a moderate-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles (●), with the PL responses being shown as open circles (○). The dashed vertical line denotes the 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 (~21% F_{IO2}). *B*: group mean response to moderate-intensity exercise in hypoxia (~13.2 F_{IO2}). *$P < 0.05$ compared with H-PL.

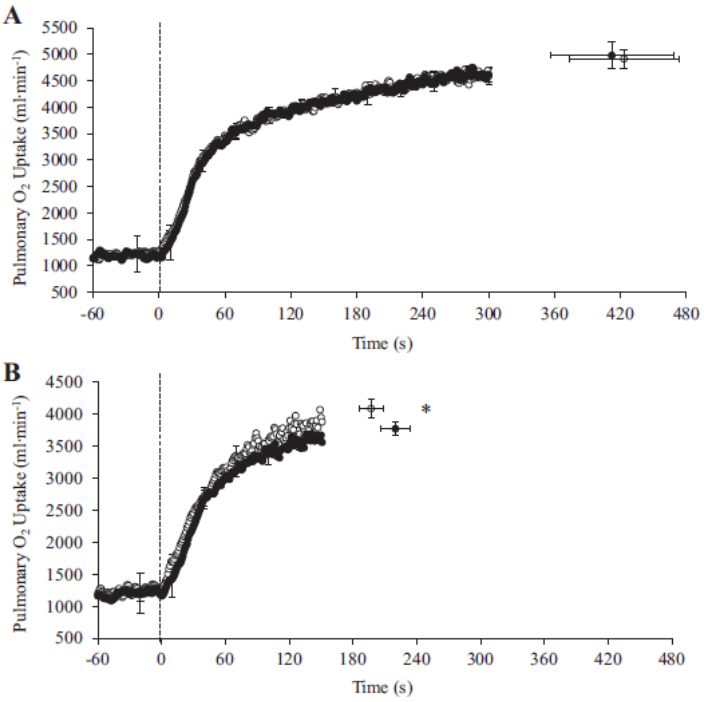

Fig. 3. Pulmonary O_2 uptake ($\dot{V}O_2$) responses and time-to exhaustion during a step increment to a severe-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles (●), with the PL responses being shown as open circles (○). The dashed vertical line denotes the abrupt step transition from baseline to severe-intensity cycling exercise. Error bars indicate the SE. *A*: group mean response to severe-intensity exercise in normoxia (~21% F_{IO2}). *B*: group mean response to severe-intensity exercise in hypoxia (~13.2 F_{IO2}). *Time to exhaustion greater in H-BR compared with H-PL ($P < 0.05$; one-tailed *t*-test).

$P = 0.50$). The increase in severe-intensity exercise tolerance was correlated with the reduction in moderate steady-state $\dot{V}O_2$ following BR supplementation in hypoxia ($r = -0.96$; $P < 0.01$).

DISCUSSION

Consistent with previous findings, the decline of plasma [NO_2^-] during exercise was greater following BR compared with PL supplementation. However, in contrast to our experimental hypothesis, the decline of plasma [NO_2^-] during exercise was similar or slightly smaller in hypoxia compared with normoxia. Nonetheless, 3 days of BR supplementation significantly speeded $\dot{V}O_2$ kinetics and lowered the steady-state $\dot{V}O_2$ 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.

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

	N-PL	N-BR	H-PL	H-BR
Moderate-intensity exercise				
$\dot{V}O_2$, ml/min				
Baseline	1102 ± 156	1010 ± 343	1167 ± 123	1056 ± 133#
End exercise	1970 ± 251	1908 ± 340	2049 ± 247	1905 ± 275#
Phase II τ, s	22 ± 10	17 ± 4#	31 ± 11	24 ± 13#
Primary amplitude	868 ± 210	899 ± 256	882 ± 214	849 ± 208
Severe-intensity exercise				
$\dot{V}O_2$, ml/min				
Baseline	1212 ± 179	1205 ± 158	1244 ± 175	1193 ± 177
End exercise	4814 ± 470	4721 ± 434	3986 ± 300†	3751 ± 249*
Phase II τ, s	30 ± 6	28 ± 9	35 ± 14	31 ± 11
Primary amplitude	2716 ± 398	2636 ± 486	2450 ± 497	2264 ± 386
Slow component amplitude	886 ± 235	881 ± 259	302 ± 290†	301 ± 274*

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

Effects of BR Supplementation on the Kinetic Profile of Plasma [NO_2^-]

Plasma [NO_2^-] increased significantly following BR supplementation compared with PL, at rest and prior to administration of the inspirate. These findings are consistent with previous research, which has consistently reported elevations in plasma [NO_2^-] (3, 4, 33, 34, 51, 62, 67), following BR supplementation.

Previous studies have suggested that baseline plasma [NO_2^-] 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_2^-] dynamics during and following exercise of different intensities in hypoxia and normoxia with and without NO_3^- supplementation. The results suggest that the metabolism of NO and its derivatives are altered by exercise and NO_3^- supplementation and, to a lesser extent, F_{IO2}. The interpretation of these data is not straightforward, however. NO_3^- can be reduced in vivo to bioactive NO_2^- and further to NO (47), and this reduction of NO_2^- to NO is expected to be facilitated in hypoxia (13). However, NO_2^- is also an oxidation product of NO generation via the NOS pathway (30) with plasma [NO_2^-] providing a sensitive marker of NO production through NOS (43). Therefore, the dynamics of plasma [NO_2^-] over the exercise bouts is

Table 3. *Near-infrared spectroscopy-derived muscle [HHb], [HbO_2], [Hb_{tot}] and TOI dynamics during moderate- and severe-intensity exercise*

	N-PL	N-BR	H-PL	H-BR
Moderate-intensity exercise				
[HHb], AU				
Baseline	7 ± 5	6 ± 5	11 ± 5†	10 ± 5*
120 s	11 ± 8	11 ± 7	18 ± 8†	17 ± 10*
End exercise	12 ± 8	11 ± 7	20 ± 8†	18 ± 10*
Time constant, s	23 ± 7	19 ± 6	22 ± 9	23 ± 7
Amplitude	5 ± 4	6 ± 4	8 ± 5†	7 ± 6*
[HbO_2], AU				
Baseline	2 ± 6	3 ± 6	2 ± 5	2 ± 7
120 s	1 ± 6	2 ± 6	−2 ± 4	−2 ± 8
End exercise	4 ± 5	5 ± 5	0 ± 3†	−2 ± 9*
[Hb_{tot}], 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				
[HHb], AU				
Baseline	5 ± 5	5 ± 5	10 ± 6†	10 ± 6*
120 s	19 ± 13	18 ± 11	25 ± 12†	24 ± 14*
End exercise	22 ± 14	21 ± 12	26 ± 12†	26 ± 14*
Time constant, s	13 ± 5	11 ± 5	11 ± 3	12 ± 6
Primary amplitude	14 ± 10	14 ± 8	14 ± 9	14 ± 10
Slow phase amplitude	3 ± 2	3 ± 2	2 ± 2†	2 ± 2*
[HbO_2], AU				
Baseline	7 ± 7	8 ± 7	6 ± 5†	5 ± 8*
120 s	−4 ± 7	−3 ± 7	−9 ± 4†	−10 ± 8*
End exercise	−7 ± 9	−7 ± 7	−11 ± 5†	−12 ± 7*
[Hb_{tot}], 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 ± 4	64 ± 4	64 ± 4
120 s	52 ± 12	51 ± 10	44 ± 9	44 ± 10
End exercise	48 ± 11	47 ± 9	41 ± 9†	41 ± 8*

Data are presented as means ± SD. Deoxygenated hemoglobin concentration ([HHb]), oxygenated hemoglobin concentration ([HbO_2]), total hemoglobin concentration ([Hb_{tot}]) and tissue oxygenation index (TOI) are shown. *$P < 0.05$ compared to N-BR. †$P < 0.05$ compared to N-PL. AU, arbitrary units.

likely reflective of the dynamic balance between NOS-derived NO and NO_2^- 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 NO_2^- 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, may be slightly greater (12).

During the 10-min passive recovery from exhaustive exercise, plasma [NO_2^-] increased in a similar fashion in H-PL and N-PL. Specifically, plasma [NO_2^-] increased after 3 min of recovery and plateaued after 10 min. The increases in plasma [NO_2^-] may represent an increase in NO oxidation (as NO is continuing to contribute to 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 Exh and 1.5Rec 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. $\dot{V}O_2$ 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) $\dot{V}O_2$ was found in H-BR compared with H-PL. These findings are consistent with previous studies that have reported reductions in submaximal cycling $\dot{V}O_2$ in varying severities of hypoxia. For example, Masschelein et al. (50) reported a 4% reduction in steady-state $\dot{V}O_2$ with an F_{IO2} of 0.11 during cycle exercise at 45% peak $\dot{V}O_2$, and Muggeridge et al. (51) reported a ~6–8% reduction in steady-state $\dot{V}O_2$ at an F_{IO2} 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 phosphocreatine (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 $\dot{V}O_2$ phase II τ during moderate-intensity exercise was reduced by BR supplementation in hypoxia. This finding is consistent with a recent study in older individuals, where the $\dot{V}O_2$ mean response time was speeded with BR supplementation (32). This may be related to the slower $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics in the young healthy participants in the present study. Specifically, the phase II τ 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 $\dot{V}O_2$ kinetics in hypoxia (29, 59). BR supplementation speeded the phase II τ in hypoxia toward values recorded in normoxia, thereby helping to reverse the detrimental effect of a reduced F_{IO2} on $\dot{V}O_2$ 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).

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 $\dot{V}O_2$ during submaximal exercise in normoxia. Previous studies have typically reported reductions in steady state $\dot{V}O_2$ of ~3–5% following several days of NO_3^- supplementation (4, 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 (4, 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 $\dot{V}O_2$ during moderate-intensity exercise were recently reported to be evident following acute supplementation with 16.8 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 $\dot{V}O_2$ response to exercise in normoxia in the present study. Furthermore, the interindividual differences in the $\dot{V}O_2$ 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 F_{IO2} during moderate-intensity exercise but BR supplementation did not significantly influence this response. Consistent with a previous study (50), [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 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 3.6% 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_2^-] difference is associated with limb vasodilatation 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 $\dot{V}O_2$ 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 $\dot{V}O_2$) 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 $\dot{V}O_2$ 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 [HHb] 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 $\dot{V}O_2$ with BR compared with PL supplementation in hypoxia of ~6%. This finding indicates the $\dot{V}O_{2\,peak}$ 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 $\dot{V}O_2$ and the improvement in exercise tolerance following BR supplementation in hypoxia ($r = -0.96$). Therefore, the lack of effect on $\dot{V}O_2$ 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 F_{IO2} and BR supplementation on plasma [NO_2^-] dynamics during moderate- and severe-intensity exercise and subsequent recovery in humans. The greater rate of decline of plasma [NO_2^-] during exercise following BR compared with PL supplementation suggests that elevating plasma [NO_2^-] 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 $\dot{V}O_2$ kinetics, and improved severe-intensity exercise

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

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, Gilchrist M, Winyard PG, Jones AM. 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 × 70 ml/day, ~9.6 mmol/day NO_3^-) or a nitrate-depleted beetroot juice placebo (PL; 2 × 70 ml/day, ~0.01 mmol/day 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 ± 9 vs. PL: 120 ± 6 mmHg; $P < 0.05$) and diastolic (BR: 70 ± 5 vs. PL: 73 ± 5 mmHg; $P < 0.05$) blood pressure. Nitrate supplementation resulted in a speeding of the $\dot{V}O_2$ mean response time (BR: 25 ± 7 vs. PL: 28 ± 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 $\dot{V}O_2$ 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_3^- supplementation, either in the form of sodium nitrate ($NaNO_3^-$) or beetroot juice, can significantly increase plasma [NO_2^-] and reduce resting blood pressure in young adults (5, 49, 76, 81). Moreover, dietary NO_3^- 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/day, containing 5.5 mmol/day of NO_3^-; Ref. 4) resulted in a significant reduction in oxygen uptake ($\dot{V}O_2$) 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 $\dot{V}O_2$-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_3^- 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_2^-] (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_3^- supplementation might enhance NO bioavailability and vascular function in older adults.

The ageing 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

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muscle blood flow (80) during exercise is attenuated with increasing age. Moreover, the distribution of blood flow in the microcirculation, capillary density, and capillary hemodynamics (7, 8, 18, 30, 59, 60, 65), as well as mitochondrial volume density and oxidative function (15, 16) are compromised with aging. There is evidence that $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics in older adults. Faster $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 ^1H 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 $\dot{V}O_2$ 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_2^-], 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 slat-belt treadmill (PPS-55 Sport, Woodway, Weil am Rhein, Germany) set at a 1% gradient (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 ($\dot{V}CO_2$) from visual inspection of individual plots of $\dot{V}CO_2$ vs. $\dot{V}O_2$; *2*) an increase in expired ventilation ($\dot{V}E$)/$\dot{V}O_2$ with no increase in $\dot{V}E/\dot{V}CO_2$; 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 step-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 keys 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 8 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 *visits 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 (Philips Gyroscan Clinical Intera). 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 nonferrous 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. ^{1}H MRS brain measurements of *N*-acetyl aspartate (NAA), creatine (Cr), choline (Ch), 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 NO_3^--rich BR (2 × 70 ml/day, organic beetroot juice, each containing ~4.8 mmol NO_3^-, Beet it, James White Drinks, Ipswich, UK) or NO_3^- -depleted PL (2 × 70 ml/day, organic beetroot juice containing ~0.01 mmol NO_3^-, 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 NO_3^- 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 beeturia (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 × systolic pressure +2/3 × 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 [NO_2^-] was used as a biomarker for NO availability (42, 52). To obtain plasma [NO_2^-], 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 [NO_2^-]. Prior to, and regularly during analysis, all glassware, utensils, and surfaces

were rinsed with deionized water to remove any residual NO_2^-. 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_2^-] 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 radiotelemetry (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 ^{31}P transmit/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 via 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_i, phosphodiester, PCr, 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_i 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_i solution, where the localized volume sampled within the muscle was the same dimensions and distance from the coil as the P_i solution. This allowed the calculation of muscle P_i concentration, following corrections for relative coil loading. Subsequently, absolute values of [PCr] and ATP concentrations were calculated via the ratio of P_i to PCr and P_i to ATP. Intracellular pH was calculated using the chemical shift of the P_i spectral peak relative to the PCr peak (72). In addition to this, ADP concentration ([ADP]) was calculated as described previously (39).

^1H MRS was performed using an eight-channel SENSE head coil in the left frontal white matter and the occipito-parietal gray matter in single voxels of 2 × 2 × 2 cm. 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 prepulse 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 prepulse 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 an Hankel Lanczos Singular Values Decomposition filter prior to peak fitting, from which the areas of the NAA, Cr, Ch, and mI 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, Ch:water, Cr:water, mI:water, NAA:Ch, NAA:Cr and NAA:(Cr + Ch) 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 × 2 mm, 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 isotopic $b = 800$ s/mm^{-2} images, such that ADC $= -(1/800)\ \ln\ (S/S_0)$, where S is the signal intensity in the selected ROI for the $b = 800$ s/mm^{-2}, and S_0 is the image intensity for the corresponding $b = 0$ image.

Data Analysis

Oxygen uptake. The breath-by breath $\dot{V}O_2$ data from each test were initially examined to exclude errant breaths caused by coughing and swallowing, with those values lying more than four 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 used to fit the data. With only two transitions and a relatively low-response amplitude, however, we elected to describe the overall $\dot{V}O_2$ 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 (O_2df) as the product of the $\dot{V}O_2$ response amplitude (baseline to exercise steady-state) and the MRT. $\dot{V}O_{2baseline}$ was defined as the mean $\dot{V}O_2$ measured over the final 90 s of the baseline period. 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 mean baseline $\dot{V}CO_2$, $\dot{V}E$, 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], [P_i], [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.

Statistical Analyses

Differences in plasma [NO_2^-]; 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_2^-] and BP

PL supplementation resulted in no significant change in plasma [NO_2^-] relative to the nonsupplemented control condition. In contrast, BR supplementation elevated plasma [NO_2^-] by 503% relative to control (CON: 206 ± 59 vs. BR: 1,037 ± 627 nM, $P < 0.01$) and by 418% compared with PL (PL: 248 ± 182 nM, $P < 0.01$).

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 $\dot{V}O_2$ 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 $\dot{V}O_2$ between PL and BR during the baseline walking period. The amplitude of the pulmonary $\dot{V}O_2$ response was not different between the two conditions (PL: 477 ± 200 vs. BR: 464 ± 200 ml/min) and the steady-state

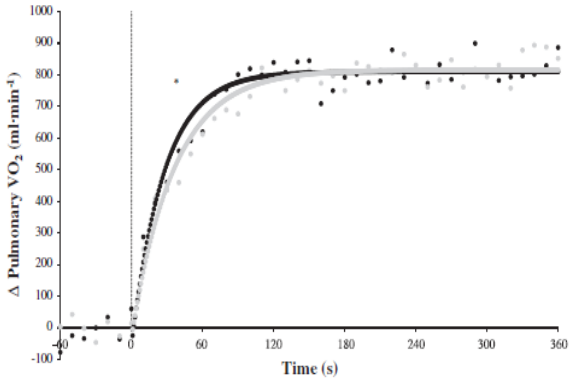

Fig. 1. Pulmonary oxygen uptake ($\dot{V}O_2$) responses during a step increment to a moderate-intensity work rate, following PL and BR supplementation, in a representative subject. Responses following BR are represented by the black line, and responses following PL are represented by the gray line. The dotted vertical line denotes the abrupt "step" transition from baseline to moderate-intensity walking exercise. Data are presented in 10-s intervals.

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

	Placebo	Beetroot
$\dot{V}O_2$		
Baseline, ml/min	518 ± 104	528 ± 87
Primary amplitude, ml/min	477 ± 200	464 ± 200
End exercise, ml/min	979 ± 269	977 ± 250
Mean response time, s	28 ± 7	25 ± 7*
Oxygen deficit, ml	225 ± 132	192 ± 137
$\dot{V}CO_2$		
Baseline, ml/min	452 ± 93	454 ± 73
End exercise, ml/min	847 ± 242	848 ± 200
$\dot{V}E$		
Baseline, l/min	15.2 ± 3.9	15.4 ± 24.9
End exercise, l/min	25.0 ± 7.4	24.9 ± 6.7
Respiratory exchange ratio		
Baseline	0.87 ± 0.06	0.86 ± 0.03
End exercise	0.89 ± 0.05	0.94 ± 0.05
Heart rate		
Baseline, bpm	78 ± 9	77 ± 9
End exercise, bpm	95 ± 12	92 ± 9
Amplitude, bpm	17 ± 12	15 ± 7

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

$\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}CO_2$, $\dot{V}E$, RER, and HR were not significantly different between conditions (Table 1).

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*

	Placebo	Beetroot
[PCr]		
Baseline, mM	32.0 ± 5.5	31.9 ± 5.0
240 s, mM	25.8 ± 5.9	26.5 ± 5.8
Amplitude, mM	6.2 ± 2.5	5.3 ± 3.0
[P_i]		
Baseline, mM	3.7 ± 1.0	3.5 ± 0.8
240 s, mM	7.9 ± 1.9	8.3 ± 1.7
Amplitude, mM	4.2 ± 1.1	4.8 ± 1.7
[ADP]		
Baseline, μM	7.4 ± 1.7	7.4 ± 2.1
240 s, μM	22.5 ± 8.4	21.5 ± 8.3
Amplitude, μM	15.1 ± 8.6	14.2 ± 8.5
pH		
Baseline	7.03 ± 0.03	7.02 ± 0.02
240 s	7.07 ± 0.04	7.06 ± 0.02
Δ Baseline – 240 s	0.04 ± 0.02	0.03 ± 0.03

Values are expressed as means ± SD.

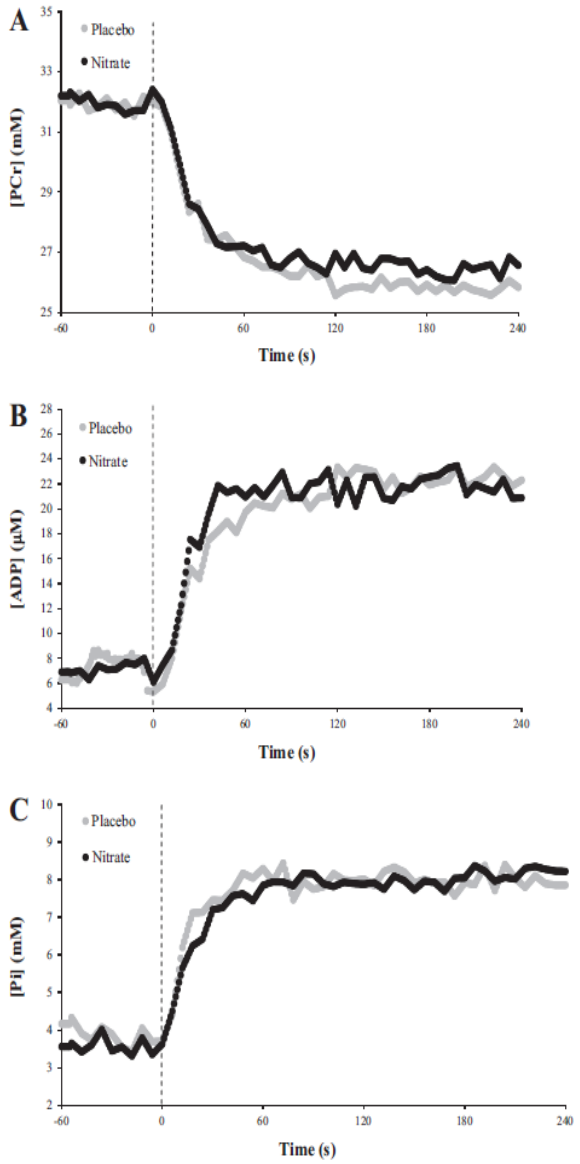

Fig. 2. Group mean muscle metabolic responses to low-intensity, leg-extension exercise following PL and BR supplementation. The change in muscle [PCr] (*A*), [ADP] (*B*), and [P_i] (*C*) from rest to steady state were unaffected by BR supplementation. The dotted vertical line denotes the abrupt "step"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

were no significant differences in the baseline or end-exercise [P_i], [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

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

	Placebo	Beetroot
[PCr]		
Baseline, mM	30.0 ± 4.2	30.6 ± 5.5
24 s, mM	21.9 ± 4.1	23.2 ± 5.5
Amplitude, mM	8.1 ± 2.7	7.3 ± 2.8
Recovery τ, s	35 ± 10	37 ± 15
[P_i]		
Baseline, mM	2.9 ± 0.9	3.1 ± 1.0
24 s, mM	8.9 ± 2.1	8.9 ± 1.9
Amplitude, mM	6.0 ± 1.7	5.8 ± 1.5
[ADP]		
Baseline, μM	8.0 ± 3.3	8.0 ± 2.2
24 s, μM	37 ± 13.6	34.1 ± 9.4
Amplitude, μM	29.4 ± 12.3	26.1 ± 10.0
pH		
Baseline	6.99 ± 0.03	7.00 ± 0.02
24 s	7.00 ± 0.03	7.00 ± 0.03
Δ Baseline – 240 s	0.01 ± 0.01	0.00 ± 0.04

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

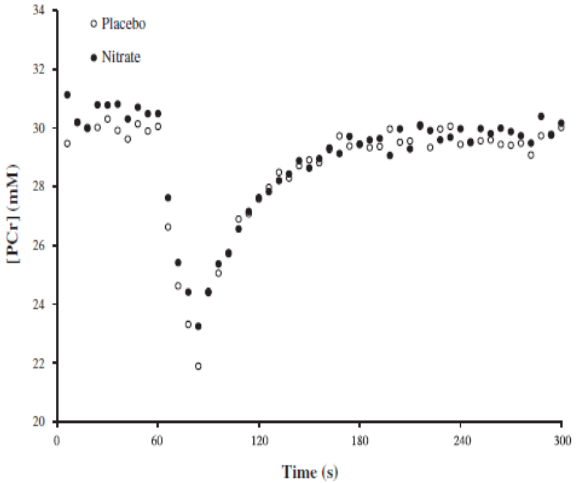

Fig. 3. Group mean intramuscular [PCr] response to 24-s high-intensity, leg-extension exercise and subsequent recovery. [PCr] responses following BR are represented as solid circles, with the PL responses being shown as open circles.

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

	Placebo	Beetroot
Serial subtractions		
3′s, correct responses in 2 min	29 ± 8	26 ± 14
7′s, correct responses in 2 min	16 ± 9	16 ± 10
Rapid visual information processing		
Correct target I.D's	21 ± 4	23 ± 4
Errors	9 ± 17	9 ± 16
Average response time, ms	599 ± 199	674 ± 194
Number recall		
Forwards correct	29 ± 8	27 ± 8
Backwards correct	22 ± 7	21 ± 7
Total correct	51 ± 14	48 ± 14

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 gray 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 [nitrite] four-fold) significantly reduced resting blood pressure and the $\dot{V}O_2$ 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 $\dot{V}O_2$ 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_2^-] and BP

Following supplementation with NO_3^--rich BR, plasma [NO_2^-] was increased to 418% of the PL value. These findings are consistent with previous studies that reported significant elevations in plasma [NO_2^-] following dietary NO_3^- supplementation (29, 50, 76). CON plasma [nitrite] values in the present study population were similar to those found in young adults. This was surprising because lower [nitrite] values may be expected in an older population (68). Moreover, it might be expected that the effect of NO_3^- supplementation on plasma [NO_2^-] might be smaller in older compared with younger adults due to age-related changes in oral bacterial colonization (63). However, the elevation of plasma [NO_2^-] 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_2^-] 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_2^- 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. *^{1}H-MRS and ADC brain scan data following placebo and beetroot juice supplementation*

^{1}H-MRS Brain Scans	Placebo	Beetroot
Gray matter		
NAA:Water	2.065 ± 0.273	2.141 ± 0.213
Cr:Water	1.024 ± 0.111	1.050 ± 0.090
Ch:Water	0.568 ± 0.117	0.559 ± 0.085
mI:Water	0.757 ± 0.193	0.803 ± 0.102
NAA:Cr	2.031 ± 0.294	2.048 ± 0.245
NAA:Ch	3.805 ± 1.054	3.908 ± 0.696
NAA:Cr+Ch	1.315 ± 0.235	1.337 ± 0.162
White matter		
NAA:Water	1.575 ± 0.263	1.637 ± 0.180
Cr:Water	0.895 ± 0.140	0.940 ± 0.078
Ch:Water	1.016 ± 0.108	0.992 ± 0.149
mI:Water	0.950 ± 0.226	1.065 ± 0.337
NAA:Cr	1.761 ± 0.118	1.742 ± 0.143
NAA:Ch	1.546 ± 0.177	1.692 ± 0.401
NAA:Cr+Ch	0.821 ± 0.069	0.850 ± 0.109
ADC, 10^{-3}		
Dorsolateral, prefrontal cortex	0.782 ± 0.033	0.783 ± 0.050
Anterior cingulated gyrus	0.753 ± 0.137	0.790 ± 0.101
Frontal lobe (deep white matter)	0.817 ± 0.052	0.841 ± 0.073

Values are expressed as means ± SD. MRS, magnetic resonance spectroscopy; NAA, *N*-acetyl aspartate; Cr, creatine; Ch, choline; mI, myo-inositol; ADC, apparent diffusion coefficient.

is unclear. Our results suggest that a high NO_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 $\dot{V}O_2$ kinetics following the onset of exercise subsequent to dietary NO_3^- supplementation. Faster $\dot{V}O_2$ 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_2 deficit was reduced by 15% following NO_3^- supplementation, as a function of the faster $\dot{V}O_2$ kinetics. Whether the small speeding of $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics following NO_3^- supplementation (5, 47, 76). Older adults typically have slower $\dot{V}O_2$ kinetics (3, 14, 22) and are more likely to evince a speeding of $\dot{V}O_2$ kinetics following interventions designed to enhance muscle O_2 delivery (66) than their younger counterparts. The MRT for $\dot{V}O_2$ kinetics for the older subjects tested in the present study was surprisingly 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_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 $\dot{V}O_2$ kinetics that we observed was linked to enhanced muscle vasodilatation and blood flow, which offset an O_2 delivery limitation to $\dot{V}O_2$ kinetics in our older subjects.

No effects on the O_2 cost of walking were evident in the present study, which is in contrast to results reported recently in younger adults (47) and to the body of literature, which indicates that NO_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_3^- supplementation on the O_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_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 $\dot{V}O_2$ kinetics, as was observed in the present study following NO_3^- supplementation, would be expected to improve performance in certain physical tasks. It is unclear why NO_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_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_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_3^- supplementation significantly reduced the amplitude of [PCr] depletion during low-intensity exercise (5). The linear relationship observed between $\dot{V}O_2$ and intramuscular [PCr], both before and after NO_3^- supplementation, suggested that the reduction in the O_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_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/or oxidative enzyme activity or, in the event of tissue hypoxia, O_2 supply (1). In the present study, NO_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_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_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_3^- supplementation. Likewise,

mI, 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 the Presley et al. study (63) 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 $\dot{V}O_2$ 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, quercetin 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_2^-], diastolic BP, MAP, and $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 may 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics in older adults. Whether this may translate into improved functional capacity in functionally impaired older adults should be considered in subsequent research.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.K., J.F., A.V., S.J.B., M.G., P.G.W., and A.M.J. conception and design of research; J.K., J.F., and O.F. performed experiments; J.K., J.F., J.R.B., O.F., and P.G.W. analyzed data; J.K., J.F., A.V., J.R.B., S.J.B., P.G.W., and A.M.J. interpreted results of experiments; J.K. prepared figures; J.K., O.F., and S.J.B. drafted manuscript; J.K., J.F., A.V., S.J.B., M.G., P.G.W., and A.M.J. edited and revised manuscript; J.K., J.F., A.V., J.R.B., O.F., S.J.B., M.G., P.G.W., and A.M.J. approved final version of manuscript.

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

Abstract

Purpose: The purpose of this study was to assess the effects of dietary nitrate (NO_3^-) supplementation on muscle metabolism and pulmonary O_2 uptake ($\dot{V}O_2$) 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 NO_3^--rich beetroot juice (8.4 mmol NO_3^-; 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 $\dot{V}O_2$ was measured at rest and during exercise and muscle biopsies were sampled before and immediately after the exercise bouts. **Results:** Muscle [NO_3^-] and plasma [NO_2^-] and [NO_3^-] were higher (P<0.05) in BR compared to PL. Neither baseline nor end-exercise $\dot{V}O_2$ 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 [NO_3^-] following BR compared to PL was positively correlated with the Δ in severe-intensity exercise tolerance (r = 0.67; P<0.05). **Conclusions:** NO_3^- supplementation elevated plasma [NO_3^-] and [NO_2^-], and muscle [NO_3^-], but this did not lead to changes in $\dot{V}O_2$ or muscle metabolic responses during moderate- and severe-intensity exercise at the group mean level. However, the higher muscle [NO_3^-] with BR compared to PL was correlated with an extended time to exhaustion, suggesting that muscle [NO_3^-] 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 (O_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 *et al*., 2008). Pulmonary O_2 uptake ($\dot{V}O_2$), which provides a close representation of muscle $\dot{V}O_2$ (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 $\dot{V}O_2$ response, the $\dot{V}O_2$ 'slow component', is evident which delays and/or prevents the attainment of a steady state. The development of the $\dot{V}O_2$ 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^+, P_i, ADP). Interventions that alter the $\dot{V}O_2$ 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 & Burnley, 2009).

Dietary nitrate (NO_3^-) supplementation in the form of NO_3^- salts or NO_3^--rich beetroot juice (BR) can have beneficial cardiovascular and metabolic effects. These effects have been attributed to the conversion of the relatively inert NO_3^- anion to bioactive nitrite (NO_2^-) 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 O_2-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 NO_3^--NO_2^--NO pathway by which ingested NO_3^- rapidly absorbed from the gut enters the entero-salivary circulation and is concentrated in the saliva (Lundberg *et al*., 2008). Anaerobic bacteria, residing in crypts on the dorsum of the tongue, reduce the NO_3^- to NO_2^- (Duncan *et al*., 1995). Once swallowed into the acidic environment of the stomach, some of this

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 $\dot{V}O_2$ phase II time constant (τ) (Kelly *et al*., 2013) and reduce the amplitude of the $\dot{V}O_2$ 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; Muggeridge *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 $\dot{V}O_2$ 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

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, $\dot{V}O_{2peak}$ = 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 $\dot{V}O_{2peak}$ 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. $\dot{V}O_{2peak}$ was

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 ($\dot{V}_E/VO_2$) with no increase in $\dot{V}_E/VCO_2$. 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% Δ (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.

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 NO_3^- supplementation before *visits 2* and *3*. The supplements were either NO_3^--rich BR (2 x 70 mL·d^{-1} of BR providing ~8.4 mmol NO_3^- per day; Beet it, James White Drinks, Ipswich, UK) or NO_3^--depleted PL (2 x 70 ml·d^{-1} of PL providing ~0.006 mmol NO_3^- 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 NO_3^- 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 ($KHCO_3$) was added.

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 NO_3^- 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 [NO_2^-] and [NO_3^-] 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 [NO_2^-] and [NO_3^-].

Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO_2^-. Plasma NO_2^- and NO_3^- were analysed by gas phase chemiluminescence analysis. This initially required NO_2^- and NO_3^- to be reduced to NO gas. For reduction of NO_2^-, undiluted plasma was injected into a glass purge vessel containing 5 ml of glacial acetic acid and 1 ml of NaI solution. For NO_3^- 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 NO_2^- and NO_3^- 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 (O_2) and infrared (CO_2) 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:

$$\dot{V}O_2\,(t) = \dot{V}O_{2baseline} + A_p\,[1 - e^{-(t-TDp/\tau p)}]$$

where $\dot{V}O_2\,(t)$ represents the absolute $\dot{V}O_2$ at a given time t; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ in the baseline period; A_p, TD_p, and τ_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. $\dot{V}O_{2baseline}$ 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.

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).

Muscle [NO_3^-]

The effect of dietary NO_3^- supplementation on muscle [NO_3^-] is illustrated in Figure 3. ANOVA revealed a main effect for supplementation on muscle [NO_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_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_3^-] remained unchanged in both BR and PL across all time-points (P>0.05). On average, muscle [NO_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).

$\dot{V}O_2$ Kinetics and HR

Group mean pulmonary $\dot{V}O_2$ responses to moderate- and severe-intensity exercise in BR and PL were not different (Table 3). During moderate-intensity exercise, baseline $\dot{V}O_2$ (BR: 1180 ± 155 vs. PL: 1183 ± 201 ml/min; P>0.05) and end-exercise $\dot{V}O_2$ (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 τ (BR: 18 ± 6 vs. PL: 20 ± 9 s; P>0.05). In the response to severe-intensity exercise, baseline $\dot{V}O_2$ (BR: 1234 ± 270 vs. PL: 1226 ± 114 ml/min; P>0.05), end-exercise $\dot{V}O_2$ (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 τ (BR: 31 ± 9 vs. PL: 30 ± 6 s; P>0.05), and the $\dot{V}O_2$ 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.

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 $[NO_2^-]$ following BR compared to the BR→PL (PL→BR: 665 ± 189 nM vs. BR→PL: 236 ± 43 nM; $P<0.05$). Steady-state $\dot{V}O_2$ during moderate-intensity exercise was reduced in the PL→BR group whereas the BR→PL group had an increase in $\dot{V}O_2$ (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 $[NO_3^-]$ compared to the BR→PL group (Table 4).

The change in muscle $[NO_3^-]$ 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 $\dot{V}O_2$ 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).

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

	Lactate (mM)		Glucose (mM)		Sodium (mM)		Potassium (mM)	
Moderate-intensity exercise	BR	PL	BR	PL	BR	PL	BR	PL
Rest	1.0 ± 0.3	0.8 ± 0.4	4.4 ± 0.7	3.9 ± 0.8	136 ± 3.6	135 ± 6.3	4.3 ± 0.9	4.1 ± 0.2
1 min	1.0 ± 0.2	0.9 ± 0.4	4.2 ± 0.4	4.0 ± 0.6	136 ± 3.6	138 ± 5.4	4.6 ± 0.8	4.4 ± 0.3
2 min	1.2 ± 0.2	1.0 ± 0.3	4.2 ± 0.6	3.9 ± 0.8	137 ± 2.2	137 ± 2.8	4.5 ± 0.5	4.4 ± 0.2
3 min	1.2 ± 0.2	1.1 ± 0.4	4.2 ± 0.4	4.0 ± 0.4	138 ± 2.4	137 ± 4.1	4.6 ± 0.8	4.6 ± 0.5
6 min	1.3 ± 0.3	1.2 ± 0.3	4.1 ± 0.5	4.0 ± 0.4	138 ± 2.6	137 ± 5.3	4.3 ± 0.2	4.3 ± 0.6
10 min	1.3 ± 0.4	1.3 ± 0.3	4.1 ± 0.5	4.0 ± 0.5	138 ± 2.6	138 ± 2.6	4.4 ± 0.3	4.6 ± 0.2
Severe-intensity exercise								
Rest	0.9 ± 0.2	0.7 ± 0.2	4.5 ± 0.4	4.1 ± 0.6	136 ± 2.6	135 ± 4.9	4.8 ± 1.3	4.6 ± 0.5
1 min	1.3 ± 0.3	1.1 ± 0.3	4.3 ± 0.3	4.3 ± 0.4	136 ± 5.5	137 ± 3.7	5.0 ± 1.4	4.7 ± 0.3
2 min	2.3 ± 1.0	2.0 ± 0.6	4.3 ± 0.3	4.2 ± 0.5	137 ± 4.5	137 ± 4.8	5.4 ± 1.6	4.8 ± 0.3
3 min	3.7 ± 1.2	3.9 ± 1.4	4.1 ± 0.5	4.2 ± 0.5	138 ± 3.9	138 ± 5.0	5.5 ± 0.9	5.2 ± 0.8
6 min	9.1 ± 2.2	8.8 ± 3.3	4.1 ± 0.6	4.2 ± 0.6	143 ± 4.8	144 ± 4.9	5.8 ± 1.0	5.6 ± 0.8
7 min	9.1 ± 2.2	8.6 ± 2.5	4.2 ± 0.7	4.2 ± 0.7	141 ± 4.2	143 ± 3.9	5.3 ± 1.3	5.0 ± 0.5
Exh	12.1 ± 2.8	10.8 ± 3.1	4.7 ± 0.8	4.8 ± 0.7	146 ± 2.6	145 ± 4.4	5.6 ± 0.7	5.7 ± 0.7
Rec 1	11.7 ± 3.0	11.0 ± 2.6	5.0 ± 1.0	5.4 ± 0.9	145 ± 3.6	144 ± 3.5	5.1 ± 1.0	4.9 ± 0.5
Rec 3	12.2 ± 2.2	11.9 ± 2.4	6.0 ± 0.9	6.3 ± 1.1	141 ± 3.5	139 ± 5.2	4.4 ± 1.1	4.0 ± 0.4
Rec 5	12.4 ± 2.3	11.8 ± 1.9	5.8 ± 1.0	6.0 ± 1.1	140 ± 3.2	139 ± 6.2	3.6 ± 0.2	3.6 ± 0.3

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' ($P < 0.05$).

	ATP (mmol/kg DW)		PCr (mmol/kg DW)		lactate (mmol/kg DW)		pH		HAD (µmol/g DW)	
	BR	PL	BR	PL	BR	PL	BR	PL	BR	PL
Rest	32.0 ± 7.1	35.6 ± 9.4	65.2 ± 11.3	67.4 ± 10.8	8.7 ± 6.1	6.4 ± 1.6	7.19 ± 0.04	7.20 ± 0.03	15.6 ± 2.8	16.0 ± 2.1
Mod	29.6 ± 7.1*	31.9 ± 7.5*	53.9 ± 16.2*	57.1 ± 15.7*	8.2 ± 3.3	7.9 ± 2.0	7.26 ± 0.05*	7.25± 0.08*	-	-
Sev	22.6 ± 4.2*	23.8 ±7.1*	18.8 ± 17.9*	17.4 ± 17.6*	93.8 ± 32.2*	89.0 ± 17.2*	6.65 ± 0.13*	6.72 ± 0.12*	-	-
Exh	21.4 ± 7.2*	24.2 ± 19*	12.7 ± 9.7*	17.2 ±17.8*	95.0 ± 17.7*	97.1 ± 29.5*	6.67 ± 0.09*	6.73 ± 0.11*	-	-

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

	PL	BR
Moderate-intensity exercise		
$\dot{V}O_2$		
Baseline, ml/min	1183 ± 201	1180 ± 155
End Exercise, ml/min	1830 ± 252	1824 ± 272
Phase II Time Constant, s	20 ± 9	18 ± 6
Primary amplitude, ml/min	648 ± 167	645 ± 153
Severe-intensity exercise		
$\dot{V}O_2$		
Baseline, ml/min	1226 ± 114	1234 ± 270
End Exercise, ml/min	4309 ± 385	4248 ± 326
Phase II time constant, s	30 ± 6	31 ± 9
Primary amplitude, ml/min	2331 ± 241	2353 ± 264
Slow Component Amplitude, ml/min	752 ± 171	661 ± 101

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.*

		Δ Plasma NO_2^- (nM)	Δ Muscle [NO_3^-] (nmol/mg DW)	Δ Moderate-intensity $\dot{V}O_2$ (ml/min)	Δ Exercise Tolerance (s)
'Responders' (n = 4)	1	821	0	-83	16
(PL→BR)	2	868	5.6	1	11
	3	406	2.2	-181	28
	4	565	27.1	-114	83
	Mean	665 ± 189 *	8.72 ± 10.78	-94 ± 65 *	35 ± 29 *
'Non Responders' (n = 4)	1	184	1.9	8	-40
(BR→PL)	2	208	0	108	-18
	3	294	17	188	-9
	4	256	-3.91	127	-18
	Mean	236 ± 43	3.84 ± 8.05	108 ± 65	-21 ± 11

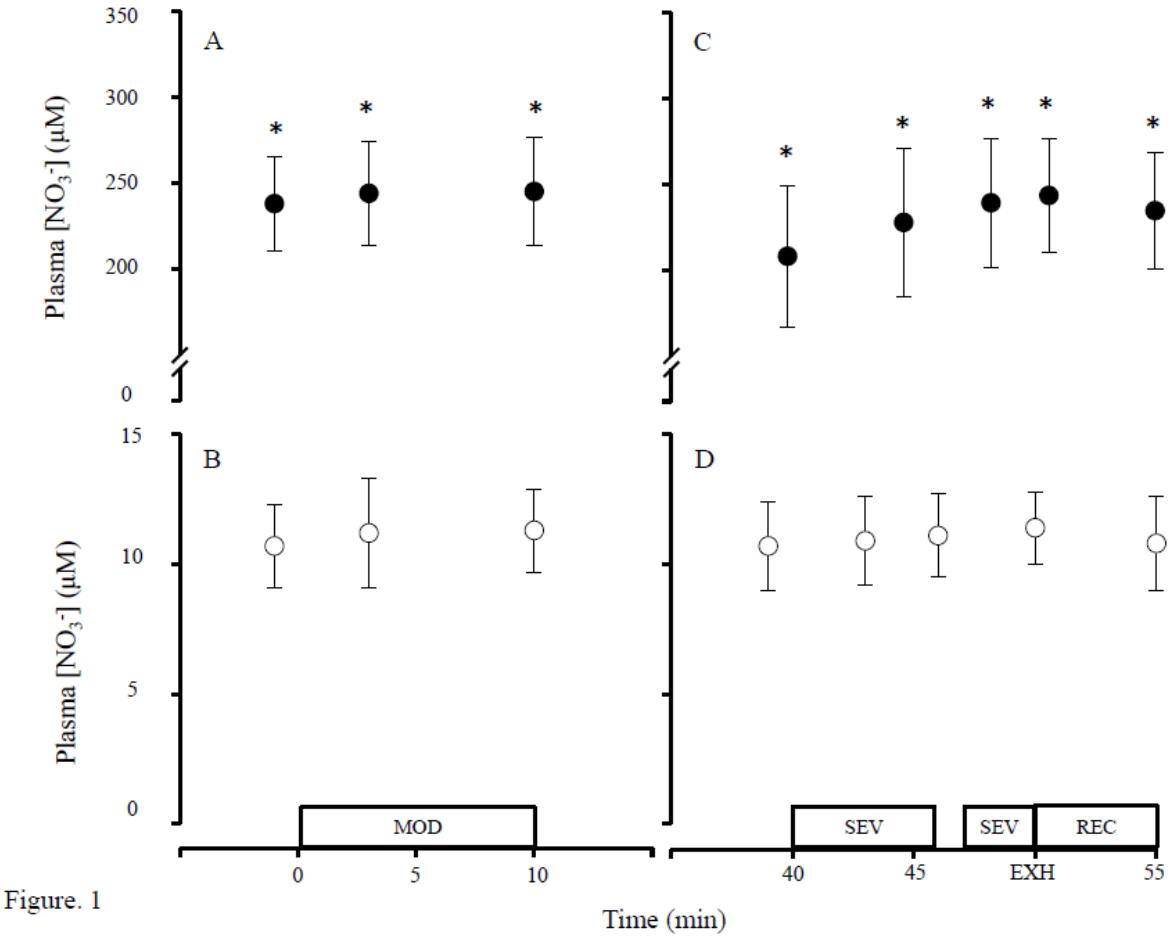

Figure. 1

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.

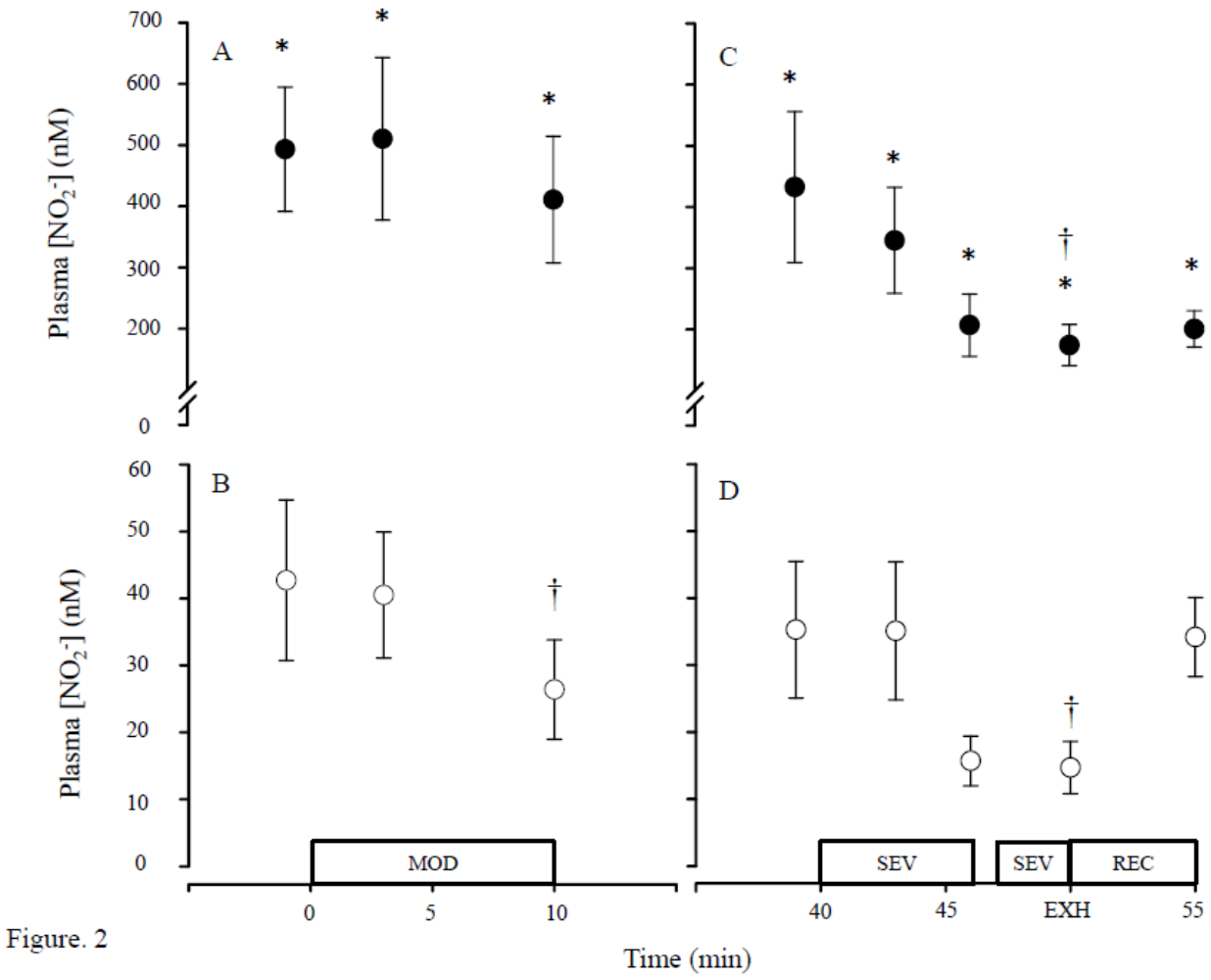

Figure. 2

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.

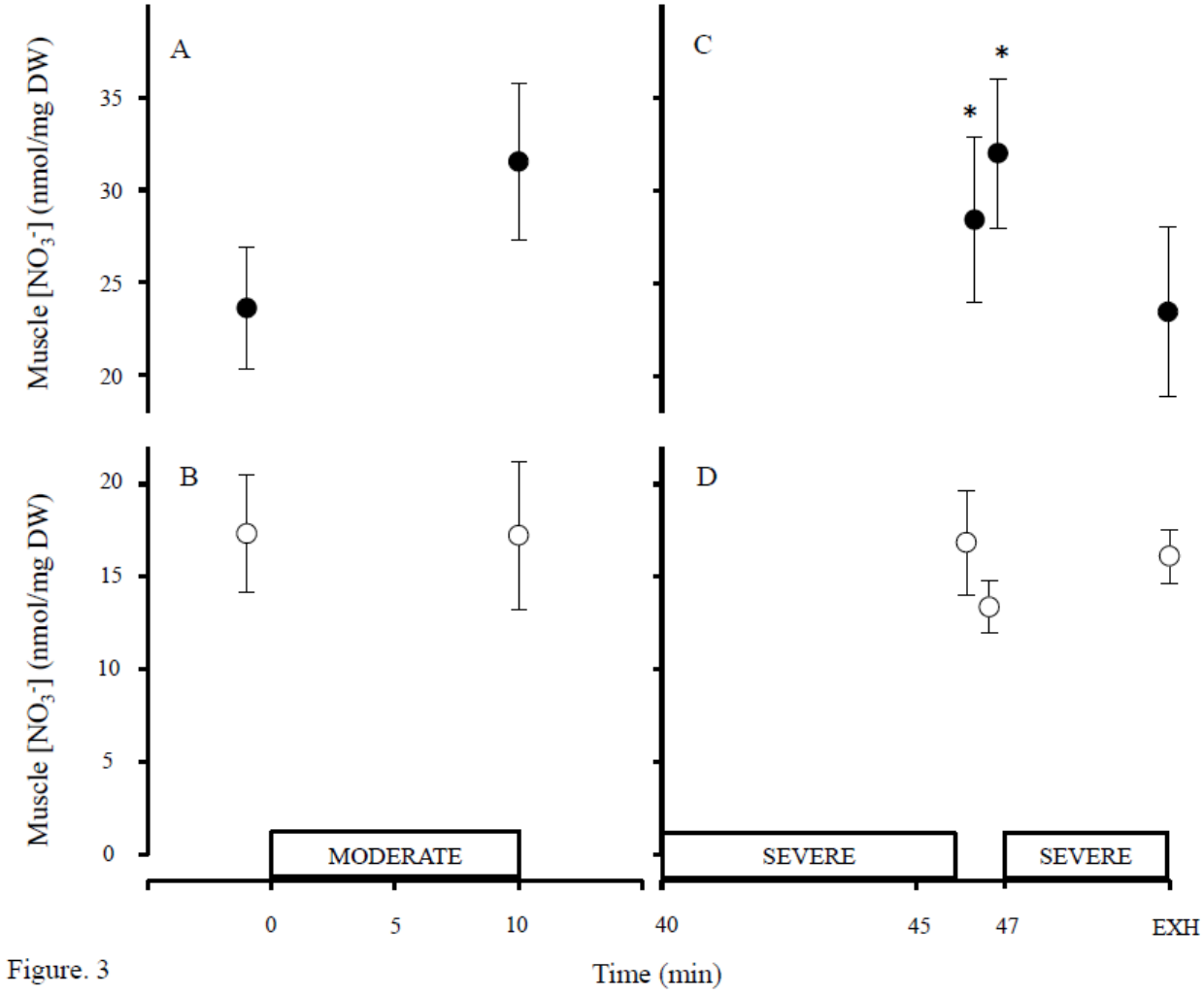

Figure. 3

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.

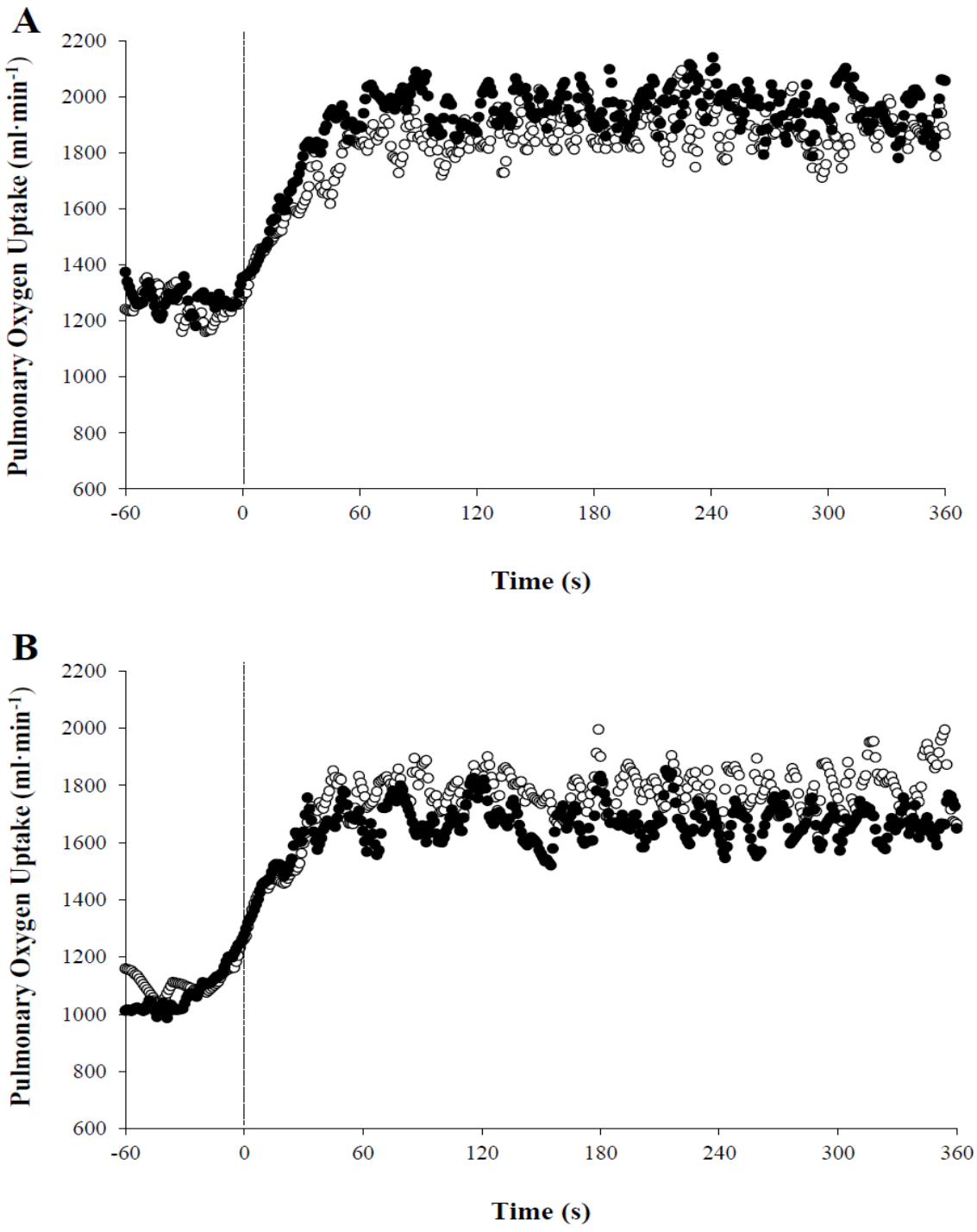

Figure 4.

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.

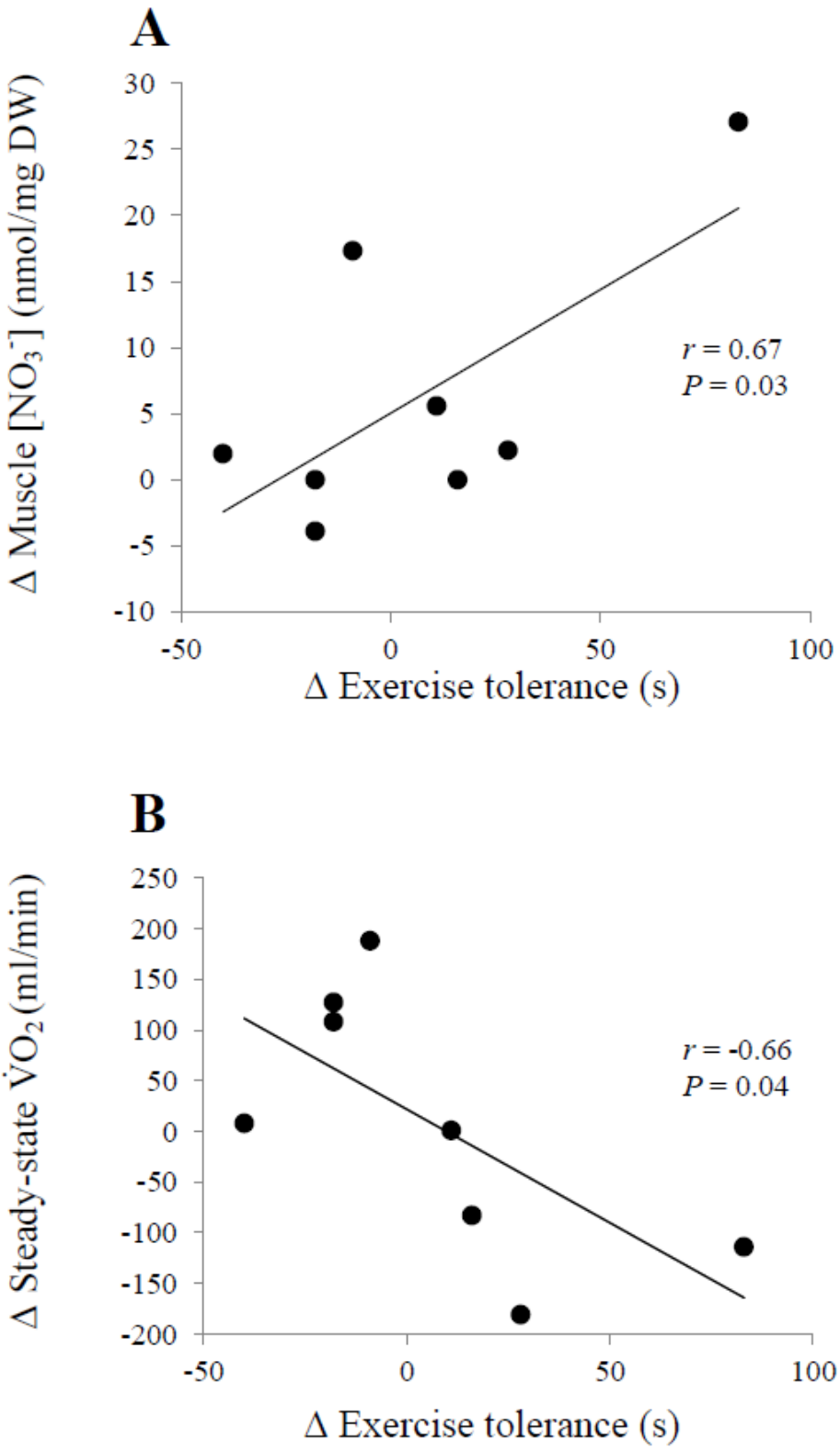

Figure 7.5. Pearson product-moment correlation coefficient between A) the change in muscle [NO_{3-}] following BR (BR-PL; nmol/mg DW) and the change in exercise tolerance following BR (BR-PL; s); and B) the change in steady-state $\dot{V}O_2$ following BR (BR-PL; ml/min) and the change in exercise tolerance following BR (BR-PL; s).

Discussion

The principal original finding of this study, consistent with our hypothesis, was that muscle [NO_3^-] was elevated following NO_3^- supplementation. In contrast to our hypothesis, NO_3^- supplementation had no significant effect on the group mean $\dot{V}O_2$ 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 NO_3^- supplementation, with the change in muscle [NO_3^-] being significantly correlated to the change in severe-intensity exercise tolerance.

Effects of NO_3^- Supplementation on Blood Variables

At rest, plasma [NO_2^-] and [NO_3^-] were elevated significantly following NO_3^- supplementation, compared with PL. These findings are consistent with previous research which has consistently reported elevations in plasma [NO_2^-] (Bailey *et al*., 2009; Kelly *et al*., 2013; Vanhatalo *et al*., 2010) and [NO_3^-] (Kapil *et al*., 2010; Larsen *et al*., 2010; Wylie *et al*., 2013a) following BR supplementation. The response profile of plasma [NO_2^-] during severe-intensity exercise was similar to data recently reported (Wylie *et al*., 2013b), with [NO_2^-] declining from baseline to exhaustion during severe-intensity exercise. Interestingly, no changes in [NO_3^-] were observed during the severe-intensity exhaustive bout in the current study, while [NO_3^-] increased significantly in the previous report (Wylie *et al*., 2013b). The ability of an individual to 'utilize' [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 [NO_2^-] in the current study was similar in BR and PL conditions (~60%), although the absolute change in [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 NO_3^- Supplementation on Muscle Variables

This is the first investigation to report the effects of NO_3^- supplementation on the [NO_3^-] of skeletal muscle. Muscle [NO_3^-] was elevated by 72% following BR compared to PL, indicating that in addition to increasing circulating plasma [NO_3^-], supplementation also

increases muscle tissue [NO_3^-]. This finding contributes to our understanding of the potential mechanistic bases behind the effects of NO_3^- supplementation on the physiological responses to exercise. Unfortunately, due to the much lower tissue concentrations of NO_2^- compared to NO_3^-, it was not possible to ascertain the influence of NO_3^- supplementation on skeletal muscle [NO_2^-] in the present study.

Using ^{31}P-MRS, Bailey et al. (2010) reported that during low-intensity single-leg knee-extensor exercise, NO_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_i. Similar effects were observed during high-intensity exercise and the positive changes in muscle metabolic and pulmonary $\dot{V}O_2$ 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 $\dot{V}O_2$ and muscle metabolic (assessed non-invasively by ^{31}P-MRS) responses were measured on separate occasions. An important strength of the present study is that the $\dot{V}O_2$ and muscle metabolic (measured via biopsy) responses were measured simultaneously during cycle ergometer exercise. In contrast to our hypothesis, NO_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 $\dot{V}O_2$ 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 ^{31}P-MRS techniques (Bailey *et al.*, 2010; Vanhatalo *et al.*, 2011).

Effects of NO_3^- Supplementation on Pulmonary O_2 Uptake

In the present study, there were no changes in the group mean $\dot{V}O_2$ 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 $\dot{V}O_2$ (Bailey *et al.*, 2009; Larsen *et al.*, 2007, Vanhatalo *et al.*, 2010) during moderate-intensity exercise following NO_3^- supplementation. Furthermore, reductions in the $\dot{V}O_2$ slow component (Bailey *et al.*, 2009) and speeding of the phase II τ (Kelly *et al.*, 2013) have been observed in response to severe-intensity exercise as a result of NO_3^- supplementation. These findings are thought to be consequent to modulations to the ATP cost of muscle force production

(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 $\dot{V}O_2$ 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 $ml \cdot kg^{-1} \cdot min^{-1}$. 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

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 $\dot{V}O_2$ 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 $\dot{V}O_{2peak}$ (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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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, $\dot{V}O_2$ 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 $\dot{V}O_2$ during moderate-intensity exercise and to enhance severe-intensity exercise tolerance.

In conclusion, short-term dietary supplementation with NO_3^--rich beetroot juice increases plasma [NO_2^-] and [NO_3^-] as well as muscle [NO_3^-]. Overall, BR supplementation had no significant effect on the muscle metabolic or $\dot{V}O_2$ 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 [NO_3^-] 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 NO_3^- - NO_2^- - NO pathway. Increasing dietary consumption of NO_3^- 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 O_2 cost of exercise and improving exercise tolerance. Scientific interest in the effects of NO_3^- 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 NO_3^- supplementation.

1) Does dietary NO_3^- supplementation modulate the power-duration relationship for severe-intensity exercise in young, healthy, recreationally active males?
2) How does dietary NO_3^- 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 NO_3^- supplementation elicited in young, healthy participants also evident in a healthy, older population?
4) How does dietary NO_3^- supplementation influence skeletal muscle [NO_3^-], pulmonary $\dot{V}O_2$ 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 NO_3^- 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 $\dot{V}O_2$ kinetics in older adults.

In order to provide further insight in to 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 $\dot{V}O_2$, 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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_3^- supplementation elevated circulating plasma [NO_3^-] and [NO_2^-]. Chapter 5 and 7 assessed changes in plasma [NO_3^-] and found a significant elevation following NO_3^- supplementation. This elevated concentration of circulating NO_3^- promotes an increase in circulating [NO_2^-], which is demonstrated in all chapters. The 197% and 400% increase in plasma [NO_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_2^-] following NO_3^- supplementation were comparable to previous research but [NO_2^-] following placebo were particularly low. Interestingly, resting control plasma [NO_2^-] in older adults in Chapter 6 was similar to that reported in young adults. This was surprising as lower [NO_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_2^-] following NO_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_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_3^-] and [NO_2^-] are clearly and consistently elevated following NO_3^- supplementation.

In Chapters 4 and 6 increases in plasma [NO_2^-] were coupled with reductions in systolic blood pressure. It is thought that increased plasma [NO_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_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_3^- supplementation, in the form of beetroot juice on [NO_3^-] of human skeletal muscle tissue. The study reported that muscle [NO_3^-] was elevated by 72% following NO_3^- supplementation compared to placebo.

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 straight forward. 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).

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 NO_3^- 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 NO_3^- 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 NO_3^- 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 [NO_3^-] following NO_3^- supplementation was positively and significantly correlated with the change in severe-intensity exercise tolerance. Chapter 7 was the first study to report muscle [NO_3^-], in humans, following NO_3^- supplementation. Further work is required to clarify the inter-relationships between changes in muscle [NO_3^-] and changes in severe-intensity exercise tolerance following NO_3^- supplementation.

Combining the findings of this thesis with existing literature, it would be fair to suggest that dietary NO_3^- 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 NO_3^- supplementation and their potential with changes in exercise tolerance.

Exercise efficiency

In 3 of the 4 experimental Chapters in this thesis the O_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_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_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_3^- had no effect upon the O_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_3^- supplementation was seen to reduce baseline (unloaded) cycling $\dot{V}O_2$ by 10% and moderate-intensity exercise $\dot{V}O_2$ by 7% in hypoxia, compared to PL. These findings are consistent with previous studies which have reported 4-8% reductions in steady state $\dot{V}O_2$ during moderate-intensity cycle exercise in hypoxia, as a result of NO_3^- supplementation (Masschelein *et al*., 2012; Muggeridge *et al*., 2014). As outlined in the literature review, the mechanistic bases behind reductions in the O_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_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_2 may be responsible, in part, for the reduced O_2 cost of exercise following NO_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_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_2 cost of submaximal exercise and changes in severe-intensity exercise tolerance following NO_3^- supplementation. Chapter 5 found a significant correlation between the reduction in steady-state $\dot{V}O_2$ and the improvement in exercise tolerance following NO_3^- supplementation in hypoxia. Similarly, Chapter 7 demonstrated that the change in steady-state $\dot{V}O_2$ during moderate-intensity exercise following NO_3^- supplementation was negatively correlated with the change in severe-intensity exercise tolerance**.** These data may suggest that NO_3^- supplementation is more effective in some individuals than others (discussed in a later section). Furthermore, why reductions in the O_2 cost of exercise or alterations in the $\dot{V}O_2$ slow component were not evident during subsequent severe-intensity exercise bouts is not clear. It could be speculated that certain underlying mechanisms of NO_3^- supplementation become more prominent at different exercise intensities. For example, during moderate-intensity exercise the most prominent effect of NO_3^- supplementation is the reduction in the O_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 $\dot{V}O_2$ 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_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 NO_3^- supplementation upon four exercise intensities spanning the severe-intensity exercise domain. The rationale for this was two-fold: 1) to establish whether NO_3^- 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 NO_3^- 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. NO_3^- 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 NO_3^- 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 NO_3^- 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.

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_3^- supplementation speeded the phase II time constant in hypoxia toward values recorded in normoxia. NO_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_3^- supplementation indicates better muscle oxygenation (Ferrari *et al*., 2004) which, as previously discussed, may be responsible for the speeding of the $\dot{V}O_2$ phase II time constant. These findings suggest that NO_3^- supplementation can help to reverse the detrimental effect of a reduced FIO_2 on $\dot{V}O_2$ kinetics, during moderate-intensity exercise. In support of this, emerging data utilizing ^{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_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_2-limited condition, but has no influence under normoxic exercise and recovery. Collectively, these data support the findings in Chapter 6 where NO_3^- supplementation reversed the potential O_2 delivery limitation on $\dot{V}O_2$ kinetics in an older population.

Considering improvements in exercise efficiency following NO_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 $\dot{V}O_2$ phase II time constant at power outputs representing 60% Δ, 70% Δ, 80% Δ or $100\%_{peak}$, following NO_3^- supplementation. However, the $\dot{V}O_2$ 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_3^- supplementation during severe-intensity exercise is a preferential distribution of O_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 $\dot{V}O_2$ 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_3^- supplementation can positively modulate the $\dot{V}O_2$ 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_3^- supplementation and may have important implications in utilising NO_3^- supplementation to improve exercise tolerance in both athletic and ageing/clinical populations.

Muscle metabolism

Chapter 6 utilised ^{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_3^- supplementation. The findings indicated a 15% attenuation of muscle [PCr] degradation during low-intensity exercise following NO_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_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_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_2 supply. Consistent with previous research in younger adults in normoxia (Lansley *et al*., 2011; Fulford *et al*., 2013; Vanhatalo *et al*., 2014), NO_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_2 delivery as a result of NO_3^- supplementation.

Chapter 7 assessed the muscle metabolic response alongside the $\dot{V}O_2$ response to moderate- and severe-intensity exercise following NO_3^- supplementation. Data obtained from muscle biopsy samples indicated that NO_3^- supplementation significantly elevated muscle [NO_3^-]. However, analyses revealed that NO_3^- 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 $\dot{V}O_2$ response or exercise tolerance.

Data from this thesis demonstrated for the first time that human muscle [NO_3^-] is significantly elevated following NO_3^- supplementation. Assessment of key fatigue-related muscle metabolites was made during exercise in the current thesis using both ^{31}P-MRS and muscle biopsy techniques. The presented data suggest that NO_3^- supplementation had no effect upon muscle metabolism. These findings were accompanied by no changes in pulmonary $\dot{V}O_2$ responses or exercise tolerance and were therefore not surprising. However, these findings contradict previous research (Bailey *et al.*, 2010; Vanhatalo *et al.*, 2014). When NO_3^- supplementation-mediated alterations in exercise efficiency (assessed by pulmonary $\dot{V}O_2$) are evident, it is expected that changes in muscle metabolism would also be present.

Therapeutic effects of NO_3^- supplementation

Blood pressure

The reduction of blood pressure is arguably one the most important physiological benefits of NO_3^- supplementation as it has potential implications for the prevention and/or treatment of hypertension. It is thought that increased plasma [NO_2^-] 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 *et al.*, 1994). Findings from the current thesis present positive results with regard to the reduction of blood pressure following NO_3^- supplementation. Chapter 4 assessed resting blood pressure in young, healthy individuals and consistent with previous research (Larsen *et al.*, 2006; Webb *et al.*, 2008; Kapil *et al.*, 2010; Bailey *et al.*, 2010; Kelly *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_3^- supplementation on blood pressure

in a healthy, older population, demonstrated significant reductions in systolic (5 mm Hg), diastolic (3 mm Hg) and mean arterial pressure (3mm 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 (H^1MRS, 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

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 NO_3^- supplementation may not always be effective.

Effectiveness of NO_3^- supplementation

Chapters 5 and 7 indicate that NO_3^- supplementation elicits beneficial effects in some individuals but not others. Chapter 5 found a significant correlation between the reduction in steady-state $\dot{V}O_2$ and the improvement in exercise tolerance following NO_3^- supplementation in hypoxia. Similarly, Chapter 7 demonstrated that the change in steady-state $\dot{V}O_2$ 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 [NO_3^-] 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 [NO_2-]; a significantly reduced steady-state $\dot{V}O_2$ 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 [NO_3^-] compared to the 'non-responders'. The mechanistic bases behind differences in the effectiveness of NO_3^- 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 NO_3^- supplementation and suggest a number of explanations for this. Some studies indicate that NO_3^- supplementation may be less effective as an ergogenic aid in highly-trained endurance athletes, at least when NO_3^- 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 [NO_2^-], 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 NO_3^- 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.

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.

Measurement of NO markers restricted to NO_2^- and NO_3^-

The main aim of administering NO_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_2^- and NO_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_2^- and NO_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_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_2^- and NO_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_2^- and NO_3^- and muscle NO_3^- provided the most suitable, appropriate and practical characterization of NO bioavailability following NO_3^- supplementation.

Constant work rate tests to exhaustion

The aim of this thesis was to assess the ergogenic and therapeutic qualities of dietary NO_3^- supplementation. A key aspect of each study was to assess the effects of NO_3^- supplementation on the $\dot{V}O_2$ 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 $\dot{V}O_2$ 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 $\dot{V}O_2$ 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

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 $\dot{V}O_2$ 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 $\dot{V}O_{2peak}$ (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 $\dot{V}O_2$ accurately represent muscle $\dot{V}O_2$?

Whilst assessing the effectiveness of NO_3^- supplementation as a therapeutic and ergogenic aid, it is important that the pulmonary $\dot{V}O_2$ signal measured is reflective of muscle $\dot{V}O_2$. A number of techniques have been employed in the present thesis to minimize the breath-by-breath variability which is inherent with measuring $\dot{V}O_2$ 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 $\dot{V}O_2$ kinetics does indeed accurately represent muscle $\dot{V}O_2$ kinetics (Barstow *et al*., 1990; Poole *et al*., 1991; Grassi *et al*., 1996; Krustrup *et al*., 2009).

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.

Cerebral physiology & cognitive function

Recent research provided promising potential with regard to the role of NO_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_3^- supplementation upon cerebral measures and cognitive function in healthy older individuals, the potential implication of NO_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_3^- supplementation is a current and relevant topic in exercise and health physiology. From elucidating the mechanisms behind alterations to the $\dot{V}O_2$ kinetic response to exercise and subsequent improved athletic performance, to quantifying the extent to which NO_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_3^- supplementation. These data demonstrate that dietary 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, 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_3^- supplementation may not *always* be effective, it *can* modulate the $\dot{V}O_2$ 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.

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