DIETARY NITRATE SUPPLEMENTATION: DOSE-RESPONSE RELATIONSHIPS AND EFFECTS ON INTERMITTENT EXERCISE PERFORMANCE

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Submitted by Lee Wylie to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Sport and Health Sciences.

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Abstract

Dietary supplementation with inorganic nitrate (NO_3^-) has been shown to lower the oxygen (O_2) cost of submaximal exercise and to improve performance during continuous endurance exercise. The objectives of this thesis were: 1) to improve understanding of the supplementation procedures in which NO_3^- is most likely to benefit exercise physiology and performance, and 2) to explore the ergogenic potential of dietary NO_3^- in intermittent exercise. Recreationally-active adult participants volunteered to participate in the original investigations presented in this thesis. These participants underwent various oral NO_3^- supplementation regimes and subsequently provided venous blood samples for the determination of plasma nitrate concentration ($[NO_3^-]$) and plasma nitrite concentration ($[NO_2^-]$), and completed different exercise tests to assess the potential for NO_3^- supplementation to improve physiological responses during exercise and exercise performance. **Chapter 4:** Following the ingestion of 70, 140 and 280 mL concentrated NO_3^--rich beetroot juice (BR: containing 4.2, 8.4 and 16.8 mmol NO_3^-, respectively) plasma $[NO_2^-]$ increased dose-dependently, with peak changes occurring at ~2-3 h post ingestion. The O_2 cost of submaximal exercise and exercise tolerance were also influenced dose-dependently 2.5 h post acute BR ingestion when compared to a NO_3^--depleted placebo (PL). Specifically, 8.4 mmol and 16.8 mmol lowered the O_2 cost of moderate-intensity exercise by 1.7% ($P = 0.06$) and 3.0% ($P < 0.05$), respectively. Exercise tolerance during severe-intensity exercise was significantly improved after ingestion of 8.4 mmol (+14%; $P < 0.05$), with no further improvement evident following ingestion of 16.8 mmol (+12%; $P < 0.05$). 4.2 mmol NO_3^- did not significantly lower the O_2 cost of submaximal exercise or improve exercise tolerance. **Chapter 5:** Plasma $[NO_2^-]$ was dose-dependently elevated 2 h and after 7 days and ~4 weeks of supplementation with 3 mmol and 6 mmol NO_3^-. Compared to pre-supplementation baseline, and PL, moderate-intensity exercise O_2 uptake ($\dot{V}_{O2}$) was not lowered with a low dose of dietary NO_3^- (3 mmol NO_3^-) acutely (2 h) or after ingesting it daily up to ~4 weeks. In contrast, ingestion of 6 mmol NO_3^- significantly lowered submaximal exercise $\dot{V}_{O2}$ by 3% after 2 h ($P = 0.06$), 7 days and ~4 weeks (both $P < 0.05$) of supplementation. Another interesting observation was that the reduction in submaximal exercise $\dot{V}_{O2}$ after ~4 weeks of supplementation with 6 mmol NO_3^- was preserved 24 h after consumption of the final NO_3^- dose despite plasma $[NO_2^-]$ having returned to baseline. **Chapter 6:** In recreational team sport players, 28 mmol dietary NO_3^- administered over ~30 h improved performance in the Yo-Yo

Intermittent Recovery Level 1 (Yo-Yo IR1) test (a test that mimics the high-intensity intermittent exercise bouts typical of team sport games) by 4.2%, compared to PL (NO_3^-: 1704 m vs. PL: 1636 m). The decline (or 'utilisation') of plasma [NO_2^-] from pre-exercise to post-exercise was greater with BR compared to PL, and the magnitude of this decline in plasma [NO_2^-] was positively correlated with the improvement in Yo-Yo IR1 performance. **Chapter 7:** Supplementation with dietary NO_3^- for 3-5 days (8.2 mmol NO_3^- per day) significantly improved performance during 24 x 6-s all-out cycling sprints interspersed with 24 s recovery, but not during 7 x 30-s all-out sprints interspersed with 240 s of recovery or 6 x 60-s self-paced maximal efforts interspersed with 60 s of recovery, in a group of recreational team sport players.

The novel findings presented in this thesis suggest that the supplementation procedure, in particular the NO_3^- dose administered, should be carefully considered by individuals wishing to elicit ergogenic effects following dietary NO_3^- supplementation. Specifically, the findings suggest that a low dose (≤4.2 mmol) of NO_3^- is not sufficient to acutely improve exercise tolerance, and that submaximal exercise $\dot{V}_{O2}$ is not lowered following acute or prolonged (~30 day) supplementation with a low NO_3^- dose. In contrast, results show that higher doses (6-8.4 mmol) of NO_3^- might be sufficient to acutely lower the O_2 cost of submaximal exercise and improve exercise tolerance. The present thesis also provides evidence to indicate that dietary NO_3^- might hold potential as an ergogenic aid for individuals participating in intermittent exercise involving brief bouts of high-intensity exercise interspersed with short recovery periods, such as team sport players. These findings are of importance as they will help inform supplementation procedures in future studies assessing the ergogenic efficacy of NO_3^- supplementation. Moreover, they suggest that NO_3^- supplementation has the potential to improve performance during intermittent exercise as well as continuous endurance exercise.

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Symbols and abbreviations

[ ]	concentration
Δ	difference, change
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
BP	blood pressure
BR	nitrate-rich beetroot juice
Ca^{2+}	calcium
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
CON	control
Cr	creatine
CV	coefficient of variation
eNOS	endothelial nitric oxide synthase
FMN	flavin mononucleotide
GET	gas exchange threshold
H^+	hydrogen ion, proton
HR	heart rate
iNOS	inducible nitric oxide synthase
K^+	potassium ion
MAP	mean arterial pressure
MRS	magnetic resonance spectroscopy
NADPH	nicotinamide adenine dinucleotide phosphate
$NaNO_3$	sodium nitrate
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
O_2	oxygen
O_2^-	superoxide
P	power output
PCr	phosphocreatine
P_i	inorganic phosphate
PL	placebo
P/O	oxygen cost of ATP resynthesis
RER	respiratory exchange ratio
SD	standard deviation
SE	standard error
TT	time trial
VCl_3	vanadium chloride
$\dot{V}_{CO2}$	pulmonary carbon dioxide output
$\dot{V}_{E}$	minute ventilation (expired)
$\dot{V}_{O2}$	pulmonary oxygen uptake
$\dot{V}_{O2max}$	maximal pulmonary oxygen uptake
$\dot{V}_{O2peak}$	peak pulmonary oxygen uptake
IR1	Intermittent recovery test, level 1
W	Watt

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

Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. Beetroot juice and exercise: pharmacokinetics and dose-response relationship. *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.

Wylie LJ, Bailey SJ, Kelly J, Blackwell JR, Vanhatalo A, Jones AM. Influence of beetroot juice supplementation on intermittent exercise performance. *European Journal of Applied Physiology, 116 (2): 415-25*

Wylie LJ, Ortiz de Zevallos J, Isidore T, Vanhatalo A, Bailey SJ, Jones AM. Dose-dependent effects of dietary nitrate on the oxygen cost of moderate-intensity exercise: acute vs. chronic supplementation. *Nitric Oxide* 57:30-39, 2016.

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, Wylie LJ, Black MI, McDonagh S, Jackman SR, 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, Intergrative and Comparative Physiology*, 307: R920-R930, 2014.

Thompson C, Wylie LJ, Fulford J, Kelly J, Black MI, McDonagh ST, Jeukendrup AE, Vanhatalo A, Jones AM. Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *European Journal of Applied Physiology*, 115: 1825-1834, 2014.

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Arnold JT, Oliver SJ, Lewis-Jones TM, Wylie LJ, Macdonald JH. Beetroot juice does not enhance altitude running performance in well-trained athletes. *Applied Physiology, Nutrition, and Metabolism*. 40 (6): 590-595, 2015.

Vanhatalo A, Black MI, DiMenna FJ, Blackwell JR, Friis Schmidt J, Thompson C, Wylie LJ, Mohr M, Bangsbo J, Krustrup P, Jones AM. The mechanistic bases of the power-time relationship: muscle metabolic responses and relationships to muscle fibre type. Journal of Physiology (in press)

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

Bailey SJ, Blackwell JR, Williams E, Vanhatalo A, Wylie LJ, Winyard PG, Jones AM. Influence of watermelon juice supplementation on endurance performance in humans (in review).

Bailey SJ, Blackwell JR, Wylie LJ, Holland T, Winyard PG, Jones AM. The improvements in blood pressure and exercise tolerance after short-term inorganic nitrate supplementation are attenuated in cigarette smokers compared to non-smoking controls (in review).

Conference Activity

Poster Presentation: Dietary nitrate supplementation lowers blood pressure and improves exercise tolerance in non-smokers, but not in cigarette smokers. Physiology 2015, Cardiff, July 2015.

Oral Presentation: Dietary nitrate supplementation lowers blood pressure and improves exercise tolerance in non-smokers, but not in cigarette smokers. 20th annual Congress of the European College of Sports Science, Malmö University, Malmö, June 2015.

Poster Presentation: Dietary nitrate supplementation lowers blood pressure and improves exercise tolerance in non-smokers, but not in cigarette smokers. BASES Student Conference, Liverpool John Moores University, Liverpool, March 2015.

Oral Presentation: Dietary nitrate supplementation improves team-specific intense intermittent exercise performance. BASES Student Conference, Cardiff Metropolitan University, Cardiff, March 2013.

Oral presentation: Beetroot juice and exercise: the pharmacokinetic-pharmacodynamic and dose-response relationships. 18th annual Congress of the European College of Sports Science, National Institute of Physical Education of Catalonia (INEFC), Barcelona, June 2013.

Dedication

I dedicate this thesis to my Mum, Dad and Nan. I hope it makes you proud.

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The research and compilation of this thesis would not have been possible without the support of a number colleagues, friends and family, to all of whom I am extremely thankful.

First and foremost, I would like to thank my director of studies, Professor Andrew Jones. Andy, your dedication, passion, productivity and attention to detail is truly remarkable and has been, and will continue to be, an inspiration to me. Thank you for always finding time for me whenever I have required your guidance and support. Being a part of your team has been a privilege, and has provided me with opportunities for which I will be eternally grateful. Thanks for everything Chief.

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Chapter 1: Introduction

Nitric oxide (NO), or nitrogen monoxide, was first identified as a clear, colorless gas by Joseph Priestly over 200 years ago. For much of the time since, this free radical has primarily been known as an air pollutant that contributes to the depletion of our planet's ozone layer. This all changed in the 1980's. NO was not only found to be synthesised by the human body from the amino acid L-arginine, but it was also identified as the endothelium derived relaxing factor (EDRF) responsible for vasodilation. These observations established NO as a mammalian physiological signalling molecule, a finding that was later deemed so important it earned the discoverers Robert F. Furchgott, Louis J. Ignarro and Ferid Murad the Nobel Prize in Physiology or Medicine in 1998. Indeed, in the years that followed these initial discoveries, researchers started exploring the role of NO in other physiological processes. Over 100,000 scientific papers later, we now know that NO is one of the most widespread signalling molecules in the human body with effects not exclusive to vascular control but also integral to neurotransmission, reproduction, host defence response, muscle contractility and mitochondrial respiration, to mention a few. Research to understand the production, regulation and biological functions of NO continues today.

Soon after endogenous NO production was discovered, it also became clear that the anions, nitrate (NO_3^-) and nitrite (NO_2^-), which had previously only been considered as toxic constituents of our diet, were also endogenously produced as oxidation products of NO. Initially, scientific interest was primarily focused on these anions as markers of NO production, with the belief that they were stable and biologically inert. However, in 1994, within a decade of the L-arginine: NO pathway discovery, it was found that NO_3^- and NO_2^- can actually undergo a serial reduction to yield NO, meaning that they are, in fact, acting as storage pools for NO production. Interestingly, this alternative pathway ensures that NO production can continue across a wide range of physiological conditions by enabling NO synthesis in hypoxic and acidic environments in which NOS activity may be reduced. Despite the exciting prospect that NO bioavailability could be 'boosted' through this pathway by ingesting dietary NO_3^-, over 10 years elapsed before the influence of NO_3^- supplementation on physiological functions in humans was studied. A series of momentous observations followed that have caused a paradigm shift in our understanding of the role of NO_3^- in human physiology, and

highlighted its potential as not only a therapeutic aid but also an ergogenic aid (i.e. an aid used for the purpose of enhancing sports performance).

The first of these discoveries came in 2006 when a research group at the Karolinska Institute, Sweden, showed that 3 days of dietary supplementation with pharmacological sodium NO_3^- ($NaNO_3$) significantly lowered resting blood pressure in healthy normotensive adults (Larsen et al. 2006). This important observation has potential implications for preventing and treating cardiovascular disease and has began to explain, in part, the well known therapeutic effects associated with a diet rich in green leafy vegetables (Joshipura et al. 1999; Joshipura et al. 2001). However, this was not the only impressive effect that accompanied $NaNO_3$ supplementation. One year later the same research group published a paper to show that 3 days of dietary NO_3^- supplementation (0.1 mmol NO_3^- $kg^{-1} \cdot d^{-1}$) again with a NO_3^- salt, lowered the O_2 cost of submaximal cycle exercise (Larsen et al. 2007). Considering that no physical, nutritional or pharmacological intervention was known to consistently alter the O_2 cost of cycle exercise, this finding was remarkable. Spotting the potential importance of this finding for the improvement of endurance exercise performance, Professor. Jones's team at the University of Exeter, UK, attempted to replicate the findings of the Karolinska group but this time using natural NO_3^- rich beetroot juice as the source of NO_3^-. They found that 3-6 days of beetroot juice supplementation (500 mL containing ~5.1 mmol NO_3^- per day) reduced the O_2 cost of submaximal exercise (see Figure 1.1), but that it could also improve exercise tolerance during high-intensity exercise in recreationally-active subjects (Bailey et al. 2009). Research to further explore the ergogenic potential of NO_3^- increased and studies demonstrated performance improvements in a cycling time trial in moderately-trained athletes after the ingestion of beetroot juice (Lansley et al. 2011a; Cermak et al. 2012a)

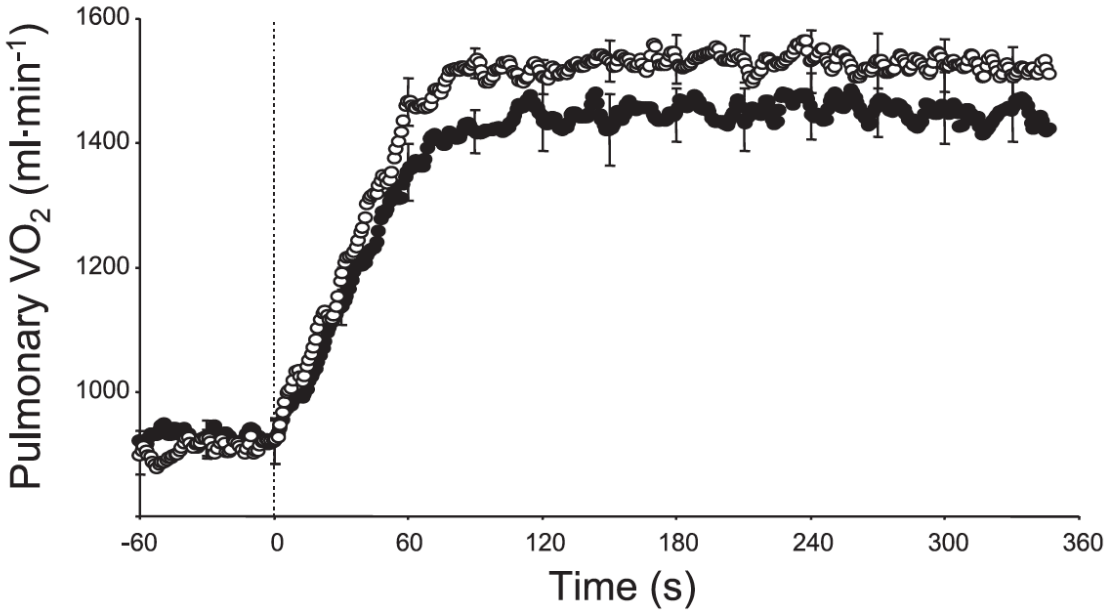

Figure 1.1: The group mean pulmonary O_2 uptake ($\dot{V}O_2$) response following 5 days of NO_3^- rich beetroot juice (closed circles) or placebo (open circles) supplementation during a step increment to a moderate-intensity work rate. Note that the O_2 cost of submaximal exercise was significantly reduced following beetroot supplementation. (From Bailey et al., 2009).

The fact that a natural vegetable juice could improve endurance exercise performance did not go unnoticed by the media, with headlines such as "Beetroot juice: The drink of champions" and "Beetroot juice may help beet your best" becoming a regular occurrence. Unsurprisingly, the use of dietary NO_3^- as an ergogenic aid also soared, with both elite and non-elite endurance athletes wishing to benefit from the potential boost in performance afforded by an increase in NO bioavailability. While research at this point had established that the ingestion of 5-8 mmol dietary NO_3^- daily for 3-15 days, or a single acute bolus of an equivalent dose could lower the O_2 cost of submaximal exercise and improve exercise performance, the optimal supplementation strategy for these effects were unknown. As with all nutritional and pharmaceutical aids, optimising the supplementation regime in terms of the timing of consumption and the dose and duration of supplementation, could have significant influence on the effects afforded by the supplement and would therefore be vital in recognising the true potential of this natural and readily available ergogenic aid.

Sport events are not limited to continuous exercise in which participants are required to cover a pre-determined distance in the shortest amount of time possible (e.g. a

cycling time trial). Some of the world's most popular sports, including Association Football, Rugby Union/League, Hockey and Basketball all require participants to repeatedly perform bouts of high-intensity exercise, interspersed with recovery periods. Training interventions for the majority of athletes also regularly involve exercise of an intermittent nature (i.e. high intensity interval training). While the early evidence that NO_3^- could improve exercise efficiency (i.e. lower the O_2 required for a given work rate) naturally led to the exploration of NO_3^- as an ergogenic aid for individuals participating in continuous endurance events, the effects of NO_3^- supplementation on intermittent exercise had not been explored. Interestingly, recent evidence from rodent experiments found that dietary NO_3^- supplementation appears to selectively improve contractile function (Hernández et al. 2012) and blood flow (Ferguson et al. 2013a) in type II 'fast-twitch' muscle. Considering the recruitment of these fibres is greater during high-intensity intermittent exercise than continuous sub-maximal endurance exercise, the ergogenic effects of dietary NO_3^- may extend to this type of exercise. If this is the case, then dietary NO_3^- can not only be considered as an ergogenic aid for endurance athletes, but also team sport players or for others completing intermittent exercise during training.

The purpose of this thesis was to establish the influence of different supplementation strategies, in particular with regard to different doses of NO_3^-, on the physiological responses of dietary NO_3^- supplementation and to examine the potential of dietary NO_3^- supplementation to improve performance during intermittent exercise. The following review of literature develops the rationale for the investigation of dose-response relationships of dietary NO_3^- and the influence of dietary NO_3^- on intermittent exercise performance in light of existing knowledge at the conception of this thesis.

Chapter 2: Literature Review

Nitric oxide

The ubiquitous free-radical gas, nitric oxide (NO), is one of the most researched molecules in physiology and medicine. This key cellular signalling molecule is known to play a critical role in a range of physiological processes including: vasodilation (Moncada and Higgs 1993), mitochondrial function (Brown and Cooper 1994), neurotransmission (Garthwaite 2008), glucose and calcium (Ca^{2+}) homeostasis (Hart and Dulhunty 2000; Viner et al. 2000; Merry et al. 2010) and skeletal muscle fatigue (Percival et al. 2010). Due to its high reactivity, NO has a short half-life (0.1 s) *in vivo* (Kelm and Schrader 1990*)*, and as such the ability to continually produce NO is of vital importance for the integrity of numerous physiological processes. Indeed, decreased NO bioavailability has been associated with several cardiovascular diseases (Förstermann 2010) and metabolic syndrome (Huang 2009). In the human body, two NO producing pathways have been described: the NO synthase (NOS)-dependent pathway and the NOS-independent nitrate (NO_3^-)-nitrite (NO_2^-)-NO pathway.

The NOS-dependent pathway

The production of NO via the NOS-dependent pathway was first discovered in the late 1980's (Moncada et al. 1989). Three NOS isoforms are currently identified which produce NO in different locations throughout the body. These include: type I (neuronal NOS; nNOS), type II (inducible NOS; iNOS) and type III (endothelial NOS; eNOS) (Moncada and Higgs 1993; Stamler and Meissner 2001). These heme-containing enzymes catalyse a complex five-electron oxidation of one of the basic guanidine nitrogen groups of the amino acid L-arginine, to yield NO and L-citrulline (Stamler and Meissner 2001). This complex reaction requires molecular oxygen (O_2) and several cofactors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4) haem and calmodulin (Alderton et al. 2001). Rapidly following its synthesis, NO is oxidised to NO_3^- by oxyhemoglobin and to NO_2^- in the plasma via a reaction catalysed by the plasma protein ceruloplasmin (Dejam et al. 2005; Shiva et al. 2006). It is now widely acknowledged that impaired NO production from the NOS-dependent pathway is associated with a reduced exercise tolerance in humans (Lauer et al. 2008)

NO_3^--NO_2^--NO pathway

This alternative pathway was discovered more recently (Benjamin et al. 1994; Lundberg et al. 1994) and involves the serial reduction of NO_3^- to NO_2^- and further to NO and other nitrogen oxides. These observations were very surprising because NO_3^- and NO_2^- had previously been considered as merely inert oxidation end-products of NO production via the NOS pathway (Moncada and Higgs 1993). It is now acknowledged that these anions serve as a storage pool for NOS-independent NO production, thereby supplementing the NO produced via the classic NOS-dependent pathway (Lundberg et al. 2004; Bryan 2006; Lundberg and Weitzberg 2009; van Faassen et al. 2009). Of particular interest is the fact that this pathway cannot only utilise endogenously produced NO_3^-, but can also be fuelled by exogenous inorganic NO_3^- from the diet. The serial reduction of NO_3^- to yield NO involves an intricate inter-organ metabolic pathway.

Enterosalivary circulation

After ingestion, 100% of inorganic NO_3^- is absorbed from the upper gastrointestinal tract into the systemic circulation within ~60 min (Lundberg and Weitzberg 2009) where it mixes with endogenously derived NO_3^-. The relatively stable NO_3^- anion has a long half-life of 5-8 h in plasma (Tannenbaum et al. 1979; Wagner et al. 1983). Up to 60% of the NO_3^- is excreted in urine (Wagner et al. 1983; Lundberg and Govoni 2004) but ~25% is actively absorbed by the salivary glands and thus enters the enterosalivary circulation, where it is concentrated up to 20-fold in saliva (Lundberg and Govoni 2004; Govoni et al. 2008; Figure 2.1.). The protein sialin has recently been identified as the major transporter of inorganic NO_3^- in salivary glands (Qin et al. 2012).

In the oral cavity, facultative anaerobic bacteria located on the dorsal surface of the tongue reduce ~20% of salivary NO_3^- (~5 % of the ingested intake) to NO_2^- by acting as an alternative electron acceptor to gain adenosine-5'-triphosphate in the absence of O_2 (Duncan et al. 1995). Following the ingestion of a NO_3^- load, salivary [NO_2^-] can rise to 1-2 mM (Lundberg and Govoni 2004). When swallowed a portion of this NO_2^- is reduced to NO in the acidic environment of the stomach (Benjamin et al. 1994; Lundberg et al. 2004), a reaction which is greatly enhanced in the presence of vitamin C and polyphenols (Weitzberg and Lundberg 1998; Gago et al. 2007). However, some NO_2^- escapes and is rapidly and efficiently absorbed to increase circulating plasma

[NO_2^-] (Webb et al. 2008). Here it has a half-life of 1-5 min (Lundberg and Weitzberg 2005) and can be reduced to NO under appropriate conditions. It is important to note that the rise in plasma [NO_2^-] following oral NO_3^- intake is attenuated following the use of antibacterial mouthwash (Govoni et al. 2008) and by spitting (Webb et al. 2008), highlighting the importance of this enterosalivary circulation for the reduction of NO_3^- to NO_2^-.

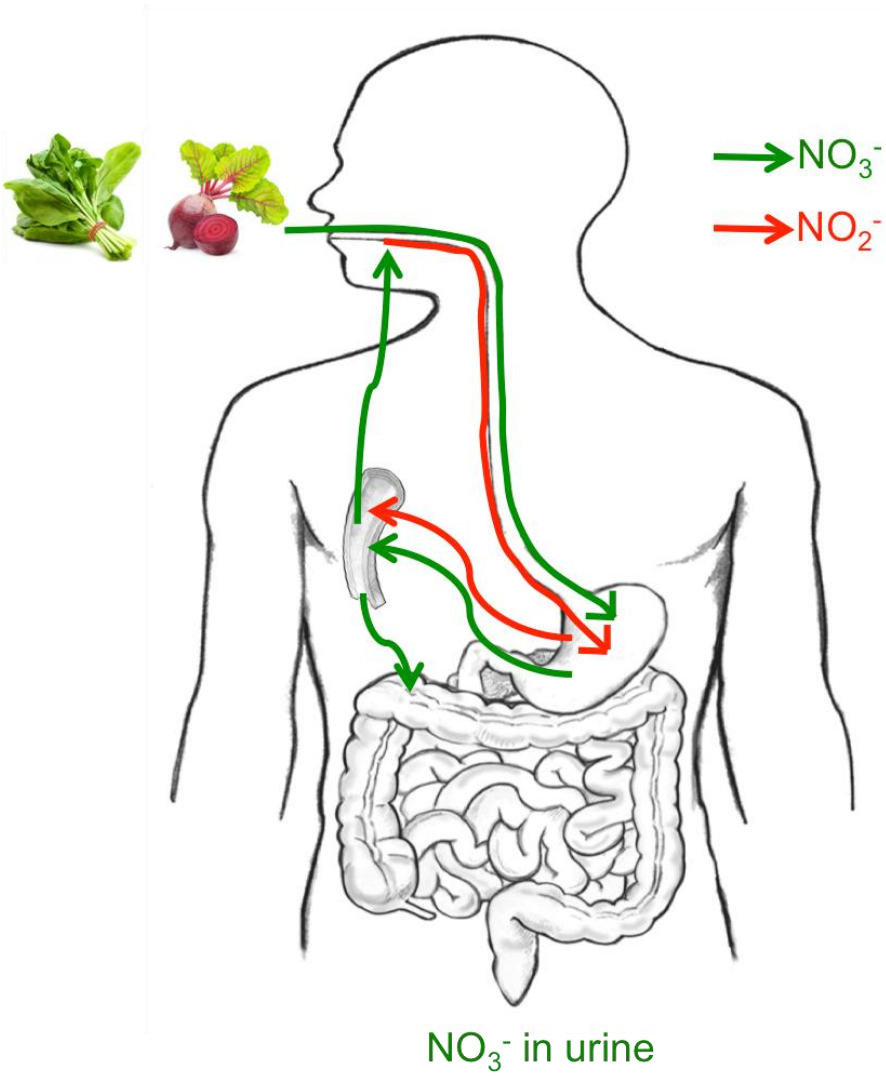

Figure 2.1. The enterosalivary circulation of nitrate. Nitrate (NO_3^-) is represented by the green arrows and nitrite (NO_2^-) by the red arrows (redrawn from Lundberg et al., 2011).

NO synthesis from NO_2^-

The final step of the NO_3^--NO_2^--NO pathway is achieved by a simple one-electron reduction of NO_2^- to yield NO. This reaction is catalysed by a number of NO_2^-

reductases including deoxyhaemoglobin (Cosby et al. 2003), deoxymyoglobin (Shiva et al. 2007a), xanthine oxidase (Zhang et al. 1998), aldehyde oxidase (Li et al. 2008), cytochrome P-450 (Kozlov et al. 2003), and the mitochondrial electron transfer complexes (Kozlov et al. 1999). It is important to note that unlike NO production from the classic NOS pathway, this reduction reaction is greatly enhanced by conditions of hypoxia (Castello et al. 2006) and acidosis (Modin et al. 2001). As such, NO production from the NO_3^--NO_2^--NO pathway may serve as a backup system to ensure NO generation when the O_2-dependant NOSs may be dysfunctional (Figure 2.2). Importantly, skeletal muscle is likely to experience conditions of low O_2 tension and pH during contraction (Richardson et al. 1995), implying that the NO_3^--NO_2^--NO pathway may be of particular importance during exercise.

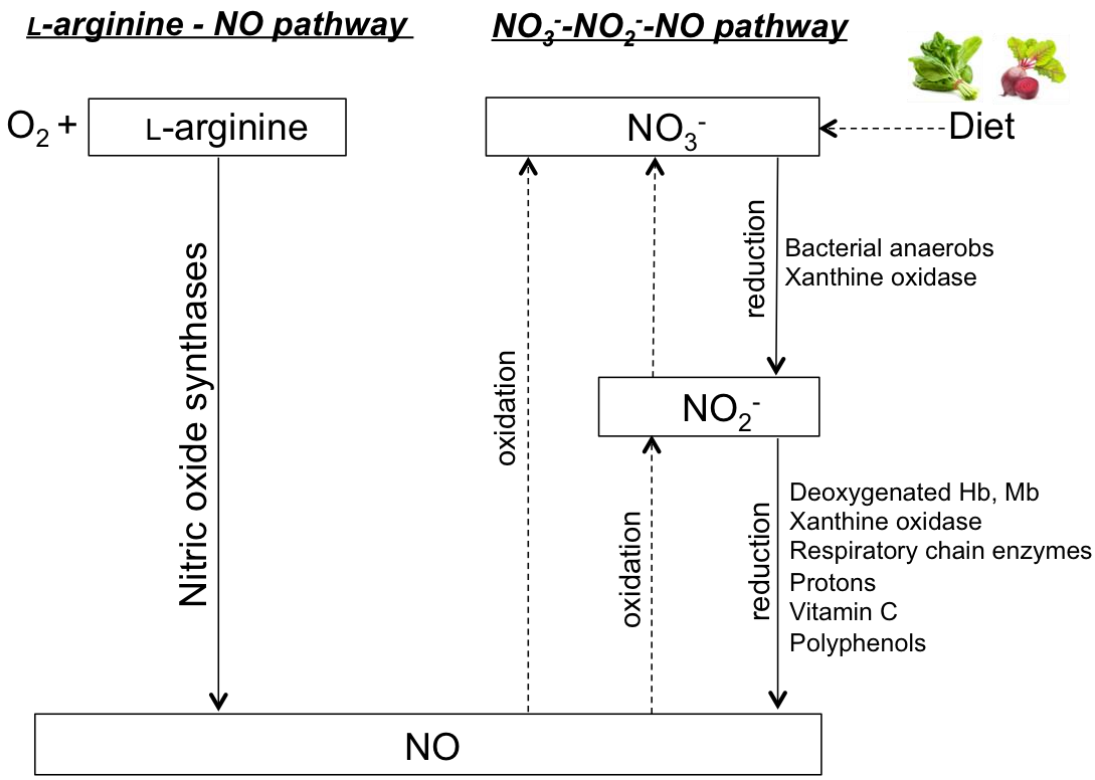

Figure 2.2. A schematic presentation of the two parallel pathways for mammalian nitric oxide (NO) synthesis. Left: The L-arginine NO pathway. Right: The nitrate (NO_3^-) – nitrite (NO_2^-) – NO pathway. Hb, haemoglobin; Mb, myoglobin (redrawn from Jones et al. 2014)

It is important to note, however, that while a greater circulating plasma [NO_2^-] following NO_3^- intake would be expected to increase NO production and associated

physiological signalling, NO_2^- itself may influence physiological processes independent of its reduction to NO via modifications to haem groups by nitrosylation, and protein thiols by S-nitrosation (Bryan et al. 2005).

Dietary inorganic NO_3^- and NO_2^- intake

Vegetables are the main source of dietary NO_3^- in the human diet and can account for 80-85% of daily NO_3^- intake (Ysart et al. 1999). Within the vegetable family, green leafy vegetables such as lettuce, spinach and beetroot are especially rich in NO_3^-, containing up to ~400 mg NO_3^- per 100 g of fresh weight produce (Wang et al., 2000). Other sources of NO_3^- include cured meats, where it is added as a preservative, and drinking water (Hord et al. 2009). Estimates on NO_3^- intake from food are ~31-185 mg/day in Europe and ~40-100 mg/day in the United States (Hord et al. 2009), whereas reported International estimates of daily NO_3^- ingestion are ~53-350 mg/day (Pennington 1998). This variability in NO_3^- intake may result from, but is not limited to, a variation in the number of servings, the species of vegetables consumed, fertilizer application, growth conditions as well as storage and transport conditions (Hord et al. 2009). Unlike NO_3^-, the content of NO_2^- in food is very low with the highest concentrations of NO_2^- found in processed and cured meats (0-0.89 mg per 100 g) where NO_2^- is added as a preservative to prevent the growth of *Clostridium botulinus* and to enhance taste and appearance (Hord et al. 2009). In contrast, the NO_2^- content of vegetables is lower with average concentrations of < 10 mg NO_2^- per kg, and maximal concentrations rarely exceeding 100 mg per kg (Pennington 1998; Santamaria 2006; Sušin et al. 2006; Hord et al. 2009). Due to the low NO_2^- content in foods, the average intake of NO_2^- is considerably lower than that of NO_3^-, with daily intake estimates of 0 to 20 mg (Pennington 1998).

Historical health concerns of NO_3^- ingestion

NO_3^- and NO_2^- have long been considered as toxic constituents of the diet due to their supposed association with the development of gastric cancer and methemoglobinemia in infants (‘blue baby syndrome’) (McKnight et al. 1999). As a result, the acceptable daily intake (ADI) of NO_3^- is set by the World Health Organization (WHO) at 3.7 mg NO_3^-/kg body mass (i.e. 4.2 – 4.7 mmol NO_3^- for a 70-80 kg human) (WHO, 2002).

Given that NO_3^- is relatively inert, the harmful effects of NO_3^- ingestion are believed to be mediated by its reduction to NO_2^-. The proposed carcinogenic effect of NO_3^- and NO_2^- originated from the observation of NO_2^--dependent *in vivo* formation of N-nitrosamines (a class of carcinogenic substance) in some rodents following ingestion of NO_2^- (Mirvish 1975). Indeed, a small increase in the number of incidences of lymphoma in rats after chronic NO_2^- ingestion has been observed (Newberne et al., 1976), although it must be acknowledged that the doses of NO_2^- administered in this study were supra-physiological. In comparison, methemoglobinemia results from the NO_2^- mediated oxidation of ferric (Fe^{2+}) iron in oxyhaemoglobin that renders the haemoglobin molecule unable to bind O_2, potentially leading to cellular hypoxia (Fan and Steinberg 1996; McKnight et al. 1999). This concern was first raised in 1945 when babies younger than 6 months old developed methemoglobinemia following ingestion of well-water containing high concentrations of NO_3^- (Comly, 1945).

Since these early reports, however, there has been a lack of evidence to confirm a link between NO_3^- intake and any harmful effects in humans. Indeed, epidemiological studies have failed to provide evidence that a high intake of NO_3^- is related to an increased risk of cancer (Forman et al. 1985; Beresford 1985; Gangolli et al. 1994) and WHO have stated recently that 'there is inadequate evidence in humans for the carcinogenicity of nitrate in food' (WHO, 2010). The role of NO_3^- on the incidence of methemoglobinemia has also been questioned. In 1999, Avery argued that the early reported cases of methemoglobinemia following well-water ingestion in babies (Comly, 1945) were likely due to bacterial contamination (Avery 1999). In addition, there has also been a lack of evidence to demonstrate the presence of methemoglobinemia following human NO_3^- or NO_2^- exposure (Cornblath and Hartmann 1948; Kortboyer et al. 1997; Dejam et al. 2007).

Instead of harmful effects, there is now a growing body of evidence indicating the therapeutic potential of NO_3^- consumption (Webb et al. 2008; Raat et al. 2009; Lundberg et al. 2010; Bailey et al. 2012). For example, NO_3^- consumption, and the NO_3^--NO_2^--NO pathway, have been shown to have positive effects on ischeamia reperfusion injury (Raat et al. 2009), blood pressure (BP) regulation (Webb et al. 2008), platelet aggregation (Richardson et al. 2002) and the physiological response to exercise (Bailey et al. 2012). Due to these observations, there are now calls from the research community

for a re-evaluation of the ADI (Hord et al. 2009; Kapil et al. 2010). Importantly, the current ADI can be exceeded by just one portion of spinach (Hord et al. 2009), and individuals who follow the influential Dietary Approach to Stop Hypertension (DASH) diet, which has been shown to reduce BP, will consume approximately 1200 mg NO_3^- every day; exceeding the current ADI by ~550% (Hord et al. 2009).

NO_3^- salts and beetroot juice

Pharmaceutical NO_3^- salts, such as sodium NO_3^- ($NaNO_3$) and potassium NO_3^- (KNO_3), administered in solution or via capsules, have been used extensively as a convenient means to administer NO_3^- in human experimental studies (Larsen et al. 2007; Kapil et al. 2010; Bescós et al. 2011). However, other researchers have opted to administer NO_3^- in the form of beetroot juice (BR), which is naturally rich in dietary NO_3^-. Along with NO_3^-, beetroot contains other compounds that may influence human metabolism. For instance, betaine has been used in the treatment of cardiovascular disease (Borsook and Borsook 1951; Van Zandt and Borsook 1951), and has been shown to elicit improvements in muscle strength, power and endurance (Hoffman et al. 2009). In addition, beetroot is rich in the polyphenols, quercetin and resveratrol, which have been linked to mitochondrial biogenesis and an associated increase in aerobic capacity (Lagouge et al. 2006; Davis et al. 2009; Ganio et al. 2010). Beetroot may also provide protection against exercise-induced oxidative stress (Kanner et al. 2001) and act to limit the formation of potentially harmful nitrogenous compounds (Santamaria 2006), due to its high antioxidant and polyphenol content (Shepherd et al. 2015). The potential of these ingredients to interfere with the interpretation of nitrate effects during experimental studies, led to the development of a NO_3^--depleted beetroot juice which is identical in colour, taste, smell and texture (Lansley et al. 2011b). This allowed for the effects of NO_3^- to be isolated from those of the other potential 'active' ingredients in BR, and also provided a means to carry out a genuinely double-blind placebo controlled experimental study.

Plasma NO_3^- and NO_2^- concentrations

In general, human plasma NO_3^- and NO_2^- levels at rest range from between 20-40 uM and 50-300 nM, respectively (Kelm 1999; Rassaf et al. 2004; Lundberg and Govoni 2004; Larsen et al. 2007; Bailey et al. 2009). However, there are exceptions, with some

studies reporting plasma NO_2^- concentrations in the μM range (Bescós et al. 2011). The variation between individuals and studies is likely a function of differences in nutritional intake, fitness status, health and the measurement technique used to determine the concentration of these NO metabolites. Indeed, regular exercise has been shown to result in higher circulating levels of NO_3^- (Jungersten et al. 1997; Lewis et al. 1999; Green et al. 2004), whilst diseases with endothelial dysfunction result in low plasma NO_3^- and NO_2^- levels (Kleinbongard et al. 2006). Many different methods are available for the determination of NO_2^- concentration in plasma, including ozone based chemiluminescence, high performance liquid chromatography and colorimetric assays (e.g. Greiss reaction) (Tsikas 2005). Whilst ozone based chemiluminescence is considered the gold standard technique and is widely used due to its high sensitivity and ability to detect nM concentrations of NO_2^-, the Griess reaction lacks sensitivity and is unable to accurately determine concentrations below the μM range (Tsikas 2005). Consideration of the participant cohort and method used to determine plasma NO_2^- must therefore be given when comparing plasma [NO_2^-] and [NO_3^-] data between studies.

Previous research has consistently reported a significant rise in plasma [NO_3^-] and [NO_2^-] following dietary supplementation with NO_3^-, administered in the form of $NaNO_3$, KNO_3 and BR (e.g. Webb et al. 2008; Bailey et al. 2009; Kapil et al. 2010; Bescós et al. 2011). Specifically, Webb et al., (2008) found that the peak elevation in plasma [NO_3^-] and [NO_2^-] occurred 1-2 h and 2-3 h, respectively, post ingestion of 500 mL BR (containing ~45 mmol NO_3^-). The same research group later found that the acute ingestion of three different doses of KNO_3 resulted in a dose-dependent elevation in plasma [NO_3^-] and [NO_2^-], with peak elevations occurring at similar time points post consumption (Kapil et al., 2010). Consistent with their half-life in the human systemic circulation, plasma [NO_3^-] and [NO_2^-] returned to baseline by 24 h post consumption in these studies (Webb et al. 2008; Kapil et al. 2010). Importantly, the time at which plasma [NO_2^-] peaked after NO_3^- ingestion in these studies has informed the timing of NO_3^- ingestion in the majority of subsequent NO_3^- supplementation studies. Specifically, physiological assessments in these studies have been made 2-3 h post NO_3^- ingestion to coincide with the peak rise in plasma [NO_2^-] (and therefore presumably NO bioavailability) (Bailey et al. 2009; Vanhatalo et al. 2010; Bescós et al. 2011; Lansley et al. 2011b). However, the pharmacokinetic response to different doses of BR has not been characterised. With the use of BR in both basic research and an applied

sports setting increasing, the time-to-peak plasma [NO_2^-] following different doses of BR will provide crucial information to guide the optimal timing of BR ingestion.

Importantly, a number of research groups have also shown that the rise in plasma [NO_2^-] after acute ingestion of NO_3^- can be maintained (neither increased nor decreased) after daily consumption of NO_3^- for up to 15 days (Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010). Interestingly, it has also been reported that maximal exhaustive exercise can result in both an increase (Rassaf et al. 2004; Allen et al. 2010) but more frequently a reduction in circulating plasma [NO_2^-] (Larsen et al. 2010; Dreißigacker et al. 2010; Bescós et al. 2011), implying that NO_2^- may be serving as an NO substrate during exercise, particularly at high intensities. However, the kinetics of plasma [NO_2^-] during intermittent exercise, which involves the repeated rapid transition from low- to high- exercise intensity, has not been determined.

The remainder of this review will summarise the established influence of an increase in plasma [NO_2^-] (and therefore presumably NO bioavailability) following dietary NO_3^- supplementation on a number of different physiological processes, along with their potential underlying mechanisms. In addition, this review will highlight intermittent exercise as a condition where NO_3^- supplementation may be particularly beneficial for exercise performance enhancement.

Established effects of dietary NO_3^- supplementation

Blood pressure

A diet rich in fruit and vegetables lowers the risk of morbidity and mortality from cardiovascular disease (Joshipura et al. 2001), a finding that may partly be attributed to the lowering of BP that accompanies such diets (Appel et al. 1997). In particular, the greatest protective effect is found in those diets with the highest consumption of green leafy vegetables (Joshipura et al. 2001) suggesting that NO_3^- may be particularly important for these health benefits.

There are now numerous reports demonstrating that dietary NO_3^- can significantly reduce resting BP after acute (Webb et al. 2008; Kapil et al. 2010; Vanhatalo et al.

2010) and chronic (3-15 days) supplementation (Larsen et al., 2007, 2010; Bailey et al., 2009, 2010; Vanhatalo et al., 2010). This suggests that dietary inorganic NO_3^- may provide an effective approach for improving BP and vascular health. Importantly, Webb and colleagues from the William Harvey Research Institute, London, have carefully characterised the reduction in BP over 24 h following the ingestion of BR. They found that acute BR ingestion (~45 mmol NO_3^-) reduced systolic (by ~10.4 mmHg) and diastolic (by ~8.1 mmHg) BP and mean arterial pressure (MAP; by ~8 mmHg) at 2.5 to 3 h after BR intake, and that this reduction was closely related to the peak rise in plasma [NO_2^-] (Webb et al. 2008). The same group later reported that the rise in plasma [NO_2^-] and the reduction in BP following consumption of three different doses (4, 13 and 24 mmol) of KNO_3 were dose-dependent, with the largest reduction in systolic and diastolic BP being 9 and 6 mmHg, respectively (Kapil et al. 2010). By 24 h these changes in BP had returned to baseline values (Webb et al. 2008; Kapil et al. 2010). The kinetics of these changes in BP following NO_3^- ingestion provide valuable information to inform the use of inorganic NO_3^- as an effective approach for improving BP and vascular health.

The BP lowering effects of NO_3^- supplementation are likely mediated by its reduction to NO_2^- and further to NO (Ignarro et al., 1987), although NO_2^- itself may also confer direct vasodilatory effects (Alzawahra et al. 2008). It is well documented that an increase in NO bioavailability can stimulate smooth muscle relaxation via stimulating the release of cyclic guanosine monophosphate (cGMP) (Ignarro et al. 1987). Considering the presence of polyphenols and vitamin C in BR (see under '*Pharmaceutical NO_3^- salts and beetroot juice'* for further details*)* and the ability for these antioxidants to enhance the reduction of NO_2^- to NO (Lundberg et al. 2010), it is possible that the dose-response relationship between changes in BP following BR ingestion is different to that following NO_3^- salt ingestion. Future studies characterising the dose-response relationship between BR intake and BP lowering over 24 h will therefore provide important information on the optimal dose of BR for lowering BP. While this has not previously been possible due to the large volumes of BR required to administered large doses of dietary NO_3^- (i.e. 1 L of non-concentrated BR contains only ~10.2 mmol NO_3^-), the advent of concentrated BR (~70 mL contains 4.1 mmol NO_3^-) now permits the dose-response relationship between BR and BP to be examined.

O_2 cost of submaximal exercise

As well as important haemodynamic effects, dietary NO_3^- supplementation has beneficial effects upon the physiological responses to submaximal exercise (e.g. Larsen et al. 2007; Bailey et al. 2009; Lansley et al. 2011b; Muggeridge et al. 2013). Following the onset of submaximal exercise performed below the gas exchange threshold (GET; analogous with the lactate threshold) (Jones and Poole 2005) O_2 uptake ($\dot{V}_{O2}$) rises exponentially and reaches a 'steady state' after 120-180 s. This steady state $\dot{V}_{O2}$ represents the metabolic cost of exercise and has long been considered to be independent of age, health and aerobic fitness, as well as unaffected by known physical (e.g. exercise training), nutritional and pharmaceutical agents (Jones and Poole 2005). Remarkably, however, in 2007, Larsen and colleagues reported that supplementation with $NaNO_3$ (0.1 mmol NO_3^- $kg^{-1}\cdot d^{-1}$) for 3 days significantly increased plasma [NO_2^-] and reduced the O_2 cost of submaximal cycling in a group of healthy untrained volunteers (Larsen et al. 2007). These findings were later corroborated by Bailey et al., (2009) who reported a reduction in the steady-state $\dot{V}_{O2}$ during moderate-intensity cycle exercise following 4-6 days of supplementation with BR (0.5 $L\cdot d^{-1}$ or 5.5 $mmol\cdot d^{-1}$ of NO_3^-; Bailey et al. 2009). The lowering of submaximal exercise $\dot{V}_{O2}$ following dietary NO_3^- supplementation has important implications for athletes because a high exercise efficiency (i.e. a low $\dot{V}_{O2}$ for a given power output) is a key determinant of exercise performance (Jones and Burnley 2009). Moreover, for senescent populations, or individuals with metabolic, respiratory or cardiovascular diseases, a reduction in the O_2 cost of daily activities might significantly improve functional capacity and quality of life.

Since these early reports, many studies have investigated the influence of dietary NO_3^- supplementation on the O_2 cost of submaximal exercise (Table 1). Although not in all studies (Breese et al. 2013; Kelly et al. 2014), supplementation with 5-26 mmol NO_3^- daily for 3-15 days in healthy, recreationally active or moderately trained participants, has been shown to significantly lower the O_2 cost of submaximal exercise in walking (Lansley et al. 2011b), running (Lansley et al. 2011b; Porcelli et al. 2014), double-legged knee-extensor exercise (Bailey et al. 2010), and cycling at various intensities (Vanhatalo et al. 2010; Cermak et al. 2012a; Thompson et al. 2014; Whitfield et al. 2016). In addition, a reduction in $\dot{V}_{O2}$ has also been evident in the same participant cohort when supplementation is continued for 15 days (Vanhatalo et al. 2010).

Importantly, a similar lowering of moderate intensity $\dot{V}_{O_2}$ has been reported acutely, 1 h following $NaNO_3^-$ ingestion (~0.033 mmol.per kg^{-1} body mass; Larsen et al. 2010) and 1.5-3 h following BR ingestion (~5-8 mmol of NO_3^-; Vanhatalo et al. 2010; Muggeridge et al. 2014; Muggeridge et al. 2013; Thompson et al. 2014) suggesting that chronic supplementation is not required to achieve this effect. In contrast to these results, studies have reported no significant reductions in the O_2 cost of submaximal exercise following acute (Bescós et al. 2011; Peacock et al. 2012; Boorsma et al. 2014; Sandbakk et al. 2015) or chronic (Christensen et al. 2013) administration of NO_3^- in highly trained/elite endurance athletes (i.e. $\dot{V}_{O2peak} \geq 60$ $ml.kg^{-1}.min^{-1}$). There is one exception, however, with Peeling et al., (2015) recently observing a reduction in steady-state $\dot{V}_{O_2}$ during laboratory kayaking in a group of National and International level kayak athletes. The reason for the between-study discrepancies in the effect of NO_3^- supplementation on submaximal exercise $\dot{V}_{O_2}$ is unclear, but intra-study differences in the supplementation regime (i.e. dose and timing of ingestion), exercise modality and the participant population investigated (trained vs. untrained) might be responsible.

Table 2.1. The effects of dietary NO_3^- supplementation on the O_2 cost of submaximal exercise.

Author	Participants	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	Supplementation regime	Exercise Mode	Protocol	NO indices	Change in the O_2 cost of submaximal exercise
Larsen et al., (2007)	9 healthy well-trained males	55 ± 4	3 days $NaNO_3$ supplementation (0.1 mmol · kg^{-1} · day^{-1})	Cycling	5 min exercise bouts at WRs equivalent to 45, 60, 70, 80, 85 and 100% $\dot{V}O_{2peak}$	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	↓ $\dot{V}O_2$ over 4 lowest WRs
Bailey et al., (2009)	8 recreationally active males	49 ± 5	6 days of NO_3^- rich BR supplementation (~5.6 mmol NO_3^- · day^{-1})	Cycling	4 x MI (80% GET) exercise bouts completed on days 4-6 of supplementation	↑ plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during MI exercise bouts
Bailey et al., (2010)	7 recreationally active males	Not available	6 days of NO_3^- rich BR supplementation (~5.1 mmol NO_3^- · day^{-1})	Two-legged knee-extensor exercise	6 x LI (15% MVC iEMG signal) exercise bouts completed on days 4-6 of supplementation	↑ plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during LI exercise bouts
Vanhatalo et al., (2010)	8 recreationally active males and females	47 ± 8	Acute (2.5 h prior to exercise), 5 d and 15 d of NO_3^- rich BR supplementation (~5.2 mmol NO_3^- · day^{-1})	Cycling	2 x MI (90% GET) exercise bouts after 2.5 h, 5 d and 15 d of supplementation	↑ plasma $[NO_2^-]$ at all time points	↓ $\dot{V}O_2$ during MI exercise bouts at all time points.
Larsen et al., (2010)	7 healthy recreationally active males and females	Not available	Acute (1 h prior to exercise) ingestion of 0.1 mmol kg^{-1} of $NaNO_3$.	Cycling	5 min LI (~84 W) exercise bout.	↑ plasma $[NO_2^-]$ ↑ plasma $[NO_2^-]$ ↑ plasma cGMP	↓ $\dot{V}O_2$ during LI exercise bouts
Larsen et al., (2011)	13 recreationally active males and females	Not available	3 days $NaNO_3$ (0.1 mmol kg^{-1} ·day^{-1}) supplementation	Cycling	Exercise bout at WRs equivalent to 50% $\dot{V}O_{2peak}$	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during exercise bouts
Lansley et al., (2011)	9 recreationally active males	55 ± 7	6 days of NO_3^- rich BR supplementation (~5.1 mmol NO_3^- ·day^{-1})	Walking/ running	2 x 6 min walking bouts (4 $km.h^{-1}$), and 4 MI running bouts (80% GET) on days 4 and 5 of supplementation.	↑ plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during walking and running exercise bouts
Bescos et al., (2011)	11 male cyclists and triathletes	65 ± 6	Acute (3 h prior to exercise) ingestion of $NaNO_3$ (10 mg kg^{-1} ·day^{-1})	Cycling	6 min at WRs equivalent to 2, 2.5, 3 and 3.5 W·kg^{-1}	↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ during exercise bouts at all intensities.
Cermak et al., (2012)	13 trained male cyclists or triathletes	58 ± 2	6 days NO_3^- rich BR supplementation (8 mmol NO_3^- ·day^{-1})	Cycling	30 min bouts at WRs corresponding to 45% and 65% maximum power output achieved during an incremental test to exhaustion.	↑ plasma $[NO_3^-]$	↓ $\dot{V}O_2$ during exercise bouts at both intensities.

Peacock et al., (2012)	10 highly trained male skiers	70 ± 5	Acute (2.5 h prior to exercise) ingestion of KNO_3 (9.9 mmol NO_3^-)	Running	5 min running bouts at 10 $km \cdot h^{-1}$ and 14 $km \cdot h^{-1}$.	↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ during exercise bouts at both intensities.
Muggeridge et al., (2013)	8 trained male kayakers	49 ± 6	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich BR (4.2 mmol NO_3^-)	Kayaking	15 min at WR equivalent to 60% of maximal WR attained during a maximal graded kayaking test.	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during exercise bout
Christensen et al., (2013)	10 elite male cyclists	72 ± 4	6 days NO_3^- rich BR supplmentation (8 mmol $NO_3^- \cdot day^{-1}$)	Cycling	3 x 6 min at WR equivalent to 70% peak power output achieved during maximal graded exercise test.	↑ plasma NOx (nitrate + nitrite)	↔ $\dot{V}O_2$ during exercise bouts
Breese et al., (2013)	9 recreationally active males and females	Males: 48 ± 6 Females: 46 ± 9	6 days of NO_3^- rich BR supplementation(8 mmol $NO_3^- \cdot day^{-1}$)	Cycling	3 x 4 min MI (90% GET) exercise bouts on days 4-6 of supplementation.	↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ during exercise bouts
Kelly et al., (2014)	12 recreationally active males	58 ± 6	6 days NO_3^- rich BR supplementation (8.4 mmol $NO_3^- \cdot day^{-1}$)	Cycling	2 x 5 min MI (80% GET) exercise bouts on days 3-6 of supplementation	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ during exercise bouts
Thompson et al., (2014)	16 recreationally active males	47± 6	Acute (90 min before exercise) ingestion of NO_3^- rich BR (5 mmol NO_3^-).	Cycling	2 x 20 min exercise bouts at WRs equivalent to 50 and 70% $\dot{V}O_{2peak}$	↑ plasma $[NO_2^-]$	Trend for ↓ $\dot{V}O_2$ during exercise bout at 50% $\dot{V}O_{2peak}$ (P = 0.110)
Muggeridge et al., (2014)	9 male trained cyclists	53 ± 4	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich gel (~8.1 mmol NO_3^-)	Cycling	10 min at WR equivalent to 60% during maximal graded exercise test.	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	Trend for ↓ $\dot{V}O_2$ (P = 0.086)
Porcelli et al., (2014)	8 untrained males 7 moderately trained males 6 highly trained males	Untrained: 28 - 44 Moderately trained: 46-57 Highly trained: 64-82	6 days $NaNO_3$ supplementation (8.4 mmol $NO_3^- \cdot day^{-1}$).	Running	4 x 6 min MI (80% GET)	All groups: ↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$ Note: the increase in plasma $[NO_3^-]$ & $[NO_2^-]$ was lower in the highly trained individuals.	Untrained: ↓ $\dot{V}O_2$ during exercise bouts Moderately trained: ↓ $\dot{V}O_2$ during exercise bouts Highly trained: ↔ $\dot{V}O_2$ during exercise bouts
Sandbakk et al., (2015)	9 male elite cross-country skiers	69 ± 6	Acute (2.5 h prior to exercise) of KNO_3 (9.9 mmol NO_3^-)	Running	2 x 5 min exercise bouts at 10 $km \cdot h^{-1}$ and 14 $km \cdot h^{-1}$	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ during exercise bouts
Boorsma et al., (2016)	8 male elite 1500 m runners	80 ± 5	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich BR (19.5 mmol NO_3^-) 8 days NO_3^- rich BR supplementation (days 1 & 8 subjects consumed 19.5 mmol	Running	5-7 min exercise bouts at WRs equivalent to 50, 65 and 80% $\dot{V}O_{2peak}$	↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ during exercise bouts at all intensities.

			NO_3^- ·day^{-1} and on day 2-7 subjects consumed 13 mmol NO_3^- ·day^{-1})				
Whitefield et al., (2016)	10 healthy, recreationally active males	50 ± 1	7 days NO_3^- rich BR supplementation (26 mmol NO_3^- ·day^{-1})	Cycling	10 min exercise bouts at WRs equivalent to 50 and 70% $\dot{V}O_{2peak}$	↑ plasma $[NO_3^-]$ ↑ plasma $[NO_2^-]$	↔ $\dot{V}O_2$ at 50% $\dot{V}O_{2peak}$ ↓ $\dot{V}O_2$ at 70% $\dot{V}O_{2peak}$

↑, significant increase; ↓, significant reduction; ↔, no significant difference; $NaNO_3$, sodium nitrate; KNO_3, potassium nitrate; $[NO_3^-]$□, nitrate concentration; $[NO_2^-]$, nitrite concentration; $\dot{V}O_2$, oxygen uptake; WRs, work rates; MI, moderate-intensity; cGMP, cyclic guanosine monophosphate; GET, gas exchange threshold; BR, beetroot juice; Δ; $\dot{V}O_{2peak}$, peak oxygen uptake

Mechanistic bases for reduced O_2 cost of submaximal exercise

A lower O_2 cost of submaximal exercise following NO_3^- administration might be a function of: 1) an inhibition of mitochondrial adenosine triphosphate (ATP) production, which would mandate a compensatory increase in anaerobic energy yield; 2) improved coupling between ATP hydrolysis and muscle force production (i.e. a lower ATP cost of force production); 3) an increase in the mitochondrial P/O ratio (i.e. a lower O_2 cost of mitochondrial ATP resynthesis); or 4) some combination of these candidate mechanisms.

In previous studies (Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011b), the reduced O_2 cost of submaximal exercise following dietary NO_3^- supplementation was not accompanied by an elevated blood [lactate], which suggests no compensatory elevation in anaerobic metabolism. However, it is acknowledged that blood [lactate] represents a very indirect and incomplete assessment of muscle anaerobic energy turnover and therefore, this potential mechanism could not be excluded based on these findings. In light of this, Bailey et al., (2010) investigated the first two of the potential aforementioned mechanisms using calibrated ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS) (Bailey et al. 2010). ^{31}P-MRS enables the *in vivo* assessment of the absolute muscle concentration changes in phosphocreatine ([PCr]), inorganic phosphate ([Pi]) and adenosine diphosphate ([ADP]), as well as pH. Therefore, this technique provided an estimation of changes in total ATP turnover rate and the proportional contribution from PCr hydrolysis, glycolysis and oxidative phosphorylation (Lanza et al. 2006; Layec et al. 2009; Bailey et al. 2010).

During low-intensity knee-extensor exercise, 4-6 days of BR supplementation resulted in a reduction in the steady-state amplitude of muscle [PCr] degradation and [Pi] and ADP accumulation, with no change observed in pH (Bailey et al. 2010). Additional calculations revealed that estimated total ATP turnover rate was significantly reduced as a consequence of reduced estimated ATP turnover rates from PCr hydrolysis and oxidative phosphorylation (Bailey et al. 2010; Figure 2.3). These data suggest that NO_3^- supplementation improved coupling between ATP hydrolysis and skeletal muscle force production. Based on existing models of respiratory control, the observed changes in [ADP], [Pi] and [PCr] following NO_3^- administration would reduce the stimuli for

increased oxidative phosphorylation (Bose et al. 2003; Brown, 1992; Chance & Williams., 1955; Mahler, 1985), and therefore may explain the reduction in the O_2 cost of submaximal exercise after NO_3^- supplementation (Bailey et al. 2010). Furthermore, the fact that NO_3^- supplementation lowered ATP turnover rate through PCr hydrolysis, but did not alter ATP turnover rate from anaerobic glycolysis (Figure 2.3) suggests that the reduced O_2 cost of muscle contraction is not a function of an elevated anaerobic energy turnover.

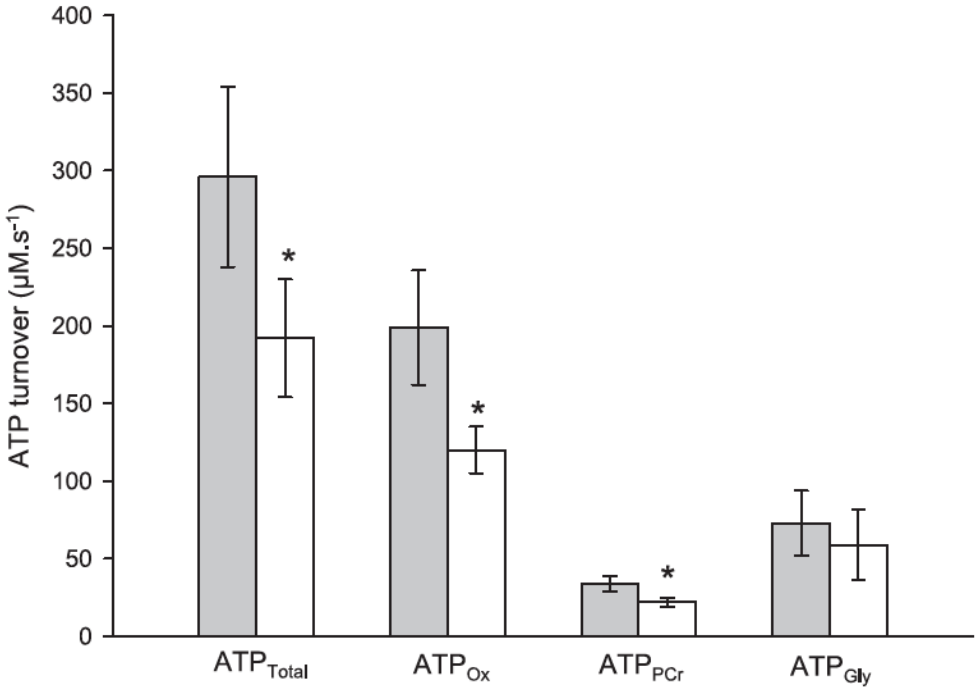

Figure 2.3. The group mean ATP resynthesis rate averaged over an entire bout of low-intensity knee-extension exercise following BR (white bars) and PL (grey bars) supplementation. The figure shows the total ATP turnover (ATP_{Total}) and ATP derived from oxidative phosphorylation (ATP_{Ox}), PCr (ATP_{PCr}) and glycolysis (ATP_{Gly}). Note the significant reduction in ATP derived from oxidative phosphorylation and PCr, but not from glycolysis. (From Bailey et al. 2010)

An increase in the mitochondrial P/O ratio following NO_3^- supplementation also has the potential to lower the O_2 cost of submaximal exercise. The P/O ratio is a classical measure of mitochondrial oxidative phosphorylation efficiency, and is quantified as the amount of O_2 consumed per ADP phosphorylation to ATP (Hinkle 2005). A number of factors including proton leak (Rolfe et al. 1994), proton slip (Groen et al. 1990), energetic cost of metabolite transport (Klingenberg 1970) and physiological uncoupling (Echtay et al. 2002) can negatively affect P/O ratio. NO_3^-, NO_2^- and NO may interact

with the mitochondrion in several different ways. These interactions may include the binding of NO to cytochrome-c-oxidase leading to partial inhibition of mitochondrial respiration (Brown and Cooper 1994) and the regulation of tissue protein expression and activity (Bryan et al. 2005).

Larsen et al., (2011) assessed the mitochondrial P/O ratio by isolating mitochondria from the *vastus lateralis* muscle of healthy humans who had supplemented their diet for 3 days with $NaNO_3$ (0.1 mmol NO_3^- $kg^{-1} \cdot d^{-1}$). To allow for mitochondrial respiration to be investigated, the resultant mitochondrial suspension was added to a reaction medium containing the substrates pyruvate and malate. With a controlled submaximal infusion of ADP, which was chosen to closely resemble the metabolic state *in vivo* (Kuznetsov et al. 1996), the P/O ratio was increased by 19% (Larsen et al. 2011). Further analyses revealed that both the respiratory control ratio (ratio between state 3 (coupled) and state 4 (uncoupled) respiration) and maximal rate of ATP production through oxidative phosphorylation were increased following $NaNO_3$ supplementation (Larsen et al. 2011). Mitochondrial respiration with substrates (pyruvate and malate) but without added ADP, provided a measure of back leakage of protons through the inner mitochondrial membrane, termed state 2 respiration. Following $NaNO_3$ administration this back leak was reduced by 45% in addition to a 48% reduction in state 4 respiration, which was the O_2 consumption when exhaustion of ADP had occurred (state 4). Taken together, these results suggest that NO_3^- supplementation increased the P/O ratio by reducing proton leakage and uncoupled respiration. Importantly, the increased P/O ratio following $NaNO_3^-$ supplementation was correlated with the reduction in $\dot{V}_{O2}$ during submaximal exercise (Larsen et al. 2011).

The mitochondrial proteins, uncoupling protein 3 (UCP-3) and adenine nucleotide translocase (ANT), are believed to be responsible for a major part of the proton leak in mitochondria (Parker et al. 2008; Bevilacqua et al. 2010). Using Western blotting, Larsen et al., (2011) reported a significant down regulation in ANT expression following 3 days of NO_3^- supplementation. This provides a possible mechanistic basis for a reduced proton leak following NO_3^- administration and also a rationale for the reduction in the O_2 cost of exercise following chronic ($\geq$3 days) NO_3^- supplementation. However, it is important to note that these changes would not occur quickly enough to explain the same effect 1-3 h post ingestion of NO_3^-. Indeed, Larsen et al., (2011)

reported no effect of acute NO_2^- exposure on P/O ratio or state 2 and 4 respiration. Notably, NO and NO_2^- have previously been reported to acutely and reversibly cause post-translational protein modifications via s-nitrosation, which may result in an alteration of mitochondrial respiration (Reid 1998). Further research is required to determine the mechanistic basis of an acute reduction in submaximal $\dot{V}_{O2}$ 1-3 h after NO_3^- ingestion.

Despite the results of Larsen et al., (2011), more recent findings suggest that an improvement in mitochondrial efficiency may not be achieved when NO_3^- is administered in the form of NO_3^- rich BR (Whitfield et al. 2016). Indeed, Whitfield et al., (2016) demonstrated that while 7 days of BR supplementation (26 mmol NO_3^- per day) did significantly lower submaximal exercise $\dot{V}_{O2,}$ measures of mitochondrial coupling, including the P/O ratio, and the expression of ANT and UCP-3 were not altered. In this case, the observed lowering of $\dot{V}_{O2}$ is likely due solely to a reduction in the ATP cost of force production, as previously shown by Bailey et al., (2010). The reason for the discrepancy between the effects of NO_3^- salts and BR on mitochondrial efficiency is not clear. It is important to acknowledged, however, that the studies of Larsen et al., (2011) and Whitfield et al., (2016) varied considerably in terms of supplementation duration (3 days vs. 7 days), dose of NO_3^- administered (~7 mmol vs. 26 mmol NO_3^- per day). Although not experimentally proven, it is possible that the different physiological effects observed in these two studies were a result of these between-study differences rather than the form of NO_3^- used *per se*. In this regard, it may be that at particular doses and durations of BR supplementation, an improvement in mitochondrial efficiency may be observed. In order to comprehensively assess the mechanistic basis for a reduction in $\dot{V}_{O2}$ following BR and $NaNO_3$ supplementation, a study in which both NO_3^- forms are compared directly (in terms of mitochondrial efficiency, the ATP cost of force production [using ^{31}P-magnetic resonance spectroscopy] and the O_2 cost of submaximal exercise) is required.

Exercise performance

In 2010, Dreißigacker et al., identified plasma [NO_2^-] as an important correlate of exercise tolerance (Dreißigacker et al. 2010) suggesting that interventions, such as dietary NO_3- supplementation, that increase plasma [NO_2^-] may have the potential to

improve exercise tolerance. In support of this hypothesis, Bailey et al., (2009) reported a significant rise in plasma [NO_2^-] and a 16% improvement in severe-intensity constant work rate exercise tolerance (at 70% Δ, where Δ refers to the difference between the work rate at the gas exchange threshold (GET) and the $\dot{V}_{O2peak}$) following 4-6 days of BR supplementation (0.5 $L \cdot d^{-1}$ containing 5.5 $mmol.d^{-1}$ of NO_3^-) (Bailey et al. 2009). Since this observation, several studies have investigated the influence of dietary NO_3^- supplementation on exercise tolerance (Table 2.2). Although not a universal finding (Kelly et al. 2014), supplementation with 5-10 mmol NO_3^- daily for 5-11 days has been shown to improve exercise tolerance in healthy, recreationally-active or moderately-trained participants during two-legged knee-extensor exercise (+ 25%; Bailey et al. 2009), treadmill running (+ 15%; Lansley et al. 2011b) and across a range of severe-intensity cycling work rates (+ 12-21%; Kelly et al. 2013; Thompson et al. 2014; Breese et al. 2013). In a similar participant cohort, an improvement in incremental exercise tolerance has also been shown during single-legged knee-extensor exercise (Lansley et al. 2011b) and cycling (Vanhatalo et al. 2010) following 3-15 days of supplementation with NO_3^- (~5-8 mmol/day). Conversely, incremental exercise was not significantly improved in trained athletes following acute $NaNO_3$ (0.16 $mmol \cdot kg^{-1} \cdot BM$) administration (Bescós et al. 2011).

While dietary NO_3^- supplementation has been reported to improve exercise tolerance by 12-25% during constant work rate exercise (i.e. time to reach exhaustion) (Bailey et al. 2009; Bailey et al. 2010; Lansley et al. 2011b; Breese et al. 2013; Kelly et al. 2013; Thompson et al. 2014), this benefit would be expected to translate into no more than a 1-2% improvement in exercise performance (i.e. time to cover a set distance, e.g. a cycling time trial) (Hopkins et al. 1999). The influence of dietary NO_3^- on exercise performance has been examined on many occasions with varying outcomes (Table 2.2). Consistent with the prediction of Hopkins et al., (1999), acute NO_3^- supplementation has been shown to enhance performance in trained cyclists during a

Table 2.2. The effects of dietary NO_3^- supplementation on exercise performance

Author	Participants	$\dot{V}O_{2peak}$ (ml.kg^{-1}.min^{-1})	Supplementation regime	Exercise Mode	Exercise protocol	NO indices	Performance
Bailey et al., (2009)	8 recreationally active males	49 ± 5	6 days of NO_3^- rich BR supplementation (~5.6 mmol $NO_3^- \cdot day^{-1}$)	Cycling	SI (70% Δ) exercise bouts completed on day 4 of supplementation	↑ plasma $[NO_2^-]$	↑ Tlim
Larsen et al., (2010)	9 healthy recreationally active males and females	3.72 ± 0.33 l.min^{-1} (note: relative $\dot{V}O_{2peak}$ data not available).	2 days $NaNO_3$ supplementation (0.1 mmol $\cdot$ kg^{-1} $\cdot$ day^{-1})	Leg and arm cycling	Incremental exercise test to Tlim	↑ plasma $[NO_2^-]$ ↑ plasma $[NO_2^-]$ ↑ plasma cGMP	Trend for ↑ Tlim (P = 0.13)
Bailey et al., (2010)	8 recreationally active males	Not available	6 days of NO_3^- rich BR supplementation (~5.1 mmol $NO_3^- \cdot day^{-1}$)	Two-legged knee-extensor exercise	HI (30% MVC iEMG signal) exercise bout completed on day 4 of supplementation	↑ plasma $[NO_2^-]$	↑ Tlim
Vanhatalo et al., (2010)	8 recreationally active males and females	47 ± 8	Acute (2.5 h prior to exercise), 5 d and 15 d of NO_3^- rich BR supplementation (~5.2 mmol $NO_3^- \cdot day^{-1}$)	Cycling	Incremental exercise test to Tlim after 2.5 h, 5 d and 15 d of supplementation	↑ plasma $[NO_2^-]$ at all time points	↑ peak power output after 15 d, but not 2.5 h or 5 d of supplementation.
Lansley et al., (2011a)	9 competitive male cyclists	56 ± 6	Acute (2-2.5 h prior to exercise) ingestion of NO_3^- rich BR (~6.2 mmol NO_3^-).	Cycling	4- and 16.1 km TT	↑ plasma $[NO_2^-]$	Improved 4- and 16.1 km TT performance
Lansley et al., (2011b)	9 recreationally active males	55 ± 7	6 days of NO_3^- rich BR supplementation (~5.1 mmol $NO_3^- \cdot day^{-1}$)	Running	SI (75% Δ) running bout and incremental single-legged, knee-extension exercise test to Tlim on days 4-6 of supplementation	↑ plasma $[NO_2^-]$	↑ Tlim during SI running and the incremental test.
Bescos et al., (2011)	11 male cyclists and triathletes	65 ± 6	Acute (3 h prior to exercise) ingestion of $NaNO_3$ (10 mg kg^{-1} $\cdot day^{-1}$)	Cycling	Incremental exercise test to Tlim	↑ plasma $[NO_2^-]$	↔ peak power output
Cermak et al., (2012)	13 trained male cyclists or triathletes	58 ± 2	6 days NO_3^- rich BR supplementation (8 mmol $NO_3^- \cdot day^{-1}$)	Cycling	10 km TT	↑ plasma $[NO_3^-]$	↑ performance
Cermak et al., (2012)	20 trained male cyclists	60 ± 1	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich BR (8.7 mmol NO_3^-).	Cycling	1 h TT	↑ plasma $[NO_2^-]$ ↑ plasma $[NO_2^-]$	↔ performance
Peacock et al., (2012)	10 highly trained skiers	70 ± 5	Acute (2.5 h prior to exercise) ingestion of KNO_3 (9.9 mmol NO_3^-)	Running	5 km TT	↑ plasma $[NO_2^-]$	↔ performance

Murphy et al., (2012)	5 male and 6 female subjects	Not available	Acute (75 min prior to exercise) ingestion of baked beetroot (~500 mg NO_3^-).	Running	5 km TT	Not measured (Note: BP was reduced following BR supplementation)	Trend for ↑ performance (P = 0.06)
Bescos et al., (2012)	13 trained male cyclists and triathletes	60 ± 7	3 days supplementation with $NaNO_3$ (10 mg ·kg^{-1}·day^{-1}).	Cycling	40 min TT	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↔ performance
Wilkerson et al., (2012)	8 well-trained male cyclists	63 ± 8	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich BR (~6.2 mmol NO_3^-)	Cycling	50 km TT	↑ plasma [NO_2^-]	↔ performance
Muggeridge et al., (2013)	8 trained male kayakers	49 ± 5	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich BR (4.2 mmol NO_3^-)	Kayaking	1 km TT	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↔ performance
Christensen et al., (2013)	10 elite male cyclists	72 ± 4	6 days NO_3^- rich BR supplementation (8 mmol NO_3^- ·day^{-1})	Cycling	400 kcal TT (post completion of a 120 min 'preload' exercise bout)	↑ plasma NOx (nitrate + nitrite)	↔ performance
Breese et al., (2013)	9 recreationally active males and females	Males: 48 ± 6 Females: 46 ± 9	6 days of NO_3^- rich BR supplementation (8 mmol NO_3^- ·day^{-1})	Cycling	SI (70% Δ) exercise bout (following transition from MI exercise bout) on day 6 of supplementation.	↑ plasma [NO_2^-]	↑ Tlim
Kelly et al., (2013)	9 active males	55 ± 8	7-12 days NO_3^- rich BR supplementation (8.2 mmol NO_3^- ·day^{-1}).	Cycling	SI exercise bouts at WRs corresponding to 60, 70, 80% Δ & 100% peak power output achieved during a ramp incremental test	↑ plasma [NO_2^-]	↑ Tlim at 60, 70, 80% Δ. ↔ Tlim at 100% peak power output
Kelly et al., (2014)	12 recreationally active males	58 ± 6	6 days of NO_3^- rich BR supplementation (8.4 mmol NO_3^- ·day^{-1})	Cycling	SI (70% Δ) exercise bout	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↔ Tlim
Muggeridge et al., (2014)	9 male trained cyclists	53 ± 4	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich gel (~8.1 mmol NO_3^-)	Cycling	16.1 km TT	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↔ performance
Thompson et al., (2014)	16 recreationally active males	47± 6	Acute (90 min before exercise) ingestion of NO_3^- rich BR (5 mmol NO_3^-).	Cycling	SI (90% $\dot{V}O_{2peak}$)	↑ plasma [NO_2^-]	↑ Tlim
Peeling et al., (2015)	Study consists of 2 sub-studies: (a) 6 national level male kayakers (b) 5 international female kayakers	Not available	(a) Acute (2.5 before exercise) ingestion of NO_3^- rich BR (4.8 mmol NO_3^-) (b) Acute (2.5 h before exercise) ingestion of NO_3^- rich BR (9.6 mmol NO_3^-).	Kayaking	(a) 4 min maximal laboratory kayak TT (b) 500 m on water kayak TT	↑ plasma [NO_2^-]	(a) ↔ performance (b) ↑ performance
Porcelli et al., (2014)	8 untrained males	Untrained: 28 - 44	6 days $NaNO_3$ supplementation (8.4 mmol NO_3^- ·day^{-1}).	Running	(a) Incremental exercise test to Tlim	All groups: ↑ plasma [NO_3^-]	Untrained: (a) ↔ performance

	7 moderately trained males 6 highly trained males	Moderately trained: 46-57 Highly trained: 64-82			b) 3 km TT	↑ plasma [NO_2^-] Note: the increase in plasma [NO_3^-] & [NO_2^-] was lower in the highly trained individuals.	(b) ↑ performance Moderately trained: (a) ↔ performance (b) ↑ performance Highly trained: (a) ↔ performance (b) ↑ performance
Sandbakk et al., (2015)	9 male elite cross-country skiers	69 ± 6	Acute (2.5 h prior to exercise) of KNO_3 (9.9 mmol NO_3^-)	Running	5 km TT	↑ plasma [NO_3^-]□ ↑ plasma [NO_2^-]	↔ performance
Boorsma et al., (2016)	8 male elite 1500 m runners	80 ± 5	Acute (2.5 h prior to exercise) ingestion of NO_3^- rich BR (19.5 mmol NO_3^-) 8 days NO_3^- rich BR supplementation (days 1 & 8 subjects consumed 19.5 mmol NO_3^-·day^{-1} and on day 2-7 subjects consumed 13 mmol NO_3^-·day^{-1})	Running	1500 m TT.	↑ plasma [NO_3^-]□ after acute ingestion and 8 days of supplementation	↔ performance at both measurement points.

↑, significant increase; ↓, significant reduction; ↔, no significant difference; $NaNO_3$, sodium nitrate; KNO_3, potassium nitrate; [NO_3^-]□, nitrate concentration; [NO_2^-], nitrite concentration; $\dot{V}O_2$, oxygen uptake; WRs, work rates; MI, moderate-intensity; SI, severe-intensity; TT, time trial; cGMP, cyclic guanosine monophosphate; GET, gas exchange threshold; Tlim, time until limit of tolerance; BR, beetroot juice; Δ, difference between power output at the gas exchange threshold and peak oxygen uptake; $\dot{V}O_{2peak}$, peak oxygen uptake

4 and 16.1 km cycling TT (Lansley et al. 2011a) and in recreationally-active participants during a 5 km running TT (Murphy et al. 2012). Furthermore, an improvement in exercise performance has been observed in trained male cyclists/triathletes during a 10 km TT following chronic NO_3^- supplementation (8 mmol NO_3^- daily for 6 days; Cermak et al. 2012a). However, several studies administering NO_3^- acutely (Wilkerson et al. 2012; Cermak et al. 2012b; Peacock et al. 2012; Bescós et al. 2012; Muggeridge et al. 2013; Muggeridge et al. 2014; Sandbakk et al. 2015) or chronically (Christensen et al. 2013; Boorsma et al. 2014) have not observed an improvement in performance during a cycling TT (Christensen et al. 2013; Cermak et al. 2012b; Wilkerson et al. 2012; Muggeridge et al. 2014), running TT (Sandbakk et al. 2015; Boorsma et al. 2014; Peacock et al. 2012) or a kayacking TT (Muggeridge et al. 2013), in trained (Muggeridge et al. 2014; Muggeridge et al. 2013) or highly-trained/elite (i.e. $\dot{V}_{O2peak} \geq 60$ ml.kg^{-1}.min^{-1}; Christensen et al. 2013; Sandbakk et al. 2015; Boorsma et al. 2014; Peacock et al. 2012; Wilkerson et al. 2012; Cermak et al. 2012b) endurance athletes. Although these result do not provide promising evidence for an ergogenic effect of dietary NO_3^- in elite athletes, a recent study by Peeling et al., (2015) has observed a 1.7% improvement during an on-water 500 m kayaking TT in National and International level kayak athletes following acute BR ingestion (~9.6 mmol NO_3^-). Furthermore, while previous studies have not reported an improvement in cycling TT performance at the group mean level in elite athletes after NO_3^- supplementation, individual 'responders' within the group have been identified (Christensen et al. 2013; Boorsma et al. 2014). Together, these results suggest that under certain circumstance, dietary NO_3^- can provide a worthwhile performance enhancement in this population.

It is likely that intra-study differences in the supplementation regime (acute vs. chronic and dose administered), exercise duration, exercise modality and participant populations (moderately trained vs. highly trained) contribute to the inconsistent influence of dietary NO_3^- on exercise performance/tolerance. Indeed, a growing body of literature now appears to demonstrate that dietary NO_3^- supplementation may be less effective at improving exercise efficiency (Bescós et al. 2011; Peacock et al. 2012; Boorsma et al. 2014; Sandbakk et al. 2015) and/or performance (Christensen et al. 2013; Sandbakk et al. 2015; Boorsma et al. 2014; Peacock et al. 2012; Wilkerson et al. 2012; Cermak et al. 2012b) in highly trained/elite athletes, particularly following acute

NO_3^- administration (see Jones 2014 and Jonvik et al. 2015 for discussion). The reason for this is unclear, but it may be related, in part, to the higher NOS activity (and therefore baseline plasma [NO_2^-]) reported in this participant population (Jungersten et al. 1997), which might reduce the potential increase in plasma [NO_2^-] from a standard NO_3^- dose and thus attenuate any accompanying benefits. In addition, these participants are likely to have greater skeletal muscle capillarisation (Jensen et al. 2004) which would potentially minimise areas of hypoxia, an environment where the reduction of NO_2^- to NO is enhanced (Castello et al. 2006). Finally, considering recent evidence suggesting the targeted effects of NO_3^- supplementation on type II muscle fibres (Hernández et al. 2012; Ferguson et al. 2013a; see *Mechanistic basis for improvement in exercise performance* for further details), the lower proportion of these fibres in endurance trained athletes (Tesch and Karlsson 1985) compared to their recreationally active counterparts may also reduce the potential benefits of NO_3^- supplementation. Therefore, when comparing the influence of dietary NO_3^- supplementation on exercise efficiency and/or tolerance/performance between studies, the participant population must be taken into consideration.

Mechanistic bases for improvement in exercise performance

Bailey et al., (2010) were the first to investigate the mechanistic bases for improvements in high-intensity exercise tolerance following NO_3^- supplementation. Specifically, the muscle metabolic (using ^{31}P-MRS) and pulmonary $\dot{V}_{O2}$ responses were measured during high-intensity knee extensor exercise following 4-6 days supplementation with BR. Together with a significant improvement in exercise tolerance (~25%), the primary [PCr] amplitude and the [PCr] slow-component amplitude were reduced, with no significant alteration in pH dynamics after NO_3^- supplementation. These changes were accompanied by a strong trend for reduced [ADP] and [Pi] accumulation. Consistent with the findings during low-intensity exercise, total ATP turnover was reduced as a result of reduced ATP turnover from PCr hydrolysis and oxidative phosphorylation. It is therefore likely that NO_3^- supplementation significantly reduced the ATP cost of muscle force production, facilitating a sparing of finite PCr stores. Accumulation of metabolites such as [ADP] and [Pi] and the rate of depletion of the finite intramuscular [PCr] reserve are known as important contributors to muscle fatigue development (Allen et al. 2008; Jones et al.

2008). Although the intramuscular [ADP], [Pi] and [PCr] were not altered at exhaustion following NO_3^- administration, the time taken to reach these critical concentrations was delayed. Therefore, improvements in exercise tolerance may be a result of an attenuated rate of decline in [PCr] and accumulation of [ADP] and [Pi], enabling high-intensity exercise to be sustained for longer before the attainment of critical values. In support, the $\dot{V}_{O2}$ slow-component amplitude was also reduced during high-intensity exercise, meaning that the attainment of $\dot{V}_{O2peak}$ was delayed and tolerable duration of exercise was extended.

An increase in mitochondrial biogenesis could also contribute to an enhancement in exercise tolerance. However, in a later study, Lansley et al (2011b) found that the maximal rate of mitochondrial ATP resynthesis, estimated from the maximal rate of PCr resynthesis, was not significantly altered after 4-6 days of NO_3^- supplementation, suggesting that mitochondrial biogenesis had not occurred. Consistent with this, Larsen et al., (2011) did not observe changes in markers of mitochondrial density or biogenesis following 3 days of NO_3^- supplementation. Together, these findings suggest that mitochondrial biogenesis does not contribute to an enhanced exercise tolerance with NO_3^- after up to 6 days of supplementation. However, this does not exclude the possibility that a longer supplementation period could stimulate mitochondrial biogenesis.

Recent studies suggest that NO_3^- supplementation may result in targeted benefits for type II ('fast-twitch') muscle fibres (Hernández et al. 2012; Ferguson et al. 2013a). Although there is significant heterogeneity between and within muscle fibre types, when compared to type I ('slow-twitch) fibres, type II fibres tend to differ in terms of calcium handling, and normally have lower mitochondrial and capillary density (Bottinelli and Reggiani 2000). Together, these factors result in type II fibres having a lower microvascular PO_2 during rest and contraction (Behnke et al. 2003; McDonough et al. 2005; Ferreira et al. 2006), a relatively greater reliance on anaerobic pathways for ATP production and a greater susceptibility to fatigue, when compared to type I fibres. Unsurprisingly, these type II fibres are recruited heavily during high-intensity exercise (Krustrup et al. 2004; Krustrup et al. 2009) and thus the preferential effects of NO_3^- supplementation in these fibres might account for some of the ergogenic effects observed at this intensity of exercise.

Hernandez et al., (2012) excised fast-twitch skeletal muscles (*m. extensor digitorum longus*, EDL) and slow-twitch muscles (*m. soleus*) from mice that had consumed NO_3^- in drinking water or drinking water without NO_3^- for 7 days. The EDL, but not the soleus muscle, manifest increased contractile force at low stimulation frequencies (those used most often for normal movement) in the mice supplemented with NO_3^-. This result was accompanied by increased expression of the Ca^{2+} handling proteins calsequestrin 1 (CASQ1) and dihydropyridine receptor (DHPR) only in the EDL muscle. Subsequent analysis of isolated fast-twitch *m. flexor digitorum brevis* (FDB) single fibres then revealed that NO_3^- supplementation increased tetanic $[Ca^{2+}]_i$ and force production. It therefore appears that NO_3^- supplementation improves contractile force by enhancing SR Ca^{2+} release via modifications to sarcoplasmic reticulum (SR) Ca^{2+} handling in fast-twitch but not slow-twitch muscles. Interestingly, when applied to an *in vivo* model, these data suggest that following NO_3^- supplementation fast-twitch muscle can be activated at a lower frequency to achieve the same force output, and that, for a given force output, the number of motor units needed to be recruited will be reduced. These data are consistent with the reduction in ATP cost of force production reported by Bailey et al., (2010) in low- and high-intensity exercise discussed above. It is important to note that the improvements in skeletal muscle Ca^{2+} handling reported by Hernandez et al., (2012) are only likely to be activated following chronic NO3- supplementation due to the time required for protein expression changes to be manifest. However, it is possible that NO and/or NO_2^- may improve skeletal muscle Ca^{2+} handling by acutely and reversibly altering SR protein function through post-translational modifications (Reid 1998).

Changes in blood flow may also contribute to enhanced high-intensity exercise performance following NO_3^- supplementation. Acute infusion of NO_2^- has been shown to increase muscle blood flow (BF) in humans completing forearm exercise (Gladwin et al. 2000). Recently, Ferguson et al., (2013a) examined the effect of 5 days BR supplementation on total, and inter- and intra-muscular hindlimb BF and vascular conductance (VC) at rest and during submaximal exercise in rats. Together with a reduction in MAP, BR supplementation resulted in higher total hindlimb muscle BF and VC during exercise, with targeted increases in the muscles and muscle parts comprising principally type II muscle fibres. Interestingly, NO has also been shown to

modulate the distribution of tissue and intracellular O_2 via quenching the metabolic activity of fibres in close proximity of the capillaries and increasing oxygenation of the fibres further away by elevating the O_2 gradient (Thomas et al. 2001). Both an increase in BF, and therefore presumably O_2 delivery, and improved distribution of this O_2, specifically in muscle compartments comprising type II muscle fibres, may improve exercise performance by, for example, increasing the relative contribution of aerobic ATP resynthesis and reducing metabolic perturbation (Bailey et al. 2010; Breese et al. 2013).

In summary, the improved exercise tolerance after NO_3^- supplementation might be linked to a blunted muscle metabolic perturbation, or improved skeletal muscle blood flow distribution and/or skeletal muscle Ca^{2+} handling, particularly in type II muscle fibres.

Dose optimisation for dietary NO_3^- supplementation

From the above review of literature, it is clear that both acute and chronic dietary NO_3^- supplementation has the potential to lower the O_2 cost of submaximal exercise and improve exercise performance, at least in recreationally-active participants. Unsurprisingly, these observations have led to the widespread use of dietary NO_3^-, particularly in the form of BR, as an aid to enhance exercise performance, and an exponential increase in researchers assessing the effects of dietary NO_3^- on a variety of exercise parameters. However, there are still unanswered questions regarding the NO_3^- intake regime that may optimise these effects, which might explain some of the inconsistencies between studies. Therefore additional research is required to improve supplementation guidelines for future NO_3^-supplementation studies.

To date, studies assessing the acute effects of dietary NO_3^-/BR on the O_2 cost of submaximal exercise and performance have administered 5-10 mmol NO_3^-/BR 1-3 h prior to assessment (Vanhatalo et al. 2010; Lansley et al. 2011a; Cermak et al. 2012b). Therefore, it is currently unclear if a dose-response relationship between NO_3^- intake and these measures exist. Resolving the dose-response relationship between NO_3^-/BR dose and exercise economy/performance responses will provide valuable information to inform the supplementation guidelines in both a research and applied sports setting.

These data may also explain why a proportion of studies administering NO_3^- acutely have observed no significant effect on exercise economy and performance (e.g. Cermak et al. 2012b). It is important to note here, that for the dose-response relationship between BR intake and exercise parameters to be determined accurately, the kinetic response of plasma [NO_2^-] following different doses of dietary BR must be considered. These data will allow for the timing of acute supplementation to be optimised for the peak elevation of plasma [NO_2^-] (see *Plasma [NO_3^-] and [NO_2^-]* for further details).

As highlighted in the review above, chronic (>3 days) NO_3^- supplementation has been shown to alter the expression of key mitochondrial and skeletal muscle Ca^{2+} handling proteins. These structural adaptations may be responsible, in part, for the reduction in submaximal exercise $\dot{V}_{O2}$ and improvements in exercise performance following chronic NO_3^- supplementation (Larsen et al. 2011; Hernández et al. 2012). However, these modifications to skeletal muscle would unlikely be manifest within 1-3 h following NO_3^- ingestion, and therefore cannot account for the same effects observed after acute NO_3^- ingestion. Given this potential benefit of a chronic NO_3^- supplementation period, it may be reasoned that the NO_3^- dose required to elicit a beneficial effect when a chronic supplementation regime is adopted is lower than that required to elicit an effect after ingestion of a single acute bolus. Examining if a period of chronic NO_3^- supplementation alters the acute dose-response relationship of NO_3^- supplementation will therefore contribute further to understanding the optimal NO_3^- supplementation strategy.

It is also important to fully understand the different independent and combined effects afforded by a chronic supplementation strategy compared to those evoked by an acute ingestion strategy. However, while several studies have assessed the influence of chronic NO_3^- supplementation on the physiological response to exercise, in all of these studies an acute bolus of dietary NO_3^- has been administered 1-3 h prior to the physiological assessment to ensure that plasma [NO_2^-] was significantly elevated (e.g. Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011b). Therefore, these studies are in fact assessing the combined effects of chronic NO_3^- loading and acute NO_3^- ingestion, not chronic NO_3^- supplementation alone. This leaves the possibility that: 1) an acute bolus of dietary NO_3^- and the accompanying rise in plasma [NO_2^-] is not required for lowering submaximal exercise $\dot{V}_{O2}$ after a period of

chronic supplementation; 2) the acute ingestion of NO_3^- may augment the effects of chronic supplementation alone; and 3) the effects of acute NO_3^- ingestion may account for some (or all) of the effects observed after chronic NO_3^- supplementation. Assessment of the chronic effects of dietary NO_3^- exposure without the acute ingestion of dietary NO_3^- is therefore warranted to improve understanding of the potential mechanisms by which NO_3^- supplementation might be ergogenic. The information would provide important practical advice to guide the optimal supplementation strategy before competition after a period of chronic NO_3^- supplementation.

Potential effects of dietary NO_3^- supplementation on intermittent exercise performance

The literature reviewed above provides encouraging evidence that, at least in moderately-trained individuals, NO_3^- supplementation has the potential to improve continuous endurance exercise performance (Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011a; Cermak et al. 2012a). However, it is less clear whether NO_3^- supplementation can improve performance during high-intensity intermittent exercise, which is a hallmark of some of the world's most popular sports, such as association football, basketball and rugby union/league. Typically, intermittent exercise involves repeated bouts of high-intensity (near maximal) exercise interspersed with periods of recovery. The requirement to repeatedly transition from a low to high metabolic rate requires a substantial contribution from both the anaerobic and aerobic energy pathways (Gaitanos et al. 1993; Bogdanis et al. 1995; Casey et al. 1996; Bangsbo et al. 2008).

During a single brief period of high intensity exercise, the rapid requirement for ATP is predominantly met via anaerobic metabolic systems, specifically PCr hydrolysis and glycolysis (Cheetham et al. 1986; Gaitanos et al. 1993; Bogdanis et al. 1995; Casey et al. 1996). For example, during a single short (~6 s) all-out sprint, PCr hydrolysis and glycolysis accounts for approximately 50% and 44% of total ATP provision respectively, with the remaining ~6% provided by the muscle's small store of ATP (Gaitanos et al. 1993). As a consequence of the rapid anaerobic metabolism of PCr and glycogen, lactate, hydrogen (H^+) ions and Pi accumulate and pH, PCr and glycogen are reduced, whilst ionic balances are also disturbed (see Glaister 2005 for review). In

intermittent exercise, the following recovery period provides time to restore metabolic homeostasis to resting conditions, but if the duration of recovery is not sufficient to achieve this, subsequent performance may be compromised (e.g. Gaitanos et al. 1993; Casey et al. 1996). Therefore, any intervention that can improve metabolic responses during the 'work' periods and/or aid metabolic recovery during the 'recovery' periods will likely benefit intermittent exercise performance.

Fatigue during intermittent exercise

Fatigue during repeated bouts of high-intensity exercise has been associated, in part, with the incomplete recovery of muscle PCr levels during the recovery period (Gaitanos et al. 1993; Bogdanis et al. 1995; Trump et al. 1996). Indeed, a reduced level of muscle PCr limits ATP provision from PCr hydrolysis and increases the relative contribution of ATP provision from oxidative phosphorylation in subsequent bouts of exercise (Bogdanis et al. 1995). As a consequence, ATP turnover is attenuated and force production (or power output) declines with repeated bouts of exercise (Gaitanos et al. 1993; Bogdanis et al. 1995). Along with a progressive increase in the contribution of aerobic metabolism to ATP resynthesis during repeated bouts of high-intensity exercise, aerobic metabolism is also essential for PCr resysnthesis during recovery (Harris et al. 1976; Haseler et al. 1999). This was neatly demonstrated in 1976, by Harris and colleagues, who reported that PCr resynthesis during recovery from intense single knee-extension exercise was completely abolished when the circulation (and therefore O_2 delivery) to the quadriceps was occluded (Harris et al., 1976). In line with this, it has since been shown that increasing muscle O_2 delivery via the inhalation of hyperoxic gas can enhance PCr resyntheisis (Haseler et al. 1999).

Together with a reduction in PCr hydrolysis, fatigue during repeated bouts of high-intensity exercise has also been associated with the reduced energy contribution from anaerobic glycolysis (Gaitanos et al. 1993; Bogdanis et al. 1995; Parolin et al. 1999), potentially as a result of the accumulation of H^+ ions and/or cytosolic citrate which may inhibit key regulatory enzymes of glycolysis (Parmeggiani and Bowman 1963; Taylor and Halperin 1973; Boscá et al. 1985). Indeed, Gaitanos et al., (1993) suggested that acidosis was responsible for the reduction in glycogen breakdown and glycolytic rate as sprint number increased during 10 x 6-s all-out sprints. Furthermore, Bishop et al.,

(2003) has reported a significant correlation between performance decrement in repeated sprints and the reduction in blood pH. The depletion of muscle glycogen also has the potential to result in glycolytic inhibition, but it has been suggested that glycogen would not fall sufficiently to cause fatigue during short-duration intermittent exercise, as long as pre-exercise intramuscular glycogen stores are within the normal range 300 mmol/kg dm, as measured in muscle homogenate (Gaitanos et al. 1993; Hultman et al., 1990). However, it is possible that during prolonged periods of intermittent exercise, where aerobic metabolism of glycogen is increased, glycogen availability may contribute to the attenuated rate of glycolysis and fatigue (Saltin 1973; Krustrup et al. 2006; Karlsson et al., 1969). Furthermore, it should be acknowledged that localised depletion of glycogen in areas of high ATP demand (e.g. SERCA and actomyosin ATPase; Nielsen et al. 2011) may contribute to fatigue development, even when glycogen concentration in muscle homogenate may appear to be sufficient.

The accumulation of Pi and changes in ionic balances may also negatively impact performance during high-intensity intermittent exercise. For instance, intracellular Pi accumulation has been shown to inhibit muscle excitation contraction coupling directly, by attenuating Ca^{+} release from the SR (Fryer et al. 1995; Kabbara and Allen 1999; Dahlstedt et al. 2000; Dahlstedt and Westerblad 2001; Allen et al. 2002). On the other hand, the accumulation of potassium (K^{+}) in the muscle interstitum during contraction has been proposed to cause fatigue during intense exercise in humans (Fitts 1994; Fitts and Balog 1996; Juel et al. 2000; Sejersted and Sjøgaard 2000) by impairing cell membrane excitability (Ruff et al. 1988).

Muscle fibre type recruitment

During intense exercise, it is well documented that type II skeletal muscle fibres contribute heavily to skeletal muscle force production (Greenhaff et al. 1994; Casey et al. 1996). It is also known that the progressive loss of force/power output with repeated bouts of exercise is associated with fatigue in these fibres (Soderlund et al. 1992; Greenhaff et al. 1994; Casey et al. 1996). For example, it has been shown that during a single all-out bout of exercise, PCr and glycogen are depleted to a greater extent in type II fibres compared to type I (Greenhaff et al. 1994; Casey et al. 1996), and that the incomplete resynthesis of PCr in these fibres during recovery is associated with reduced

performance during a subsequent bout of exercise (Casey et al. 1996). Furthermore, given their expected recruitment during high-intensity intermittent exercise, metabolic byproducts such as Pi and H^+ are likely to accumulate predominantly in type II fibres (Greenhaff et al. 1994; Casey et al. 1996).

Nitrate supplementation and high-intensity intermittent exercise

The fact that type II muscle fibres are recruited heavily during high-intensity intermittent exercise, and that NO_3^- supplementation has recently been found to have a preferential effect on these fibres, suggests that dietary NO_3^- may be an effective ergogenic aid for individuals participating in this type of exercise. Indeed, as discussed earlier (see *Mechanistic bases for improvement in exercise performance*), NO_3^- supplementation has been found to improve perfusion of type II muscle (Ferguson et al. 2013a) and contractile function in type II muscle fibres (Hernández et al. 2012). Both of these have the potential to improve high-intensity intermittent exercise performance. For example, an improvement in skeletal muscle oxygenation (through an improvement in perfusion) specifically in type II muscle portions has the potential to improve performance by: 1) increasing the proportional contribution of aerobic metabolism to ATP resynthesis (Bangsbo et al. 2001) and thus blunt the rate of PCr decline and accumulation of metabolic byproducts (i.e. H^+, Pi, ADP); and 2) improve recovery of type II muscle fibres by improving muscle oxygenation during recovery periods (Paganini et al. 1997; Vanhatalo et al. 2011). It is also important to highlight that the proportionally high O_2 demand relative to O_2 delivery during this type of exercise is likely to result in a hypoxic and acidic environment, particularly in type II fibres, and, as discussed previously, this is an environment which accelerates the reduction from NO_2^- to NO (Castello et al. 2006; Modin et al. 2001; see section *NO synthesis from NO_2^-* for more information).

Together, these observations provide a strong rationale for the efficacy of dietary NO_3^- supplementation to improve high-intensity intermittent exercise performance. While little was known about the effects of dietary NO_3^- on intermittent exercise performance at the time this thesis was conceived, Bond et al (2012) first observed a higher mean power output during 6 x 500 m rowing ergometer repetitions with 90 s recovery following NO_3^- supplementation. Although promising, the intensity and duration of

these bouts of exercise, along with the duration of recovery, was not applicable to team sport game play. Therefore, more research was required to determine whether NO_3^- supplementation could be ergogenic in team sport specific intermittent exercise.

Intermittent exercise during team sports

The majority of team sports (e.g. association football, field hockey, rugby union/league) require participants to repeatedly produce high intensity efforts over a prolonged period of time (1-2 h) (Bishop et al. 2003; Bishop and Edge 2005), requiring a substantial energy contribution from both the anaerobic and aerobic energy pathways. In these sports, the mean duration of high-intensity efforts has been reported to range from 1-7 s (Mayhew and Wenger 1985; Docherty et al. 1988; Bangsbo et al. 1991; Withers et al. 1991). Whilst many of these efforts are separated by relatively long rest intervals (>1 min), a series of repeated bouts interspersed with short recovery periods (< 30 s) are often required (Spencer et al. 2005). Unsurprisingly, the ability of players to repeatedly perform these intense periods of exercise is associated with performance in these sports. For example, the standard of a soccer player has been positively correlated with the amount of high-intensity running performed throughout a game (Ekblom 1986; Bangsbo et al. 1991) (Mohr & Bangsbo 2001). A variety of laboratory/field tests have been designed in an attempt to reproduce the physiological and metabolic demands of these intermittent sports, including the Yo-Yo Intermittent Recovery Test (Bangsbo et al. 2008).

Yo-Yo Intermittent Recovery Test

The Yo-Yo Intermittent Recovery Test Level 1 (IR1) was specifically designed to mimic the high-intensity bouts typical of team sport gameplay (Bangsbo et al. 2008) and to assess fatigue resistance during intermittent exercise taxing both the aerobic and anaerobic energy systems (Krustrup et al. 2003). The test requires participants to complete repeated 2 x 20 m shuttle runs interspersed with a 10-s period of active recovery, at increasing speeds controlled by audio signals from an audio player (Bangsbo et al. 2008; Figure 2.4). Participants are required to run until they are not able to maintain the required speed, and the distance covered at that point is the test result (Bangsbo et al. 2008). Assessments of the physiological response to the Yo-Yo IR1 test confirm that both the aerobic and anaerobic energy systems are heavily utilised during

the test (Bangsbo et al. 2008). Indeed, the heart rate (HR) at the end of the test has been reported to be ~99% of the peak HR achieved during a treadmill test in which participants reached their maximum $\dot{V}_{O2}$ ($\dot{V}_{O2max}$; Krustrup et al. 2003). Furthermore, exhaustion during the Yo-Yo IR1 test is associated with a significant reduction in muscle [PCr], [glycogen] and pH, as well as a significant increase in muscle [lactate] (Krustrup et al. 2003), which are all consistent with a significant contribution of anaerobic metabolism to ATP turnover. It has also been reported that, at exhaustion, glycogen depletion is greater in type II fibres compared to type I, demonstrating the significant activation of these fibres during the test (Krustrup et al. 2003), as would be expected in intermittent exercise (see above).

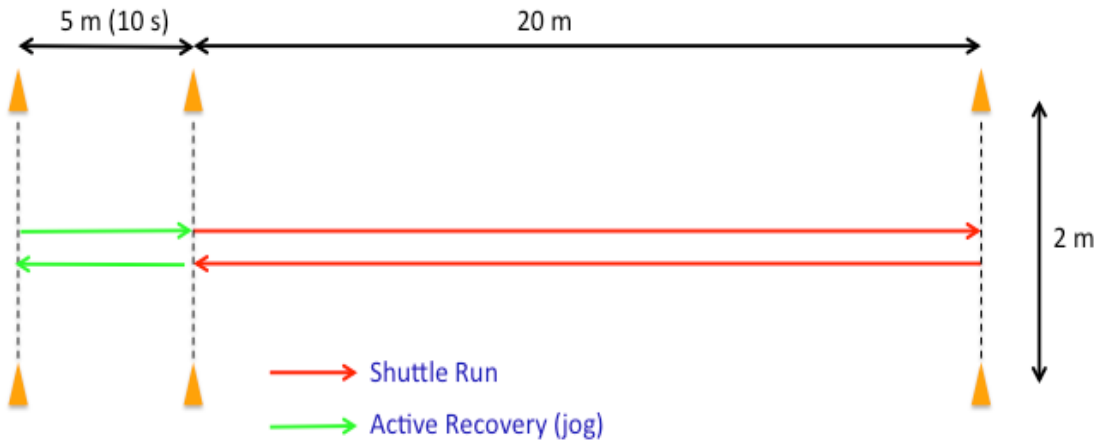

Figure 2.4. Schematic diagram of the Yo-Yo Intermittent Recovery Test.

Importantly, the Yo-Yo IR1 test has been shown to have a high level of sensitivity and ecological validity (see Bangsbo et al. 2008 for review). Performance during the test has been closely correlated with the high-intensity running during soccer games (Krustrup et al. 2003) and has been shown to discriminate between players of different ability levels in different team sports, including; rugby league (Atkins 2006), association football (Bangsbo et al. 2008), Australian football (Veale et al. 2010), and basketball (Vernillo et al. 2012). Furthermore, it is clear that the test is sensitive to training interventions (Iaia et al., 2008; Krustrup and Bangsbo 2001; Mohr et al. 2007). For example, Krustrup and Bangsbo (2001) observed a 31% improvement in performance during the IR1 test after 12-weeks of training, which was associated with 23% more high-intensity work during a game as well as a significant reduction in the fall in high-intensity exercise towards the end of the game in soccer referees (Krustrup

and Bangsbo 2001). Together, these observations support the notion that an improvement in the Yo-Yo IR1 test would positively impact performance during invasion games.

Given its physiological demands, sensitivity and ecological validity, the Yo-Yo IR1 test will serve as a valid test to examine the influence of dietary NO_3^- supplementation on team-sport specific high-intensity exercise. In carrying out this assessment, important information on the potential of dietary NO_3^- to be an ergogenic aid for team sport athletes would be assessed.

Variations of intermittent exercise

Intermittent exercise can vary in work and rest intensities, work and rest durations, work-to-rest ratio, and number of work intervals. An alteration in any or a combination of these variables inevitably has an impact on the physiological demands of intermittent exercise. For instance, extending the duration of all-out exercise or reducing the intensity of exercise would be expected to increase the relative contribution of aerobic ATP provision, and, in turn, reduce the relative contribution from the faster anaerobic energy systems (Withers et al. 1991; Gaitanos et al. 1993; Casey et al. 1996; Esbjörnsson-Liljedahl et al. 1999). In line with this, the relative contribution of type II muscle fibres to force production will also be lowered (Casey et al. 1996; Esbjörnsson-Liljedahl et al. 1999; Karatzaferi et al. 2001; Soderlund et al., 1992). Extending the recovery period can also have a significant impact on subsequent exercise performance by allowing more time for the metabolic homeostasis to return (Nevill et al. 1997; McMahon and Jenkins 2012). For example, following a 6 s sprint, PCr was restored to 55% of pre-sprint levels by 10 s, and to 90% by 3 min, post exercise (Dawson et al. 1997).

As discussed above, team sports generally involve repeated bouts of short duration (1-6 s) high-intensity or all-out sprint exercise interspersed with brief (< 30 s) periods of recovery (Mohr et al. 2003; Spencer et al. 2004; King et al. 2009). However, in contrast, the use of intermittent exercise with longer periods of exercise and recovery are regularly used by athletes during training. For example, the so-called Burgomaster protocol (Burgomaster et al. 2006) involves repeated 30 s all-out cycling sprints

interspersed with 4 min of recovery whereas a classic high-intensity interval training session may consist of repeated bouts of prolonged high-intensity exercise interspersed with an equal duration of rest (i.e. 60 s exercise and 60 s rest; Weston et al. 2014). Given the between-protocol variation in physiological demand, a study examining the effects of NO_3^- supplementation on different intermittent protocols is warranted. In order to accurately compare effects between protocols, the same participant population, exercise modality and NO_3^- supplementation procedures should be used to limit potential for these factors to influence the effects of NO_3^- supplementation. The findings from such a study would provide important information to ascertain the efficacy of dietary NO_3^- to improve performance in a range of different intermittent exercise protocols, thereby enhancing guidance for the use of dietary NO_3^- as an ergogenic aid during intermittent exercise.

Summary

Both chronic and acute dietary NO_3^- supplementation have the potential to significantly lower the O_2 cost of submaximal exercise and improve exercise performance during continuous endurance exercise. However, the optimal supplementation strategy to elicit these effects is currently unknown. Elucidating the optimal supplementation regime would require: 1) determining the optimal acute dose of NO_3^- for beneficial effects; 2) understanding how the duration of supplementation alters the NO_3^- dose required to elicit an effect; and, 3) elucidating the independent effects of acute NO_3^- ingestion and chronic NO_3^- supplementation. Moreover, while the influence of dietary NO_3^- supplementation on continuous endurance exercise has been widely investigated, understanding of the influence of NO_3^- supplementation on intermittent exercise performance is comparatively poor. It is therefore necessary to elucidate the efficacy of dietary NO_3^- supplementation in enhancing performance during this type of exercise.

Aims

The aims of this project were twofold. Firstly, to understand the optimal supplementation strategies for dietary NO_3^- to improve physiological responses during exercise and exercise performance, with a particular focus on dose-response relationships, and, secondly, to provide a thorough examination of the potential for NO_3^- supplementation to improve performance during intermittent exercise.

The following research questions will be addressed:

1) What are the pharmacokinetic responses of plasma [NO_3^-] and [NO_2^-] following different doses of BR and do the acute physiological and performance effects of BR/NO_3^- intake improve dose-dependently?
    - What is the influence of acute NO_3^- doses of 4.2, 8.4 and 16.8 mmol, consumed as 70, 140 and 280 mL concentrated BR, on plasma [NO_3^-] and [NO_2^-] and BP over a 24 h period?
    - What are the physiological responses to moderate- and severe-intensity exercise, 2.5 h postingestion of the same NO_3^- doses?

2) How does the duration of supplementation influence the dose-response relationship of NO_3^- supplementation on exercise economy, and what are the effects of chronic supplementation without the acute ingestion NO_3^- pre-exercise?
    - What are the effects of acute (2 h), 7 d and 30 d supplementation with a low dose (3 mmol) and standard dose (6 mmol) of dietary NO_3^- on the O_2 cost of moderate intensity exercise?
    - What is the effect of chronic (~30 d) NO_3^- supplementation on the O_2 cost of submaximal exercise without the intake of dietary NO_3^- 2-3 h prior to testing?

3) What are the physiological and performance effects of dietary NO_3^- supplementation on exhaustive team-specific intermittent exercise?
    - Can dietary NO_3^- supplementation increase the distance covered in the Yo-Yo IR1 test?
    - What is the kinetic response of plasma [NO_3^-] and [NO_2^-] during intermittent exercise?

4) Does dietary NO_3^- supplementation improve performance during a variety of intermittent exercise tests consisting of different work-to-rest ratios?

- Can dietary NO_3^- supplementation improve performance during: twenty four 6-s all-out sprints interspersed with 24 s recovery; seven 30-s all-out sprints interspersed with 4 min of recovery; and, six 60-s self-paced maximal efforts interspersed with 60 s of recovery?

Hypotheses

This thesis will test the following hypotheses:

1) The effects of acute dietary inorganic NO_3^- ingestion on plasma [NO_3^-] and [NO_2^-], BP, the O_2 cost of moderate-intensity exercise, and exercise tolerance (assessed as the time-to-task failure) during severe-intensity exercise will be improved dose dependent.

2) Supplementation with 3 mmol NO_3^- and 6 mmol NO_3^- will result in improved exercise efficiency at 7 d and 28-30 d when an acute bolus of NO_3^- is consumed prior to assessment. Secondly, any effects observed will still be present, albeit to a lesser extent, when an acute bolus of NO_3^- is not consumed prior to the assessment.

3) Dietary NO_3^- supplementation will increase the distance covered in the Yo-Yo IR1 test. Plasma [NO_2^-] will fall as exercise intensity increases and to a greater extent after NO_3^- ingestion.

4) NO_3^- supplementation will improve performance in all three intermittent exercise tests and performance would be enhanced to the greatest extent when maximal-intensity intermittent exercise was accompanied by the shortest recovery duration (i.e. in the 24 x 6-s test).

Chapter 3: General Methods

The four experimental Chapters (Chapters 4-7) that comprise this thesis required 396 exercise tests to be conducted. A further 40 laboratory visits were conducted for the assessment of plasma [NO_3^-] and [NO_2^-] kinetics in Chapter 4. All of the tests in Chapters 4, 5 and 7 were conducted in an air conditioned exercise physiology laboratory at sea level with an ambient temperature of 18-22ºC. Exercise tests in Chapter 5 were conducted in a sports hall on a wooden surface. Prior to the commencement of data collection, the University of Exeter Ethics Committee approved all experimental procedures employed in each of these experimental Chapters.

Health and Safety

During all experimental testing, health and safety guidelines established by the School of Sport and Health Sciences at the University of Exeter were closely followed. Great care was taken to ensure that the laboratory and sports hall provided a clean and safe environment for the assessment of human participants. This included the regular cleaning of trolleys, ergometers, work surfaces and floors using dilute Virkon disinfectant. All respiratory apparatus was disinfected according to manufacturers' recommendations. Experimenters wore disposable nitrile gloves during blood sampling and analysis. All biohazard material and sharps were disposed appropriately for later incineration.

Participants

Volunteers for all experiments were recruited from the student and staff University population. All participants were non-smokers, free from disease and were not using dietary supplements at the time of data collection. Participants in Chapters 4 and 5 were recreationally active, and participated in regular structured exercise and/or competitive sport. Participants in Chapters 6 and 7 were recreational team sport players, competing in either Associate Football or Rugby Union/League games on a regular basis. No participants in any experimental Chapters were elite-level athletes. Participants in Chapter 4 were instructed to arrive at the laboratory in a well-hydrated state, following an overnight fast. All other participants were instructed to arrive at the laboratory (Chapters 5 and 7) or sports hall (Chapter 6) well hydrated, at least 3 hours postprandial in a rested state. All participants were also asked to avoid strenuous exercise and

alcohol for 24 h, and caffeine for 6 hours, preceding each exercise test. Participants in all experimental Chapters were also asked not to use antibacterial mouthwash for the duration of the experiment. For each participant, all tests were conducted at the same time of day (±2 h).

Informed consent

Prior to any experimentation, participants were given a study information sheet that provided a comprehensive description of the experimental procedures and what they would be required to do. All potential risks and benefits of participation were also explained in this information sheet, and participants were informed that, while their anonymity would be preserved and their data stored safely, data of the participants may be published in academic journals or presented at national/international conferences. It was stressed to participants that they had the right to withdraw from the investigation at any time with no disadvantage to themselves. When the participants were happy that they understood the requirements of the study, participants gave written informed consent to participate.

Supplementation

In Chapters 4, 6 and 7, inorganic NO_3^- was administered in the form of concentrated beetroot juice (Beet it, James White Drinks, Ipswich, UK). In each of these experimental Chapters, participants were instructed to consume either NO_3^--rich beetroot juice (BR) or NO_3^--depleted beetroot juice (PL), in a double-blind randomised, crossover study design (with exception of the pharmacokinetic and pharmacodynamics experiment in Chapter 4; see details below). A washout period of at least 72 hours separated each supplementation period. The NO_3^--depleted placebo beverage was created by passage of the juice through a column containing an ion-exchange resin that selectively removes NO_3^- ions while leaving the juice identical in appearance, taste, smell and texture (Lansley et al. 2011b). A brief description of the supplementation regime for each experimental Chapter is provided below.

In Chapter 4, volunteers for the pharmacokinetics and pharmacodynamics experiments consumed a single bolus of 70, 140 or 280 mL NO_3^--rich BR (containing ~4.2, ~8.4, or ~16.8 mmol NO_3^-, respectively) or 140 mL water (control). Volunteers for the dose-

response exercise testing experiments in Chapter 4 consumed a single bolus of either 70, 140, or 280 mL NO_3^--rich BR (containing ~4.2, ~8.4, or ~16.8 mmol NO_3^-, respectively) or 70, 140, or 280 mL NO_3^--depleted PL (containing ~0.04, ~0.08, or 0.12 mmol NO_3^-) 2.5 h prior to exercise testing.

In Chapter 6, participants were asked to consume 2 x 70 mL of NO_3^--rich BR (containing ~4.1 mmol NO_3^- per 70 mL) or NO_3^--depleted PL (containing ~0.04 mmol NO_3^- per 70 mL) in the morning and 2 x 70 mL in the evening on the day before each experimental trial. On each experimental day, participants consumed an additional 2 x 70 mL 2.5 h prior, and 1 x 70 mL 1.5 h prior, to the onset of testing procedures.

In Chapter 7, participants were asked to consume 2 x 70 mL NO_3^--rich BR (containing ~4.1 mmol NO_3^- per 70 mL) or NO_3^--depleted PL (containing ~0.04 mmol NO_3^- per 70 mL) on days 1 and 2 of supplementation (non-experimental days). On experimental days (days 3, 4 and 5 of supplementation), participants consumed 2 x 70 mL prior to the onset of testing procedures. On experimental days 1 and 2 for BR and PL (days 3 and 4 of supplementation), participants consumed a further 1 x 70 mL dose 3 h post-completion of testing procedures.

In Chapter 5, inorganic NO_3^- was administered in the form of dry beetroot extract diluted in water (PepsiCo, USA). Participants were assigned in a group-matched fashion to receive either 3 mmol NO_3^- (9.75 g of dry beetroot extract diluted in 50 mL of water), 6 mmol NO_3^- (19.5 g of dry beetroot extract diluted in 50 mL of water), or a placebo (containing 0.001 mmol NO_3^-, administered as 9.5 g of sucrose and red shade colouring diluted in 50 ml water [PepsiCo, USA]) per day, for 30 days. A novel supplementation procedure was adopted on days 28-30 of this supplementation period in order to assess the influence of chronic NO_3^- supplementation with and without the acute consumption of NO_3^- prior to exercise testing. Further details of this procedure are provided in the methods section of Chapter 5.

In all experimental Chapters, participants were asked to replicate their diet in the 24 h preceding each laboratory visit. Participants were warned that supplementation might cause a harmless side effect of temporary beeturia (red urine) and red stools. In all

experimental Chapters the supplementation regime was tolerated well by all participants.

Measurement procedures

Descriptive data

Prior to any testing, participants' height and mass were measured and recorded together with the participants' age.

Heart rate

During Chapters 4 and 6, heart rate (HR) was recorded every 5 s throughout all exercise tests using short-range telemetry (Polar RS400, Polar Electro Oy, Kempele, Finland).

Blood pressure

In Chapter 4, blood pressure of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro; GE Medical Systems, Tampa, FL), with the participants in a seated position. Following 10 min of rest in an isolated room, four measurements were recorded, and the mean of the final three measurements was used for data analysis.

The reliability of measuring systolic blood pressure was determined by repeat assessments on two separate days in 10 participants. On each visit, subjects were instructed to arrive in a fasted state and then asked to remain seated in an isolated room for 10 min before four blood pressure measurements were made. The inter-study variation was calculated by comparing the average of the final 3 systolic blood pressure measurements recorded on each day. The coefficient of variation (CV) for inter-study variation was 1.7% at an overall systolic blood pressure of 118 mmHg.

Plasma NO_3^- and NO_2^- concentration

In Chapters 4, 5 and 7, venous blood samples were obtained from the antecubital fossa by venepuncture, while in Chapter 6 venous blood samples were obtained from an antecubital vein through an intravenous cannula (see *Blood lactate and glucose, and plasma potassium and sodium concentration* for more details). These blood samples

were collected into 7.5 ml lithium-heparin tubes (Vacutainer, Becton-Dickinson, New Jersey, USA) and centrifuged at 4,000 rpm and 4ºC for 5-8 min, within 3 min of collection. Plasma was extracted and immediately frozen at -80ºC. In Chapters 4, 5 and 6 these samples were later analysed for plasma [NO_3^-] and [NO_2^-] whereas samples collected in Chapter 7 were used for the determination of plasma [NO_2^-] only.

Plasma NO_2^- and NO_3^- were analysed via gas-phase chemiluminescence. This technique is dependent on the reduction of NO_2^- and NO_3^- to NO gas. To reduce NO_2^-, neat plasma was injected into a gas sealed purge vessel containing 5 mL glacial acetic acid and 1 mL sodium iodide (NaI; 4% w/v) at 35ºC. Before the determination of plasma [NO_3^-], neat plasma was first deproteinized using zinc sulfate ($ZnSO_4$)/sodium hydroxide (NaOH). In short, 200 µL plasma, 400 µL $ZnSO_4$ (10% w/v) and 400 µL 0.5 M NaOH were added together and vortexed for 30 s before being left to stand at room temperature for 15 min. Thereafter, samples were centrifuged at 4,000 rpm for 5 min, and the supernatant was removed for the analysis of NO_3^-. For NO_3^- reduction, this supernatant was injected into the gas sealed purge vessel containing 0.8% (w/v) vanadium trichloride in 1 M HCl at 95ºC.

The NO content was measured using a NO analyser (Sievers NOA 280; Analytix Ltd, Durham, UK). The reaction of NO with ozone in the chemiluminescent reaction chamber yielded nitrogen dioxide, which on creation emits light at the infra-red region of the electromagnetic spectrum. This luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed within the NO analyser, and amplified producing an analog mV output signal. [NO_2^-] and [NO_3^-] were determined by plotting signal area against a calibration plot of 50 nM to 1.25 µM $NaNO_2^-$ and 250 nM to 10 µM $NaNO_3^-$, respectively. For duplicate samples of NO_3^- and NO_2^- using these techniques, the coefficients of variation were 1.6% and 9.0%, respectively.

Blood lactate and glucose, and plasma potassium and sodium concentration

In Chapters 4, 5 and 7, fingertip blood samples were obtained to determine the whole blood [lactate]. Prior to puncturing the skin, the tip of the finger was cleaned thoroughly with an alcohol swab and left to dry. A disposable safety lancet (Safety-Lanzette,

Sarstedt) was used to make a small puncture. Initial drops of blood were wiped away and approximately 20-25 μL of free flowing blood was collected into a heparinized microvette (Microvette CB 300, Sarstedt). Samples were either analysed immediately (Chapter 4) or placed on ice and analysed within 5 min of collection (Chapters 5 and 7), using an automated blood lactate and glucose analyser (YSI 1500, Yellow Sprints Instruments, Yellow Springs, OH, USA).

In Chapter 6, venous blood samples were obtained before, during and after exercise testing, via a 20 gauge cannula (Insyte-W^{TM} Becton-Dickinson, Madrid, Spain), which was inserted into the antecubital forearm vein. The infusion of 0.9% saline using a syringe driver (Terumo NV, Leuven, Belgium) kept the cannula patent. Blood samples were collected into 5 mL heparin syringes (Terumo NV, Leuven, Belgium). 200 uL of blood was immediately haemolyzed in 200 μL of ice-cold Triton X-100 buffer solution (Triton X-100, Amresco, Salon, OH) on ice, and analysed to determine blood [lactate] and [glucose] within 5 min of collection (YSI 1500, Yellow Sprints Instruments, Yellow Springs, OH, USA). The remaining whole blood was then centrifuged at 4,000 rpm for 3 min (Hettich EBA 20, Germany) before plasma was extracted and stored on ice for ~30 min before being frozen at -80ºC. Plasma [K^+] and [Na^+] were later determined using an automated ion-selective electrode analyser (9180 Electrolyte Analyzer, F. Hoffman-La Roche, Basel, Switzerland).

Calibration of both the lactate and glucose analyser and the electrolyte analyser was completed regularly (i.e. hourly or after every 10 samples) and daily maintenance was carried out by a laboratory technician in accordance with the manufacturer's guidelines.

Pulmonary gas exchange

With the exception of the exercise tests conducted in the sports hall in Chapter 6, pulmonary gas exchange was measured breath-by-breath during all exercise tests. This gas analysis was conducted using a metabolic cart system that comprised of a bidirectional "Triple V" digital transducer and differential paramagnetic (O_2) and infrared absorption (CO_2) analysers (Jaegar Oxycon Pro, Hoechberg, Germany). Before each exercise test the gas analyser was calibrated with gases of known concentration while the volume sensor was calibrated using a 3-litre syringe (Hans Rudolph, Kansas

City, MO). Participants wore a nose clip and breathed through a low resistance (0.75 mmHg $l^{-1}.s^{-1}$ at 15 l/s) turbine volume transducer (Triple V, Jaeger, The Netherlands), which had a low dead space of 90 mL. Gas was continuously drawn down a capillary line and sampled for $\dot{V}_{O2}$, carbon dioxide ($\dot{V}_{CO2}$) and minute ventilation ($\dot{V}_{E}$) with values being displayed breath-by-breath on-line. After the completion of each test, raw breath-by-breath gas exchange and ventilation data were exported for later analysis.

The reliability of measuring pulmonary gas exchange was determined by repeat assessments on two separate days, in 10 participants. On each visit, participants completed two constant work rate step exercise tests consisting of 3 min baseline pedalling at 20 W, followed by an abrupt increase in work-rate to 80% of the work-rate associated with the gas exchange threshold (GET) for 5 min (interspersed by 10 min of rest). Before inter-study variation was calculated by comparing the estimated steady-state $\dot{V}_{O2}$ for the tests performed on separate days, breath-by-breath pulmonary gas exchange data collected during the two identical moderate-bouts on each day were averaged over 10-s periods, and time aligned to the start of exercise and ensemble-averaged. The steady-state $\dot{V}_{O2}$ response was found to be highly reproducible with coefficient of variation of 1.5% at an absolute power output of 92 W.

Cycle ergometry

All exercise tests in Chapters 4, 5 and 7 were conducted on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), which can administer work rate in various functions. In the series of experiments in these Chapters, the proportional, step and linear work rate forcing functions of the ergometer were used. The proportional function permits work rate to increase or decrease linearly as a function of time. This function was employed during the ramp incremental tests in Chapters 4, 5 and 7. The step function permits work rate to be rapidly (1000 $W.s^{-1}$) increased or decreased, from one constant work rate to another in a stepwise manner for a predetermined duration. This function was employed in Chapters 4 and 5, for all step exercise tests of various exercise intensities. The linear work rate function was employed for all three intermittent high-intensity exercise tests in Chapter 7. In comparison to the step and proportional functions that administer the external power output independent of pedal cadence by instantaneously adjusting the flywheel

resistance via electrical braking, the linear function is a cadence dependent system of work rate imposition and is given by the following equation:

Linear factor = Power Output ÷ Cadence^2

This work rate function administers a fixed work rate such that the attainment of a particular cadence will elicit a known power output. In line with manufacturers guidelines, a laboratory technician regularly calibrated the cycle ergometer and calibration equipment.

Exercise testing procedures

Incremental ramp test

In Chapters 4, 5 and 7, the first visit to the laboratory was used for the completion of a ramp incremental test to the limit of tolerance. This test consisted of a 3 min period of baseline pedaling at 20 W, following which the work rate was increased at a rate of 30 W.min^{-1} in a linear (ramp) fashion until the participant was unable to continue. The test was terminated when the pedal rate fell by >10 rpm. Participants cycled at a constant self selected pedal rate (70-90 rpm), and this pedal rate, along with saddle and handle bar height and configuration was recorded and reproduced in subsequent tests. Pulmonary gas exchange was continuously collected breath-by-breath throughout these incremental ramp tests.

Determination of $\dot{V}_{O2peak}$ and GET

The pulmonary gas exchange data from the preliminary ramp incremental test was used to calculate both the $\dot{V}_{O2peak}$ (Chapters 4, 5 and 7) and gas exchange threshold (GET; Chapters 4 and 5). The breath-by-breath pulmonary gas exchange data were averaged over consecutive 10-s periods, and $\dot{V}_{O2peak}$ was defined as the highest 30-s mean value attained before the participants volitional exhaustion. GET was estimated from 10 s average $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ measurements. A cluster of measures were used to identify the GET, including: 1) the first disproportionate increase in $\dot{V}_{CO2}$ from visual inspection of individual plots of $\dot{V}_{CO2}$ vs. $\dot{V}_{O2}$ (V-slope method; Beaver et al. 1986); an increase in $\dot{V}_E/\dot{V}_{O2}$ with no increase in $\dot{V}_E/\dot{V}_{CO2}$; and 3) an increase in end-tidal O_2 tension with no fall in end-tidal CO_2 tension.

Constant-load step tests and calculation of work-rates

In Chapters 4 and 5, constant work rate step exercise tests were used to assess the participant's physiological responses to exercise. These tests involve the abrupt transition from a lower to higher work rate. In both Chapters, these step tests were performed at work rates calculated on the basis of the GET and peak work rates attained in the ramp incremental tests. It is pertinent to note that when prescribing work rates based on pulmonary gas exchange data from incremental tests the $\dot{V}_{O_2}$ 'lag' time (mean response time) must be corrected for. This 'lag' time was assumed to approximate two-thirds of the ramp rate during incremental exercise (Whipp et al. 1981). Consequently, the GET and peak work rates used to normalize exercise intensity reflect work rates 20 W less than the work rates that coincide with the appearance of the GET and $\dot{V}_{O_{2peak}}$.

In Chapters 4 and 5, the moderate-intensity work rates used were calculated as 80% of the GET. For Chapter 4, the severe-intensity work rates were calculated as 75% Δ (i.e. a work rate equal to the difference between the power output at GET and $\dot{V}_{O_{2peak}}$ plus the power output at GET).

Reliability of exercise performance/tolerance measurements

In Chapter 4, exercise tolerance was assessed as the time-to-exhaustion during severe-intensity constant work rate cycling exercise. The reliability of this measure was determined by repeat assessments on two separate days, in 9 participants. The measure was shown to be very reliable with a coefficient of variation of 5.9%.

In Chapter 7, the total distance covered during the Yo-Yo Intermittent Recovery Test (level 1) was used to assess team-sport specific intermittent exercise performance. The reliability of this measure was determined by repeat assessments on two separate days, in 7 participants. The coefficient of variation was found to be 3.0%.

In Chapter 8, 3 different cycling intermittent exercise tests were used to assess intermittent exercise performance. Specifically, these 3 tests were: twenty four 6-s all-out sprints interspersed with 24 s recovery (24 x 6 s); seven 30-s all-out sprints interspersed with 4 min of recovery (7 x 30 s); and, six 60-s self-paced maximal efforts

interspersed with 60 s of recovery (6 x 60 s). For each exercise bout the mean power output (MPO) was determined. Subsequently, the mean of all the exercise bouts within each protocol were calculated (MPO_{mean}). To determine the reliability of MPO_{mean}, it was assessed in 4 participants on two separate days. For the 24 x 6-s, 7 x 30-s and 6 x 60-s protocols, the coefficient of variation was found to be 3.3%. 3.1% and 2.8%, respectively.

Data analysis procedures

Information on the data analysis procedures applied is provided in the specific experimental Chapters.

Statistical Analysis

All statistical analysis within the experimental Chapters was conducted with the Statistical Package for Social Sciences (SPSS). Detailed information regarding the specific statistical analysis conducted is given within each experimental Chapters. The data were screened for normal distribution using standard procedures before any statistical analysis was carried out. Data are presented as mean ± SD unless otherwise stated. Statistical significance was accepted if $P < 0.05$.

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Beetroot juice and exercise: pharmacodynamic and dose-response relationships

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Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *J Appl Physiol* 115: 325–336, 2013. First published May 2, 2013; doi:10.1152/japplphysiol.00372.2013.—Dietary supplementation with beetroot juice (BR), containing approximately 5–8 mmol inorganic nitrate (NO_3^-), increases plasma nitrite concentration ($[NO_2^-]$), reduces blood pressure, and may positively influence the physiological responses to exercise. However, the dose-response relationship between the volume of BR ingested and the physiological effects invoked has not been investigated. In a balanced crossover design, 10 healthy men ingested 70, 140, or 280 ml concentrated BR (containing 4.2, 8.4, and 16.8 mmol NO_3^-, respectively) or no supplement to establish the effects of BR on resting plasma $[NO_3^-]$ and $[NO_2^-]$ over 24 h. Subsequently, on six separate occasions, 10 subjects completed moderate-intensity and severe-intensity cycle exercise tests, 2.5 h postingestion of 70, 140, and 280 ml BR or NO_3^--depleted BR as placebo (PL). Following acute BR ingestion, plasma $[NO_2^-]$ increased in a dose-dependent manner, with the peak changes occurring at approximately 2–3 h. Compared with PL, 70 ml BR did not alter the physiological responses to exercise. However, 140 and 280 ml BR reduced the steady-state oxygen (O_2) uptake during moderate-intensity exercise by 1.7% ($P = 0.06$) and 3.0% ($P < 0.05$), whereas time-to-task failure was extended by 14% and 12% (both $P < 0.05$), respectively, compared with PL. The results indicate that whereas plasma $[NO_2^-]$ and the O_2 cost of moderate-intensity exercise are altered dose dependently with NO_3^--rich BR, there is no additional improvement in exercise tolerance after ingesting BR containing 16.8 compared with 8.4 mmol NO_3^-. These findings have important implications for the use of BR to enhance cardiovascular health and exercise performance in young adults.

nitrate; nitrite; nitric oxide; blood pressure; exercise economy; O_2 uptake; exercise tolerance

NITRIC OXIDE (NO) IS A GASEOUS signaling molecule that modulates human physiological function via its role in, for example, the regulation of blood flow, neurotransmission, immune function, glucose and calcium homeostasis, muscle contractility, and mitochondrial respiration (9, 36).[1] NO is generated through the oxidation of the amino acid L-arginine in a reaction catalyzed by NO synthase (NOS), with nitrite (NO_2^-) and nitrate (NO_3^-) being oxidation products of NO (30). It is now appreciated that under appropriate physiological conditions, NO can also be produced via the reduction of NO_2^-, a process that may be particularly important in situations where oxygen (O_2) availability is low, and/or NOS function is impaired (12). Interestingly, administration of dietary inorganic NO_3^- has been shown to increase plasma NO_2^- concentration ($[NO_2^-]$) and to produce NO-like bioactivity (19, 23, 39). Up to 25% of ingested NO_3^- enters the enterosalivary circulation and is concentrated in the saliva, whereupon facultative, anaerobic bacteria in the oral cavity reduce the NO_3^- to NO_2^- (30). When swallowed into the acidic environment of the stomach, some of the NO_2^- is converted further into NO, whereas the remainder is absorbed to increase circulating plasma $[NO_2^-]$. This NO_2^- may be reduced further to NO and other reactive nitrogen intermediates, particularly in tissues that may be relatively hypoxic, such as contracting skeletal muscle (30).

We (3, 37) and others (19, 23, 39) have demonstrated that NO_3^- ingestion, either in the form of NO_3^- salts or via the consumption of high NO_3^- vegetable products, such as beetroot juice (BR), reduces resting blood pressure (BP) profoundly and consistently. Consequently, dietary NO_3^- supplementation has emerged as a potential nutritional agent for the prevention and treatment of hypertension and cardiovascular disease (30). Webb et al. (39) assessed the effects of acute BR consumption (~23 mmol NO_3^-) on plasma $[NO_2^-]$ and BP over 24 h. Plasma $[NO_2^-]$ peaked 3 h postingestion, remained close to peak values until 5 h postingestion, and returned to baseline after 24 h (39). The systolic and diastolic BP and the mean arterial pressure (MAP) were reduced significantly, by ~10, ~8, and ~8 mmHg, respectively, at 2.5–3 h after BR intake. The same research group later reported a dose-dependent increase in plasma $[NO_3^-]$ and $[NO_2^-]$ and reduction in BP following ingestion of potassium NO_3^- (KNO_3) (19). In this study, plasma $[NO_2^-]$ rose by ~1.3-, approximately two-, and approximately fourfold following consumption of 4, 12, and 24 mmol KNO_3, respectively. The peak rise in plasma $[NO_2^-]$ was accompanied by significant reductions in both systolic BP (of ~2, ~6, and ~9 mmHg, respectively) and diastolic BP (of ~4, ~4, and ~6 mmHg, respectively). However, since BR contains polyphenols and antioxidants, which can facilitate the synthesis of NO from NO_2^- in the stomach (30), it is unclear whether BP is similarly impacted when different doses of BR are ingested compared with equivalent doses of NO_3^- salts. Given the growing interest in dietary NO_3^- supplementation in the form of BR amongst athletes and the general population, it is important to determine the pharmacokinetic-pharmacodynamic relationship between different volumes of BR consumption and changes in plasma $[NO_2^-]$ and BP to establish an optimal dose for beneficial effects.

Recent investigations suggest that dietary NO_3^- supplementation has the potential to influence human physiology beyond

[1]This article is the topic of an Invited Editorial by L. Burke (5a).

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the above hemodynamic effects (3, 26). Specifically, we (2, 3, 22) and others (6, 24–26) have demonstrated that 3–6 days of dietary NO_3^- supplementation reduces the O_2 cost of moderate-intensity exercise and may enhance exercise tolerance in healthy, young adults. It appears that these effects are related to NO_2^- or NO-mediated enhancements of muscle contractile function (2, 17) and/or mitochondrial efficiency (24) and/or enhanced muscle blood flow, especially to *type II* fibers (14). Importantly, a reduction of the O_2 cost of exercise (25, 37) and improved exercise performance (21) has also been reported as early as 2.5 h following a single dose of dietary NO_3^-, which is consistent with the time required for the peak plasma $[NO_2^-]$ to be attained (39). However, since all exercise-performance studies completed to date with BR have administered approximately 5–8 mmol NO_3^-, it is unclear whether a dose-response relationship exists between acute NO_3^- intake and the physiological responses to exercise. The establishment of the dose-response relationship between NO_3^- intake and the physiological responses to exercise and the ascertainment of the optimal NO_3^- dose for enhancing exercise performance are important, given the increasing popularity of BR supplementation in both basic research and applied exercise settings.

Therefore, the purpose of the present study was twofold: firstly, to characterize the plasma $[NO_3^-]$ and $[NO_2^-]$ pharmacokinetics and the changes in BP after ingestion of three different quantities of NO_3^--rich BR; and secondly, to investigate the dose-response relationship between BR/NO_3^- intake and the physiological responses to exercise. In two separate experiments, we administered a BR concentrate that enabled a substantial NO_3^- load to be ingested quickly and easily. We investigated: *1*) the influence of acute NO_3^- doses of 4.2, 8.4, and 16.8 mmol consumed in 70, 140, and 280 ml concentrated BR on plasma $[NO_3^-]$ and $[NO_2^-]$ and BP over a 24-h period; and *2*) the physiological responses to step transitions to moderate- and severe-intensity exercise, 2.5 h postingestion of the same NO_3^- doses. We hypothesized that the effects of dietary inorganic NO_3^- on plasma $[NO_3^-]$ and $[NO_2^-]$, BP, the O_2 cost of moderate-intensity exercise, and exercise tolerance (assessed as the time-to-task failure) during severe-intensity exercise would be dose dependent.

METHODS

The study was conducted in two phases [*study 1* (S_1), pharmacokinetics; and S_2, dose response], with the results generated in S_1 used to inform the experimental design in S_2. There was distinct subject recruitment for each experiment. Ten healthy, recreationally active men volunteered for each experiment [mean ± SD: S_1, age 23 ± 5 yr, height 1.79 ± 0.07 m, body mass (BM) 79 ± 9 kg; S_2, age 22 ± 5 yr, height 1.77 ± 0.05 m, BM 74 ± 8 kg]. None of the subjects in S_1 and S_2 was a tobacco smoker or user of dietary supplements. All subjects recruited for S_2 were fully familiar with laboratory exercise-testing procedures, having participated previously in studies using cycle ergometry in our laboratory. The procedures used in S_1 and S_2 were granted full ethics approval by the Institutional Research Ethics Committee. All subjects gave their written, informed consent to participate after the experimental procedures, associated risks, and potential benefits of participation had been explained in detail.

All subjects in S_1 and S_2 were instructed to keep a food and physical-activity diary in the 24 h preceding their first laboratory visit and to replicate food consumption and physical activity in the 24 h preceding subsequent visits. The subjects were required to arrive at the laboratory in a rested and fully hydrated state, following an overnight fast, and to avoid strenuous activity in the 24 h preceding each testing session. Subjects were instructed to refrain from caffeine and alcohol-containing drinks for 6 and 24 h before each laboratory visit, respectively, and to abstain from using antibacterial mouthwash and chewing gum throughout the study, because these are known to eradicate the oral bacteria that are necessary for the conversion of NO_3^- to NO_2^- (16).

S_1: Pharmacokinetics and Pharmacodynamics

Procedures. All subjects reported to the laboratory on four separate occasions over a period of 3 wk. Upon arrival to the laboratory, resting BP was measured, and a venous blood sample was obtained for the measurement of plasma $[NO_2^-]$ and $[NO_3^-]$. Subjects then consumed an acute dose of 70, 140, or 280 ml NO_3^--rich BR (organic BR containing ~4.2, ~8.4, or ~16.8 mmol NO_3^-, respectively; Beet It; James White Drinks, Ipswich, UK) or 140 ml water [control (CON)], in addition to a standardized breakfast (72 g porridge oats with 180 ml semiskimmed milk). BP was measured, and a venous blood sample was obtained, 1, 2, 4, 8, 12, and 24 h postingestion. For each 24-h period of data collection, subjects were provided with a standardized, low NO_3^- diet. The quantity and timing of food and drink intake were recorded on *visit 1* and replicated in subsequent visits. A washout period of at least 3 days separated the laboratory visits.

Measurements. The BP of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro; GE Medical Systems, Tampa, FL), with the subjects in a seated position. After arrival at the laboratory and following 10 min of rest in an isolated room, four measurements were recorded, and the mean of the final three measurements was used for data analysis.

Venous blood samples were drawn into lithium-heparin tubes (7.5 ml Monovette lithium heparin; Sarstedt, Leicester, UK). Samples were centrifuged at 4,000 rpm and 4°C for 7 min, within 1 min of collection. Plasma was subsequently extracted and immediately frozen at −80°C for later analysis of $[NO_2^-]$ and $[NO_3^-]$.

All glassware, utensils, and surfaces were rinsed with deionized water to remove residual $[NO_2^-]$ and $[NO_3^-]$ before blood analyses. The $[NO_2^-]$ of the undiluted (nondeproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and 4% (w/v) aqueous sodium iodide. The spectral emission of electronically excited nitrogen dioxide product, from the NO reaction with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube, housed in a Sievers gas-phase chemiluminescence NO analyzer (NOA; Sievers NOA 280i; Analytix, Durham, UK). The $[NO_2^-]$ was determined by plotting signal (mV) area against a calibration plot of 100 nM–1 μM sodium NO_2^-. Before determination of $[NO_3^-]$, samples were deproteinized using zinc sulfate ($ZnSO_4$)/sodium hydroxide (NaOH) precipitation. Aqueous $ZnSO_4$ [400 μl 10% (w/v)] and 400 μl 0.5 M NaOH were added to 200 μl of sample and vortexed for 30 s before being left to stand at room temperature for 15 min. Thereafter, samples were centrifuged at 4,000 rpm for 5 min, and the supernatant was removed for subsequent analysis. The $[NO_3^-]$ of the deproteinized plasma sample was determined by its reduction to NO in the presence of 0.8% (w/v) vanadium trichloride in 1 M HCl. The production of NO was detected using the chemiluminescence NOA, as described above.

To determine more precisely the time-to-peak plasma $[NO_2^-]$ following NO_3^- ingestion, a one-compartment model with first-order absorption and elimination kinetics was used, as described in the following equation

$$Y = \left(\exp(-Ke \times X/(Ke/Ka)\right) - \exp\left(-Ke \times X\right))/(Ke/Ka - 1)$$

where Y represents fraction absorbed; X represents time; and, Ka and Ke represent the first-order absorption and elimination rate constants, respectively.

Statistical analysis. Two-way repeated-measures ANOVA was used to assess the difference across conditions (4.2, 8.4, and 16.8

mmol NO_3^- and CON) and across time (0, 1, 2, 4, 8, 12, and 24 h) for plasma [NO_2^-] and [NO_3^-] and BP. Significant main or interaction effects were analyzed further using simple contrasts. One-way repeated-measures ANOVA was used to assess the differences in time-to-peak plasma [NO_2^-]. Relationships between plasma [NO_2^-] and BP were analyzed using Pearson product moment correlation coefficients. Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± SD unless stated otherwise.

S_2: Dose Response

Protocol. Subjects were required to report to the laboratory on seven separate occasions, over a 4- to 5-wk period. During the first visit to the laboratory, subjects completed a ramp incremental exercise test for determination of peak O_2 uptake ($\dot{V}o_{2\,peak}$) and gas-exchange threshold (GET). All tests were performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Initially, each subject completed 3 min of "unloaded" baseline cycling; then, the work rate was increased by 30 W/min until the subject was unable to continue. The subjects cycled at a self-selected pedal rate (70–90 rpm), and this pedal rate along with the saddle and handlebar height and configuration were recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 10-s periods. $\dot{V}o_{2\,peak}$ was taken as the highest 30-s mean value attained before the subject's volitional exhaustion. The GET was determined as described previously (3, 37). The work rates that would require 80% of the GET (moderate-intensity exercise) and 75% of the difference between the power output at GET and $\dot{V}o_{2\,peak}$ plus the power output at GET, i.e., severe-intensity exercise (Δ) were subsequently calculated.

On test days, subjects arrived at the laboratory at ~8 AM. A venous blood sample was drawn for measurement of plasma [NO_2^-] and NO_3^-. Subjects then ingested 70, 140, or 280 ml NO_3^--rich BR (containing 4.2, 8.4, or 16.8 mmol NO_3^-, respectively; Beet It) or 70, 140, or 280 ml NO_3^--depleted BR as a placebo (PL70, PL140, or PL280; containing ~0.04, ~0.08, or ~0.12 mmol NO_3^-; Beet It). All BR and PL doses were administered using a randomized, double-blind crossover design. Subjects were asked to consume the beverage within a 5-min period and, after doing so, were served a standardized breakfast (72 g porridge with 180 ml semiskimmed milk). A washout period of at least 72 h separated each visit.

After ingestion of the beverage, subjects were given a period of 2.5 h, during which they were allowed to leave the laboratory but were asked to refrain from strenuous physical activity. Subjects were also asked to fast during this time, although water was permitted ad libitum. Following this 2.5-h period, a second venous blood sample was drawn for measurement of plasma [NO_2^-] and [NO_3^-]. Subjects then completed "step" exercise tests, from a 20-W baseline to moderate-intensity (93 ± 11 W) and severe-intensity (258 ± 23 W) work rates for the determination of pulmonary $\dot{V}o_2$ dynamics. On each visit, subjects completed two, 5-min bouts of moderate-intensity exercise and one bout of severe-intensity exercise that was continued until task failure as a measure of exercise tolerance. All bouts of exercise on each day were separated by 5 min of passive rest. The time-to-task failure was recorded when the pedal rate fell by >10 rpm below the self-selected pedal rate. In the severe-intensity bouts, the subjects were verbally encouraged to continue for as long as possible.

Measurements. During all exercise tests, pulmonary gas exchange and ventilation were measured breath by breath, with subjects wearing a nose clip and breathing through a low dead-space (90 ml), low-resistance (0.75 $mmHg\,l^{-1}\cdot s^{-1}$ at 15 l/s) mouthpiece and impeller turbine assembly (Jaeger Triple-V; Jaeger GmbH, Hoechberg, Germany). The inspired and expired gas volume and gas concentration signals were sampled continuously at 100 Hz—the latter using paramagnetic (O_2) and infrared [carbon dioxide (CO_2)] analyzers (Oxycon Pro; Jaeger GmbH) via a capillary line connected to the mouthpiece. These analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-liter syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in capillary gas transit and analyzer rise time relative to volume signal. O_2 uptake, CO_2 output, and minute ventilation were calculated using a standard formula and displayed breath by breath. Heart rate (HR) was measured using short-range radiotelemetry (model RS400; Polar Electro Oy, Kempele, Finland).

Capillary blood samples were collected from the fingertip into a capillary tube during the baseline, preceding each step transition in work rate; during the final 30 s of each moderate-intensity exercise bout; and following exhaustion in the severe-intensity exercise bout. These samples were analyzed immediately to determine blood lactate concentration ([lactate]; model YSI 1500; Yellow Springs Instrument, Yellow Springs, OH). Venous blood samples were treated and analyzed as described in S_1.

The breath-by-breath data from each exercise test were linearly interpolated to provide second-by-second values, and the two identical, moderate-intensity repetitions performed on each visit were time aligned to the start of exercise and ensemble averaged. Baseline $\dot{V}o_2$ ($\dot{V}o_{2baseline}$), expired CO_2 at baseline ($\dot{V}co_{2baseline}$), and respiratory exchange ratio (RER) at baseline were defined as the mean values measured over the final 90 s of baseline pedaling. The end-exercise $\dot{V}o_2$, $\dot{V}co_2$, and RER were defined as the mean values measured over the final 30 s of exercise. The amplitude of the $\dot{V}o_2$ response was calculated by subtracting $\dot{V}o_{2baseline}$ from $\dot{V}o_2$ at the end of exercise. Subsequently, the functional gain of the entire response was calculated by dividing the $\dot{V}o_2$ amplitude by the change (Δ) in work rate. The amplitude of the $\dot{V}o_2$ slow component during the severe-intensity exercise bout was estimated by subtracting the mean $\dot{V}o_2$ at 2 min from the mean $\dot{V}o_2$ at 6 min.

Statistical analysis. Two-way repeated-measures ANOVA was used to assess the difference in pulmonary gas-exchange variables, blood [lactate], and HR across dose (70, 140, and 280 ml) and treatment (PL and BR). Differences in pre- and postplasma [NO_2^-] and [NO_3^-] were assessed separately in PL and BR, across dose and time (pre and post) using two-way repeated-measures ANOVAs. Significant main and interaction effects were analyzed further using simple contrasts. Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± SD unless stated otherwise.

RESULTS

Ingestion of BR was tolerated well by all subjects in S_1 and S_2. Subjects did, however, report beeturia (red urine) and red stools, consistent with previous studies (3, 39). The absolute NO_3^- doses used in S_1 and S_2 (4.2, 8.4, and 16.8 mmol) were equivalent to ~0.05 ± 0.01 (range: 0.05–0.07), ~0.11 ± 0.01 (range: 0.09–0.13), and ~0.22 ± 0.03 mmol (range: 0.19–0.26) NO_3^-/kg BM, respectively.

S_1: Pharmacokinetics and Pharmacodynamics

The effects of different volumes of BR (and therefore, different amounts of ingested NO_3^-) on plasma [NO_3^-] and [NO_2^-] are presented in Fig. 1. There were significant main effects by dose and time and an interaction effect for both plasma [NO_3^-] (Fig. 1*A*; all $P < 0.01$) and plasma [NO_2^-] (Fig. 1*B*; all $P < 0.01$).

At resting baseline, before the ingestion of any beverage, plasma [NO_3^-] was not significantly different between doses (Fig. 1*A*; all $P > 0.05$). ANOVA analyses revealed significant dose-dependent increases in plasma [NO_3^-] following BR supplementation ($P < 0.05$). The peak elevation above baseline in plasma [NO_3^-] occurred 1 h postadministration of 4.2 (160 ±

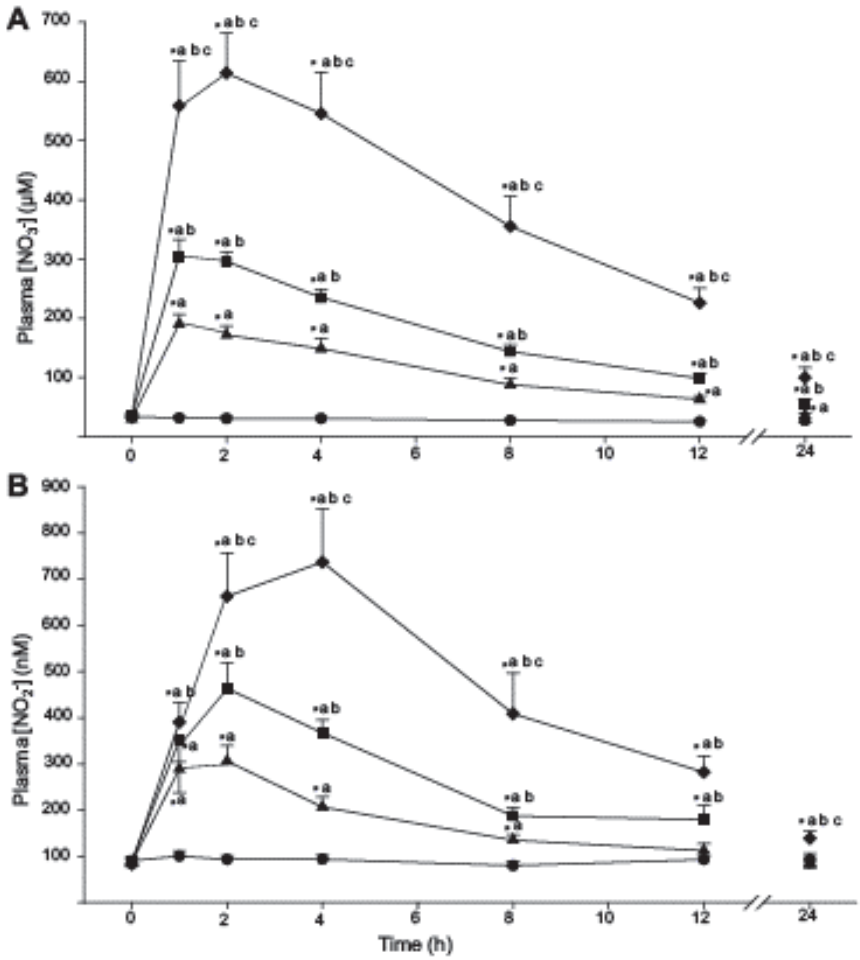

Fig. 1. Plasma nitrate concentration ([NO_3^-]; *A*) and nitrite concentration ([NO_2^-]; *B*) following consumption of water (control; ●) and 4.2 (▲), 8.4 (■), and 16.8 (♦) mmol NO_3^- (group mean ± SE). Plasma [NO_3^-] and [NO_2^-] rose significantly in a dose-dependent manner. See text for further details. *Significant difference from presupplemention baseline ($P < 0.05$); [a]significant difference from control ($P < 0.05$); [b]significant difference from 4.2 mmol NO_3^- ($P < 0.05$); [c]significant difference from 8.4 mmol NO_3^- ($P < 0.05$).

43 μM) and 8.4 mmol NO_3^- (269 ± 92 μM) and 2 h postadministration of 16.8 mmol NO_3^- (581 ± 209 μM; Fig. 1*A*; all $P < 0.05$). Plasma [NO_3^-] remained elevated above baseline and CON at all time points after administration of 4.2, 8.4, and 16.8 mmol NO_3^- ($P < 0.05$).

At baseline, before ingestion of any beverage, plasma [NO_2^-] was not significantly different between doses (Fig. 1*B*; $P > 0.05$). ANOVA analyses revealed significant dose-dependent increases in plasma [NO_2^-] following BR supplementation ($P < 0.05$). The peak elevation above baseline in plasma [NO_2^-] occurred 2 h postadministration of 4.2 (220 ± 104 nM) and 8.4 mmol NO_3^- (374 ± 173 nM) and 4 h postadministration of 16.8 mmol NO_3^- (653 ± 356 nM; Fig. 1*B*; all $P < 0.05$). Kinetic analyses revealed that plasma [NO_2^-] peaked significantly later (198 ± 64 min; range: 130–367 min) following ingestion of 16.8 mmol relative to both 8.4 mmol (146 ± 38 min; range: 77 ± 213 min; $P < 0.05$) and 4.2 mmol BR (106 ± 39 min; range: 63–192 min; $P < 0.05$). Peak plasma [NO_2^-], following ingestion of 8.4 mmol, tended to occur later compared with 4.2 mmol ($P = 0.06$). Plasma [NO_2^-] remained elevated above baseline and CON at 1, 2, 4, and 8 h after administration of 4.2, 8.4, and 16.8 mmol NO_3^- (all $P < 0.05$). At 12 h, plasma [NO_2^-] remained elevated above baseline and 4.2 mmol BR following ingestion of 8.4 and 16.8 mmol NO_3^- (all $P < 0.05$). In addition, plasma [NO_2^-] remained elevated at 24 h following administration of 16.8 mmol NO_3^- compared with all other doses ($P < 0.05$).

The effects of different volumes of BR (and therefore, different amounts of ingested NO_3^-) on systolic and diastolic BP and MAP are presented in Fig. 2. The changes in systolic BP across all conditions are presented in Fig. 2*A*. There were significant main effects by dose and time and an interaction effect on systolic BP (all $P < 0.05$). Systolic BP at baseline, before administration of any beverage, was lower ($P < 0.05$) in the 16.8-mmol NO_3^- condition (118 ± 5 mmHg) relative to CON (121 ± 5 mmHg) but not relative to 4.2 (119 ± 6 mmHg) and 8.4 mmol NO_3^- (120 ± 6 mmHg). Compared with baseline, systolic BP was lowered significantly following ingestion of 4.2, 8.4, and 16.8 mmol NO_3^- (all $P < 0.05$). The peak reduction in systolic BP occurred 4 h postadministration of 4.2 (5 ± 5 mmHg), 8.4 (10 ± 5 mmHg), and 16.8 mmol NO_3^- (9 ± 4 mmHg), respectively, relative to baseline (all $P < 0.05$). Systolic BP was reduced relative to baseline, CON, and 4.2 mmol NO_3^-, at 2, 4, and 8 h postadministration of 8.4 mmol and 16.8 mmol NO_3^- (all $P < 0.05$). There were no differences in systolic BP between 8.4 and 16.8 mmol NO_3^- at any time point ($P > 0.05$). At 24 h, systolic BP remained significantly lower (by 5 ± 5 mmHg) than baseline, following consumption of 16.8 mmol NO_3^- ($P < 0.05$). In contrast, systolic BP was not significantly different than CON or baseline at 24 h postad-

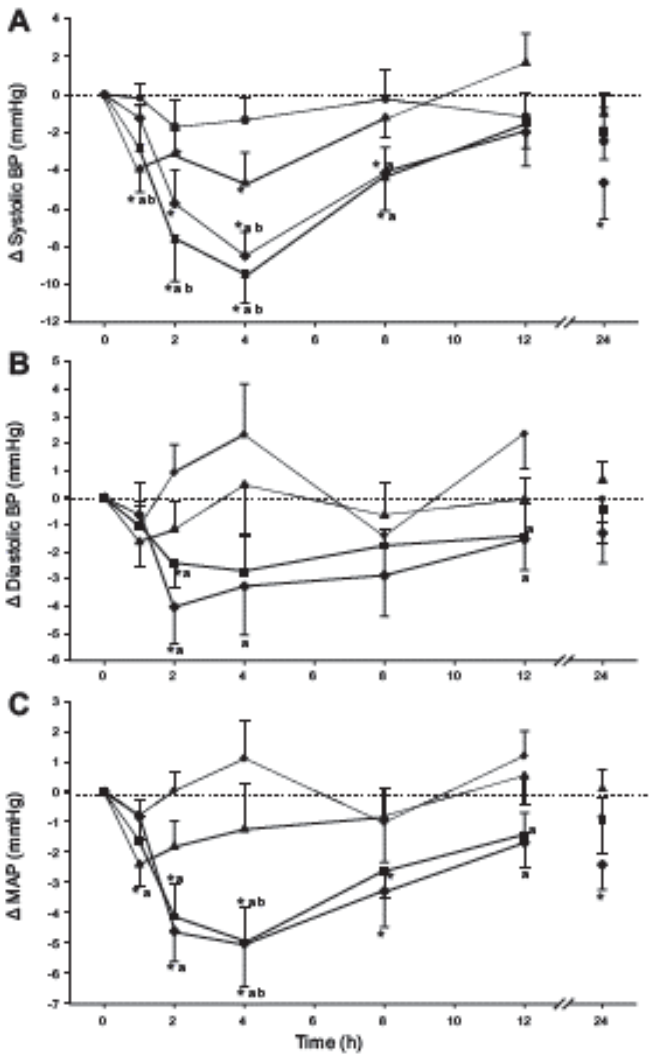

Fig. 2. Change (Δ) relative to presupplementation baseline in systolic blood pressure (BP; *A*), diastolic BP (*B*), and mean arterial pressure (MAP; *C*) following consumption of water (control; ●) and 4.2 (▲), 8.4 (■), and 16.8 (◆) mmol NO_3^- (group mean ± SE). *Significant difference from presupplementation baseline ($P < 0.05$); [a]significant difference from control ($P < 0.05$); [b]significant difference from 4.2 mmol NO_3^- ($P < 0.05$).

ministration of 4.2 and 8.4 mmol NO_3^- ($P > 0.05$). Overall, the mean systolic BP across 24 h, relative to CON, was lowered dose dependently by ~3, ~4, and ~6 mmHg after administration of 4.2, 8.4, and 16.8 mmol NO_3^-, respectively (all $P < 0.05$). The change in systolic BP was correlated with the change in plasma [NO_3^-] ($r = -0.27$; $P < 0.05$) and the change in plasma [NO_2^-] ($r = -0.37$; $P < 0.05$). The peak reduction in systolic BP was not correlated with the baseline systolic BP.

The changes in diastolic BP following the ingestion of different doses of NO_3^--rich BR are presented in Fig. 2*B*. There was a significant interaction effect (dose × time) on diastolic BP ($P < 0.05$). Diastolic BP at baseline was not significantly different among conditions (CON: 67 ± 5; 4.2 mmol: 68 ± 4; 8.4 mmol: 68 ± 6; 16.8 mmol: 67 ± 6 mmHg; $P > 0.05$). Follow-up tests revealed that ingestion of 8.4 and 16.8 but not 4.2 mmol NO_3^- reduced diastolic BP significantly, relative to baseline and CON (all $P < 0.05$). The peak reduction in diastolic BP from baseline occurred at 4 h postadministration of 8.4 mmol NO_3^- (3 ± 3 mmHg) and 2 h postadministration of 16.8 mmol NO_3^- (4 ± 4 mmHg; both $P < 0.05$) relative to baseline (both $P > 0.05$) and returned to near-baseline values by 24 h ($P > 0.05$). There were no differences in diastolic BP between 8.4 and 16.8 mmol NO_3^- at any time point ($P > 0.05$). The change in diastolic BP was correlated with the change in plasma [NO_3^-] ($r = -0.35$; $P < 0.05$) and the change in plasma [NO_2^-] ($r = -0.39$; $P < 0.05$). Moreover, the peak change in diastolic BP was correlated with the baseline diastolic BP ($r = -0.49$; $P < 0.05$).

The changes in MAP following the ingestion of different doses of NO_3^--rich BR are presented in Fig. 2*C*. There were significant main effects by dose and time and an interaction effect on MAP (all $P < 0.05$). At baseline, before the ingestion of any beverage, MAP was not significantly different among conditions (CON: 85 ± 4; 4.2 mmol: 85 ± 4; 8.4 mmol: 85 ± 5; 16.8 mmol: 84 ± 5 mmHg; $P > 0.05$). MAP was significantly lower following ingestion of 4.2, 8.4, and 16.8 mmol NO_3^- relative to baseline and CON (all $P < 0.05$). Following ingestion of 4.2 mmol NO_3^-, the peak reduction (2 ± 2 mmHg) in MAP occurred at 1 h, and MAP remained reduced by ~2 mmHg at 2 h relative to baseline ($P < 0.05$). In contrast, the peak reduction in MAP (5 ± 3 mmHg) occurred 4 h postadministration of 8.4 and 16.8 mmol NO_3^- relative to baseline ($P < 0.05$). MAP was not different between 8.4 and 16.8 mmol NO_3^- at any time point ($P > 0.05$). Overall, the mean MAP across 24 h, relative to CON, was reduced dose dependently by ~1, ~2, and ~4 mmHg after administration of 4.2, 8.4, and 16.8 mmol NO_3^-, respectively (all $P < 0.05$). The change in MAP was correlated significantly with the change in plasma [NO_3^-] ($r = -0.35$; $P < 0.05$) and the change in plasma [NO_2^-] ($r = -0.41$; $P < 0.05$).

S_2: Dose Response

Plasma [NO_3^-] and [NO_2^-]. The group mean plasma [NO_3^-] and [NO_2^-] responses in the BR and PL conditions are illustrated in Fig. 3, *A* and *B*, respectively. Presupplementation plasma [NO_3^-] was not significantly different between conditions ($P > 0.05$), and no significant change in plasma [NO_3^-] was observed following PL supplementation ($P > 0.05$). ANOVA analyses revealed a significant dose-dependent increase in plasma [NO_3^-] at 2.5 h following BR supplementation ($P < 0.05$). An elevation in plasma [NO_3^-] above baseline was apparent following 4.2 (130 ± 17 μM; $P < 0.05$), 8.4 (282 ± 54 μM; $P < 0.05$), and 16.8 mmol NO_3^- (580 ± 89 μM; $P < 0.05$). Presupplementation plasma [NO_2^-] was not significantly different among conditions ($P > 0.05$), and no significant change in plasma [NO_2^-] was observed following PL supplementation ($P > 0.05$). ANOVA analyses revealed a significant dose-dependent increase in plasma [NO_2^-] at 2.5 h following BR supplementation ($P < 0.05$). Following administration of 4.2, 8.4, and 16.8 mmol NO_3^-, plasma [NO_2^-] was elevated above baseline by 150 ± 73 nM, 291 ± 145 nM, and 425 ± 225 nM, respectively (all $P < 0.05$). Plasma [NO_2^-] was significantly greater after ingestion of 16.8 mmol compared with 4.2 mmol NO_3^- ($P < 0.05$) and tended to be greater compared with 8.4 mmol NO_3^- ($P = 0.06$). Plasma [NO_2^-] was

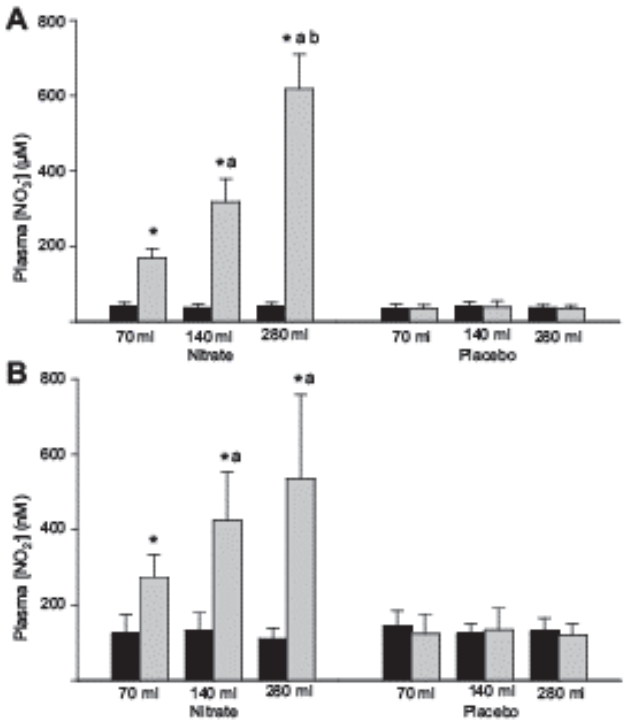

Fig. 3. Mean ± SE plasma [NO_3^-] (*A*) and [NO_2^-] (*B*) preingestion (black bars) and 2.5-h postingestion (gray bars) of 70, 140, and 280 ml NO_3^--rich beetroot juice (BR) (NO_3^-) or NO_3^--depleted BR [placebo (PL)]. See text for further details. *Significant difference from baseline ($P < 0.05$); [a]significant difference postconsumption of 70 ml NO_3^--rich BR ($P < 0.05$); [b]significant difference from postconsumption of 140 ml NO_3^--rich BR ($P < 0.05$).

significantly greater following ingestion of 8.4 mmol NO_3^- compared with 4.2 mmol NO_3^- ($P < 0.05$).

Moderate-intensity exercise. The pulmonary gas exchange and ventilatory responses to moderate-intensity exercise across all doses and conditions are summarized in Table 1. The $\dot{V}O_2$ measured during the period of baseline cycling at 20 W was not affected by dose or condition ($P > 0.05$). However, the absolute end-exercise $\dot{V}O_2$, measured over the final 30 s of moderate-intensity exercise, was altered significantly by BR ingestion ($P < 0.05$; Fig. 4*A*). Follow-up tests indicated that end-exercise $\dot{V}O_2$ was lowered significantly by ~3% following administration of 16.8 mmol NO_3^- relative to the respective PL (PL280: 1.65 ± 0.19 vs. BR280, 1.60 ± 0.23 l/min; $P < 0.05$). In addition, there was a trend toward a significant reduction (~2%) in end-exercise $\dot{V}O_2$ following administration of 8.4 mmol NO_3^- relative to the respective PL (PL140: 1.67 ± 0.21 vs. BR140, 1.64 ± 0.23 l/min; $P = 0.06$). The change in plasma [NO_2^-] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO_3^- was correlated with the change in end-exercise $\dot{V}O_2$ ($r = -0.47$; $P < 0.05$). There was no significant difference in end-exercise $\dot{V}O_2$ following ingestion of 4.6 mmol NO_3^- (BR70) compared with PL70 ($P > 0.05$).

The amplitude of the $\dot{V}O_2$ response (end-exercise − $\dot{V}O_{2baseline}$; Table 1) was affected by dose ($P < 0.05$) and tended to be affected by condition ($P = 0.07$). Follow-up tests revealed that there was a trend toward a significant reduction in the $\dot{V}O_2$ amplitude (by ~6%) after administration of 16.8 mmol NO_3^- compared with 8.4 mmol NO_3^- (BR140: 0.70 ± 0.16 vs. BR280: 0.66 ± 0.16 l/min; $P = 0.06$). The change in plasma [NO_2^-] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO_3^- was correlated with the change in $\dot{V}O_2$ amplitude ($r = -0.38$; $P < 0.05$). There was no significant difference in $\dot{V}O_2$ amplitude between PL and BR at any dose ($P > 0.05$).

The $\dot{V}CO_{2baseline}$, measured over the last 90 s of 20 W pedaling, and the end-exercise $\dot{V}CO_2$, measured over the last 30 s of exercise, were affected by dose ($P < 0.05$ for both) but not condition ($P > 0.05$ for both; Table 1). Follow-up tests revealed that $\dot{V}CO_{2baseline}$ was increased significantly, as the volume of supplement ingested increased ($P < 0.05$), irrespective of the condition (i.e., PL or BR). Specifically, $\dot{V}CO_{2baseline}$ was increased by ~7% and ~5% following consumption of 280 ml of supplement relative to 70 and 140 ml, respectively ($P < 0.05$ for both). There were no significant differences in $\dot{V}CO_2$ between the ingestion of 70 and 140 ml of supplement ($P > 0.05$). Furthermore, post hoc analysis revealed that the end-exercise $\dot{V}CO_2$ was significantly higher following ingestion of both 140 and 280 ml of supplement relative to 70 ml ($P < 0.01$ for both). There was, however, no significant difference in end-exercise $\dot{V}CO_2$ between ingestion of 140 and 280 ml of supplement ($P > 0.05$).

Baseline and end-exercise RER were affected by dose ($P < 0.05$ for both) but not condition ($P > 0.05$). The follow-up tests indicated that RER increased as the volume of supplement ingested increased ($P < 0.05$; Table 1). Specifically, RER at baseline was increased by ~5% and ~4%, following consumption of 280 ml of supplement relative to 70 and 140 ml, respectively ($P < 0.05$ for both). Although there was no significant interaction effect or main effect by condition, baseline RER tended to be higher (by ~3%) following administration of 16.8 mmol NO_3^- compared with the respective PL ($P = 0.08$). End-exercise RER was increased significantly by ~4% and ~3%, following consumption of 280 ml compared with 70 and 140 ml of supplement, respectively ($P < 0.05$ for both). In addition, the ingestion of 140 ml increased end-exercise RER compared with ingestion of 70 ml of supplement ($P < 0.05$). The baseline, end-exercise, and change in blood [lactate] and HR were not altered significantly by dose or condition (Table 2; $P > 0.05$).

Severe-intensity exercise. The pulmonary gas exchange and ventilatory responses to severe-intensity exercise across all doses and conditions are summarized in Table 1. In contrast to the effects observed for moderate-intensity exercise, the $\dot{V}O_2$ and $\dot{V}CO_2$ measured at baseline and at task failure were not altered by dose or treatment (all $P > 0.05$). Moreover, neither the dose nor the treatment altered the $\dot{V}O_2$ slow component amplitude ($P > 0.05$ for both). There was a trend toward significant main effects by dose ($P = 0.09$) and treatment ($P = 0.08$) but no interaction effect on RER at baseline ($P > 0.05$). Follow-up tests revealed that there was a trend toward significant increases in RER at baseline by ~4% and ~3% following consumption of 280 ml of supplement compared with the consumption of 70 ($P = 0.06$) or 140 ml ($P = 0.08$) of supplement, respectively. RER, at task failure, was not altered by dose or treatment ($P > 0.05$). The baseline, end-exercise, and change in blood [lactate] and HR were not altered significantly by dose or condition (Table 2; $P > 0.05$).

There was a significant main effect by condition ($P < 0.05$) but not dose ($P > 0.05$) on time-to-task failure (Table 1 and

Table 1. *Pulmonary gas-exchange variables during moderate- and severe-intensity exercise following supplementation with 3 different volumes of beetroot juice and placebo*

	70 ml		140 ml		280 ml	
	Placebo	Nitrate, 4.2 mmol	Placebo	Nitrate, 8.4 mmol	Placebo	Nitrate, 16.8 mmol
Moderate-intensity exercise						
$\dot{V}O_2$						
Baseline, l/min	0.94 ± 0.10	0.93 ± 0.09	0.92 ± 0.12	0.94 ± 0.13	0.95 ± 0.12	0.94 ± 0.08
End-exercise, l/min	1.64 ± 0.21	1.61 ± 0.21	1.67 ± 0.21[a]	1.64 ± 0.23	1.65 ± 0.19	1.60 ± 0.18[b]
Primary amplitude, l/min	0.70 ± 0.16	0.68 ± 0.16	0.74 ± 0.16	0.70 ± 0.16	0.70 ± 0.14	0.66 ± 0.16
Primary gain, ml · min^{-1} · W^{-1}	9.5 ± 1.0	9.2 ± 1.1	10.1 ± 0.9	9.5 ± 0.9	9.6 ± 0.6	9.0 ± 1.1[c]
$\dot{V}CO_2$						
Baseline, l/min	0.82 ± 0.07	0.81 ± 0.05	0.82 ± 0.09	0.83 ± 0.12	0.86 ± 0.07[a]	0.89 ± 0.07[a,d]
End-exercise, l/min	1.48 ± 0.17	1.45 ± 0.17	1.51 ± 0.17	1.50 ± 0.17	1.52 ± 0.14[a]	1.52 ± 0.17[d]
$\dot{V}_E$						
Baseline, l/min	23 ± 3	22 ± 2	23 ± 3	23 ± 4	24 ± 3	23 ± 2
End-exercise, l/min	37 ± 5	36 ± 5	37 ± 5	37 ± 5	38 ± 5	37 ± 4
RER						
Baseline	0.88 ± 0.05	0.88 ± 0.04	0.89 ± 0.04	0.89 ± 0.04	0.91 ± 0.05	0.94 ± 0.04[c,d]
End-exercise	0.91 ± 0.04	0.90 ± 0.04	0.91 ± 0.03	0.92 ± 0.05	0.93 ± 0.04	0.95 ± 0.04[c,d]
Severe-intensity exercise						
$\dot{V}O_2$						
Baseline, l/min	1.00 ± 0.10	0.99 ± 0.11	0.99 ± 0.13	0.99 ± 0.11	0.99 ± 0.11	0.97 ± 0.11
End-exercise, l/min	3.89 ± 0.40	3.97 ± 0.34	3.96 ± 0.38	3.99 ± 0.40	3.98 ± 0.35	3.94 ± 0.28
Overall gain, ml · min^{-1} · W^{-1}	12.1 ± 0.8	12.5 ± 1.0	12.5 ± 0.9	12.6 ± 1.2	12.6 ± 0.9	12.5 ± 0.8
Slow-phase amplitude, 6–2 min; l/min	0.66 ± 0.14	0.65 ± 0.15	0.67 ± 0.17	0.62 ± 0.17	0.75 ± 0.09	0.69 ± 0.11
$\dot{V}CO_2$						
Baseline, l/min	0.91 ± 0.06	0.90 ± 0.08	0.89 ± 0.08	0.91 ± 0.09	0.92 ± 0.08	0.93 ± 0.11
End-exercise, l/min	4.16 ± 0.36	4.17 ± 0.27	4.21 ± 0.38	4.18 ± 0.32	4.20 ± 0.25	4.20 ± 0.31
RER						
Baseline	0.91 ± 0.05	0.91 ± 0.06	0.91 ± 0.06	0.92 ± 0.06	0.94 ± 0.05	0.96 ± 0.05
End-exercise	1.07 ± 0.06	1.05 ± 0.05	1.06 ± 0.05	1.05 ± 0.05	1.06 ± 0.05	1.07 ± 0.05
Time-to-task failure(s)	470 ± 81	508 ± 102	498 ± 113	570 ± 153[e]	493 ± 114	552 ± 117[b]

Values are means ± SD. $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, expired carbon dioxide; $\dot{V}_E$, ventilation; RER, respiratory exchange ratio. [a]Significantly different from placebo (PL)70 ($P < 0.05$); [b]significantly different from PL280 ($P < 0.05$); [c]Significantly different from beetroot juice (BR)140 ($P < 0.05$); [d]significantly different from BR70 ($P < 0.05$); [e]significantly different from PL140 ($P < 0.05$).

Fig. 4*B*). Follow-up tests revealed that consumption of 8.4 mmol NO_3^- (BR140) and 16.8 mmol NO_3^- (BR280) resulted in a significant increase in time-to-task failure by 71 ± 77 s and 59 ± 61 s, respectively, relative to PL140 and PL280 ($P < 0.05$; Fig. 4*B*). There was no difference in time-to-task failure between BR70 and PL70 ($P > 0.05$). The change in plasma [NO_2^-] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO_3^- was correlated significantly with the change in time-to-task failure ($r = 0.55$; $P < 0.05$). There was no significant difference in time-to-task failure among 4.2, 8.4, and 16.8 mmol BR (all $P > 0.05$) or among PL70, PL140, and PL280 ($P > 0.05$).

In terms of positive changes in time-to-task failure, there were three "nonresponders" in the 4.2-mmol condition, two in the 8.4-mmol condition, and one in the 16.8-mmol condition. Individual subjects who did not respond at lower doses did respond at higher doses. The increase in plasma [NO_2^-] from baseline to pre-exercise for the nonresponders was similar to the other subjects who did respond. For example, the three nonresponders at the lowest NO_3^- dose had an increase in plasma [NO_2^-] of 140, 208, and 161 nM compared with a group mean increase of 150 nM. In addition, the nonresponders did not have high baseline values of plasma [NO_2^-] (70–121 nM) compared with the group mean.

DISCUSSION

This study is the first to characterize the pharmacokinetic-pharmacodynamic effects of NO_3^--rich BR ingestion and to investigate the dose-response relationship between BR ingestion and the physiological responses to exercise. Specifically, we studied how acute ingestion of three different BR volumes (and thus three different NO_3^- doses) impacted on plasma [NO_3^-] and [NO_2^-], resting BP, the pulmonary gas-exchange responses to moderate- and severe-intensity exercise, and exercise tolerance. Our principal findings were that plasma [NO_3^-] and [NO_2^-] increased dose dependently up to 16.8 mmol NO_3^- with there being a dose-dependent peak reduction in BP up to 8.4 mmol NO_3^-. A NO_3^- dose of 16.8 mmol was required to elicit a significant reduction in the O_2 cost of moderate-intensity cycle exercise, although there was a trend ($P = 0.06$) for a reduction with 8.4 mmol. A significant improvement in time-to-task failure during severe-intensity exercise was evident after ingestion of 8.4 mmol NO_3^-, with no further benefits observed following the ingestion of 16.8 mmol NO_3^-.

S_1: BR Pharmacokinetics and Pharmacodynamics—Effects on Plasma [NO_3^-], [NO_2^-], and BP

The results of S_1 demonstrated that concentrated BR consumption causes dose-dependent increases in plasma [NO_3^-] and [NO_2^-]. Plasma [NO_3^-] increased by approximately five- and eightfold, 1 h after the ingestion of 4.2 and 8.4 mmol NO_3^-, and by ~18-fold, 2 h after the ingestion of 16.8 mmol NO_3^-. In contrast, the increase in plasma [NO_2^-] occurred later, peaking at approximately 2–2.5 h postadministration of 4.2 and 8.4 mmol NO_3^- and ~3 h postadministration of 16.8 mmol NO_3^-.

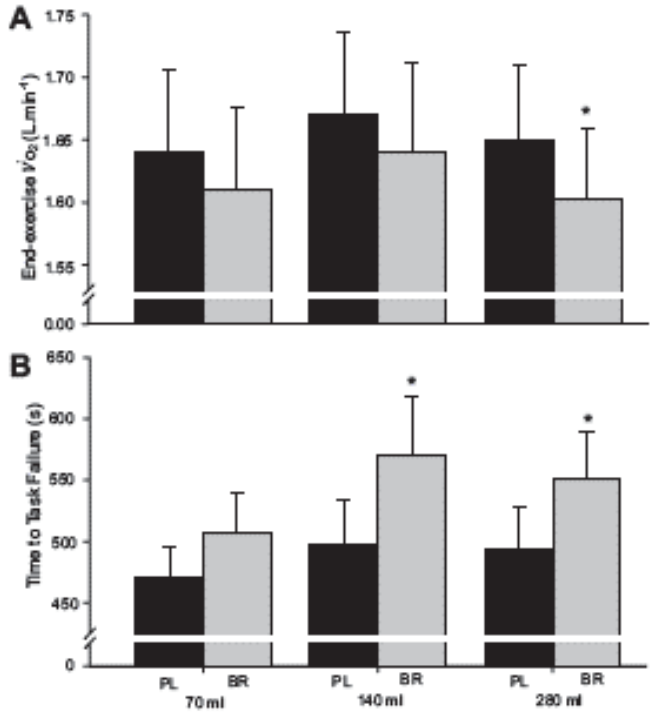

Fig. 4. Mean ± SE steady-state oxygen consumption ($\dot{V}O_2$) during moderate-intensity exercise (*A*) and time-to-task failure during severe-intensity exercise (*B*), following consumption of 70, 140, and 280 ml NO_3^--rich BR (gray bars) or NO_3^--depleted BR (PL; black bars). End-exercise $\dot{V}O_2$ during moderate-intensity exercise was reduced significantly following the ingestion of 280 ml BR. Time-to-task failure during severe-intensity exercise was extended after consumption of 140 ml BR with no further increase following 280 ml BR. *Significant difference from PL ($P < 0.05$).

As expected, the rise in plasma $[NO_2^-]$ was smaller compared with plasma $[NO_3^-]$, with peak increases of ~2.5-fold, approximately fourfold, and approximately eightfold, respectively. The delayed peak increases in plasma $[NO_2^-]$ compared with plasma $[NO_3^-]$ reflect the importance of the enterosalivary circulation and subsequent reduction of NO_3^- to NO_2^- by lingual bacteria (16, 39). These pharmacokinetic responses to BR supplementation are consistent with those reported previously following acute ingestion of KNO_3 (19). Together, these data suggest that the pharmacokinetics of plasma $[NO_3^-]$ and $[NO_2^-]$ are dose dependent when NO_3^- is administered, either as NO_3^- salt or in the form of a natural vegetable supplement.

Ingestion of concentrated BR dose dependently lowered systolic BP and MAP up to an intake of 8.4 mmol NO_3^-. More specifically, acute ingestion of 4.2, 8.4, and 16.8 mmol inorganic NO_3^-, administered in the form of BR, resulted in peak reductions of systolic BP of ~5, ~10, and ~9 mmHg and peak reductions of MAP of ~2, ~5, and ~5 mmHg, respectively. Moreover, BR ingestion resulted in a similar "threshold" effect on diastolic BP, with peak reductions of ~3 and ~4 mmHg following administration of 8.4 and 16.8 mmol NO_3^-; however, ingestion of 4.2 mmol NO_3^- did not reduce diastolic BP significantly. These reductions in BP are similar to those reported by Kapil et al. (19) following acute administration of KNO_3, except that Kapil et al. (19) reported a dose-dependent reduction in BP up to 24 mmol KNO_3. The reason for this discrepancy between studies is unclear. Interestingly, compared with Kapil et al. (19), who reported 6 mmHg and 9 mmHg reductions in systolic BP following the consumption of 12 mmol and 24 mmol KNO_3, respectively, we observed larger reductions in BP following the consumption of BR (e.g., a peak reduction of 10 mmHg in systolic BP with 8.4 mmol NO_3^- contained in 140 ml BR). It is possible that this apparent greater potency of BR compared with NO_3^- salt in reducing BP is related to the polyphenols and other antioxidants present in BR, which may facilitate a more efficient conversion of NO_3^- to NO_2^- (30). Interestingly, although the peak reduction in BP was not significantly different between 8.4 and 16.8 mmol NO_3^-, the mean reduction in BP over 24 h was dose dependent, with MAP, for example, reduced by 1, 2, and 4 mmHg following administration of 4.2, 8.4, and 16.8 mmol NO_3^-, respectively.

The results of the present study suggest that BR (and presumably other NO_3^--rich vegetable) consumption can provide a natural approach to maintaining or improving BP and

Table 2. *Heart rate and blood lactate responses to moderate- and severe-intensity exercise following supplementation with 3 different volumes of beetroot juice and placebo*

	70 ml		140 ml		280 ml	
	Placebo	Nitrate, 4.2 mmol	Placebo	Nitrate, 8.4 mmol	Placebo	Nitrate, 16.8 mmol
Moderate-intensity exercise						
Heart rate, beats/min						
Baseline	89 ± 9	89 ± 8	88 ± 8	88 ± 8	89 ± 8	89 ± 6
End-exercise	116 ± 11	116 ± 12	115 ± 10	116 ± 8	115 ± 9	115 ± 10
Blood [lactate], mM						
Baseline	1.1 ± 0.3	1.1 ± 0.5	1.1 ± 0.4	1.0 ± 0.4	1.0 ± 0.3	1.1 ± 0.3
End-exercise	1.2 ± 0.2	1.2 ± 0.5	1.2 ± 0.5	1.1 ± 0.4	1.1 ± 0.4	1.2 ± 0.5
Δ	0.1 ± 0.2	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.2	0.1 ± 0.3	0.1 ± 0.2
Severe-intensity exercise						
Heart rate, beats/min						
Baseline	99 ± 9	100 ± 8	99 ± 9	100 ± 10	100 ± 10	99 ± 8
End-exercise	186 ± 11	186 ± 12	185 ± 12	187 ± 10	186 ± 11	185 ± 10
Blood [lactate], mM						
Baseline	0.9 ± 0.4	0.9 ± 0.4	0.9 ± 0.4	0.9 ± 0.6	1.0 ± 0.3	0.9 ± 0.2
Task failure	9.7 ± 1.4	9.4 ± 1.6	9.4 ± 1.6	9.6 ± 1.8	9.5 ± 1.5	9.5 ± 1.1
Δ	8.7 ± 1.2	8.5 ± 1.7	8.5 ± 1.7	8.7 ± 1.3	8.5 ± 1.4	8.6 ± 1.2

Values are means ± SD. [lactate], lactate concentration; Δ, change.

vascular health in young adults. The reductions in BP, evident in the present study, are noteworthy. For example, it has been suggested that lowering systolic BP by 10 mmHg may reduce the risk of ischemic heart disease by ~25% and the risk of stroke by ~35% (27–29, 31). The beneficial hemodynamic effects of NO_3^- supplementation are thought to be due to the reduction of NO_3^- to NO_2^- and then to NO within the blood vessel (13), resulting in arterial dilatation and a reduced peripheral resistance (39). However, it is possible that NO_2^- itself may also exert a direct effect on the vascular system, independent of NO formation (1). There are several advantages to using inorganic rather than organic NO_3^- for the prevention or treatment of hypertension (33). These include a slow and controlled increase in plasma $[NO_2^-]$ following inorganic NO_3^- intake (due to NO_3^- uptake into the enterosalivary circulation) compared with the more abrupt changes in plasma $[NO_2^-]$ (perhaps to toxic levels) and BP, which can occur with organic NO_3^- administration (33). Moreover, unlike the chronic administration of organic NO_3^-, inorganic NO_3^- does not appear to lead to the development of tolerance (37) and endothelial dysfunction (33).

S_2: Dose Response

The results of S_2 confirm that concentrated BR consumption causes a dose-dependent increase in plasma $[NO_3^-]$ by 334%, 778%, and 1,556% and plasma $[NO_2^-]$ by 121%, 218%, and 338%, 2.5 h postingestion of 4.2, 8.4, and 16.8 mmol NO_3^-, respectively. The magnitude of the increase in plasma $[NO_2^-]$ following consumption of 8.4 and 16.8 mmol NO_3^- in the present study was much larger than the approximate 15–150% rise in plasma $[NO_2^-]$, reported previously, following acute (approximately 4–6 mmol) (5, 21, 25, 37) and chronic (approximately 5–6 mmol/day) (2, 3, 22, 26, 37) dietary NO_3^- supplementation. This finding is likely a consequence of the relatively higher NO_3^- doses (8.4 and 16.8 mmol NO_3^-) administered in the present study. Interestingly, the group mean plasma $[NO_3^-]$ and $[NO_2^-]$ reported in S_2 are somewhat lower than those reported at 2–4 h postingestion of BR in S_1. Given that there was distinct subject recruitment for S_1 and S_2, it is likely that this discrepancy is due to individual variations in the pharmacokinetic response to BR consumption. For example, when the individual plasma $[NO_2^-]$ responses to the ingestion of 16.8 mmol NO_3^- in S_1 are considered, peak concentrations ranged from 493 to 1,523 nM, and the time-to-peak concentration ranged from 130 to 367 min. The cause of this wide interindividual variability in the response of plasma $[NO_2^-]$ to NO_3^- ingestion is unclear, although it may depend, in part, on salivary flow rate; also, it is known that the reduction of NO_3^- to NO_2^- is highly dependent on the activity of oral bacteria (16, 39). Another consideration is that the absolute NO_3^- doses administered in the present study (4.2, 8.4, and 16.8 mmol in 1, 2, and 4 BR shots, respectively) resulted in somewhat different NO_3^- doses when expressed relative to BM (0.05–0.07, 0.09–0.13, and 0.19–0.25 mmol NO_3^-/kg BM, respectively).

Dose Response: Moderate-Intensity Exercise

This is the first study to assess the acute dose-dependent physiological responses to exercise following dietary NO_3^- supplementation in humans. We assessed the acute response to three different doses of BR at 2.5 h postingestion, based on the significant dose-dependent elevation in plasma $[NO_2^-]$ observed at 2–3 h postingestion in S_1 (Fig. 1*B*). The steady-state $\dot{V}O_2$ measured over the final 30 s of moderate-intensity cycle exercise was unaffected by 4.2 mmol NO_3^-, tended to be lower (~30 ml/min) following administration of 8.4 mmol NO_3^-, and was reduced significantly (by ~50 ml/min) following administration of 16.8 mmol NO_3^-.

The reduction in steady-state $\dot{V}O_2$ (~3%), observed following acute ingestion of 16.8 mmol NO_3^- (~0.23 mmol/kg BM), is similar to that reported 2.5 h postingestion of 5.2 mmol NO_3^- (~0.07 mmol/kg BM) in the form of nonconcentrated BR (37) but is smaller than the 6% reduction reported 1 h postingestion of 0.033 mmol/kg BM sodium nitrate (25). In contrast to acute ingestion, longer-term BR supplementation (3–6 days at approximately 5–7 mmol NO_3^-/day) resulted in an approximate 5–7% reduction in steady-state $\dot{V}O_2$ during moderate-intensity cycling (3, 26) and running (22).

Previous studies have indicated that the lowering of submaximal exercise $\dot{V}O_2$, following dietary NO_3^- supplementation, may result from improved mitochondrial efficiency (25) and/or a reduction in the ATP cost of muscle force production (4). Alterations in protein expression have been proposed as the mechanistic basis for these effects (17, 24); however, it is unlikely that these alterations occur quickly enough to explain the effects observed so soon (1–2.5 h) after NO_3^- ingestion (25, 37). Alternatively, NO may acutely and reversibly impact protein function through post-translational protein modifications. For instance, S-nitrosation of adenine nucleotide translocase or other mitochondrial or calcium-handling proteins (35) may contribute to the acute reduction in O_2 cost of exercise following BR ingestion. The mechanistic basis for the acute changes in the O_2 cost of exercise following BR ingestion warrants further investigation.

An interesting observation was the dose-dependent increase in baseline and end-exercise $\dot{V}CO_2$, irrespective of condition (i.e., PL or BR). This small but significant rise in $\dot{V}CO_2$ led to a dose-dependent increase in RER that was more pronounced during baseline cycling compared with the exercising steady-state. An elevation in RER is indicative of a shift in substrate use toward a relatively greater reliance on carbohydrate and is likely due to the sugar content of the concentrated BR and PL beverages (~16 g/70 ml).

Dose Response: Severe-Intensity Exercise

A novel finding of the present study was that 8.4 and 16.8 mmol NO_3^-, but not 4.2 mmol NO_3^-, administered acutely in the form of concentrated BR, significantly improved the time-to-task failure by 14% and 12%, respectively, during severe-intensity exercise. These findings are similar to the 14–16% improvement in exercise tolerance reported previously following 5–6 days of BR supplementation at a lower dose (5–6 mmol NO_3^-) (3, 22). Although the mechanism(s) responsible for the ergogenic potential of NO_3^- supplementation remain uncertain, they are believed to be mediated via a biochemical reduction of ingested NO_3^- to biologically active NO_2^- and NO (4).

NO has been linked to the efficiency of aerobic respiration (9) and the regulation of muscle contraction (35). Indeed, both more efficient mitochondrial oxidative phosphorylation, via a reduced proton leak across the inner mitochondrial membrane

(24) and a reduced ATP and phosphocreatine cost of muscle force production (2, 15), has been reported following dietary NO_3^- supplementation. In addition, recent evidence suggests that BR supplementation results in a marked increase in muscle blood flow during exercise in rats, with the blood flow preferentially distributed to muscle groups that principally contain *type II* fibers, which are recruited during severe-intensity exercise (14). Furthermore, NO_3^- supplementation has been shown to increase muscle force production in mice via modulation of intracellular calcium ion (Ca^{2+}) handling in fast-twitch fibers (17). It is possible that these mechanisms operate simultaneously and/or synergistically, resulting in enhanced exercise tolerance. It is, however, important to note that the studies that demonstrated effects of NO_3^- supplementation on muscle metabolic and vascular control mechanisms (2, 14, 17, 24) used chronic, rather than acute, NO_3^- supplementation protocols. On the other hand, Cosby et al. (10) reported acutely increased blood flow to exercising forearm muscle following infusion of NO_2^- into the brachial artery. It is possible that the improved time-to-task failure that we observed with 8.4 and 16.8 mmol NO_3^- was related to improved blood flow to muscle or to a NO-mediated enhancement of local matching of O_2 delivery to metabolic rate. This would be consistent with reports that BR supplementation results in a preferential distribution of blood flow to *type II* fibers (14) and improves oxidative function in hypoxic muscle (38). The lack of a further improvement in time-to-task failure with 16.8 mmol compared to 8.4 mmol NO_3^- mirrors the lack of an additional effect of consuming the higher NO_3^- dose on the peak reduction in BP that we observed in S_1, suggesting that the acute effects of BR ingestion on exercise tolerance may be related, at least in part, to effects on the vasculature. Further studies are needed to establish which mechanisms may be responsible for the ergogenic potential of NO_3^-, at least at high doses, as early as 2.5 h after ingestion of BR.

The results of the present study indicate a dose-dependent effect of BR supplementation on exercise tolerance up to 8.4 mmol, with no further benefit (indeed a small reduction in exercise tolerance compared with 8.4 mmol) following ingestion of 16.8 mmol NO_3^-. A possible explanation for this threshold might be a NO-dependent reduction in skeletal muscle force via modulation of excitation-contraction coupling. It has been reported that the opening of the Ca^{2+} release channels of the sarcoplasmic reticulum (SR) is inhibited by NO (32, 35) and highly related to NO availability (35). In addition, Ca^{2+} transport (35), SR Ca^{2+}-ATPase activity (18), and cytochrome c-oxidase inhibition (9) may be influenced by NO and contribute to a dose-dependent modulation of excitation-contraction coupling. Therefore, whereas an increase in NO bioavailability may result in a more efficient mitochondrial function (24) and changes to *type II* fiber contractility (17) and blood flow (14), it is possible that these positive effects may be offset by impairments of mitochondrial or contractile function at higher NO levels that might promote nitrative stress. These suggestions are naturally speculative and await further investigation.

The improvements in time-to-task failure during severe-intensity exercise, following ingestion of 8.4 and 16.8 mmol NO_3^- in the present study, were evident without any significant changes in the $\dot{V}O_2$ response to exercise. Neither the amplitude of the $\dot{V}O_2$ slow component nor the end-exercise $\dot{V}O_2$ was influenced by acute ingestion of up to 16.8 mmol NO_3^-. This finding is consistent with some (20) but not all previous reports (3, 22). For example, Bailey et al. (3) reported that 3 days of BR supplementation reduced the $\dot{V}O_2$ slow-component amplitude by 23% and improved exercise tolerance by ~16%. In contrast, Kelly et al. (20) reported that 3 days of BR supplementation improved exercise tolerance at three different severe intensities by 12–17%, without any accompanying changes in the $\dot{V}O_2$ response. We found no difference in end-exercise $\dot{V}O_2$ between BR and PL at any dose. In the severe exercise-intensity domain, the $\dot{V}O_2$ at the point of volitional exhaustion would be expected to equal the maximum $\dot{V}O_2$ ($\dot{V}O_{2\,max}$) (11). Our results are therefore consistent with some (3, 37) but not all (5, 25) previous studies that indicate that NO_3^- supplementation does not reduce $\dot{V}O_{2\,max}$. Interestingly, there was a disconnect between the effects of BR on steady-state $\dot{V}O_2$ during moderate-intensity exercise (where the greatest reduction occurred at the highest dose of NO_3^-) and the effects of BR on exercise tolerance (where the increased time-to-task failure was similar with 8.4 and 16.8 mmol NO_3^-). Collectively, these results appear to indicate that the effects of BR on severe-intensity exercise performance may be independent from the effects of BR on the O_2 cost of submaximal exercise.

It should be noted that while an approximate 12–14% extension of time-to-task failure during severe-intensity, constant work-rate exercise, following acute BR ingestion, may appear impressive, this is likely to translate into no more than a 1–2% reduction in the time to complete a given distance, for example, during a short endurance time-trial (TT) event (34). This is similar to the magnitude of improvement in performance reported previously for 4 km and 16.1 km TT after acute BR ingestion (21) and for 10 km TT following 6 days of BR supplementation (6). A 1% improvement in performance is highly meaningful in elite sport. For example, it could improve 1,500-m running performance by ~2 s or 3,000-m running performance by approximately 4–5 s in international standard athletes. It remains unclear, however, whether elite athletes may confer a performance benefit from NO_3^- supplementation. Several studies now indicate that at least when NO_3^- is ingested acutely, TT performance is not enhanced in highly trained endurance athletes (7, 8, 40). This may be related to factors such as greater NOS activity, better muscle oxygenation and mitochondrial efficiency, and a lower fraction of *type II* fibers in the muscles of highly endurance trained compared with moderately trained subjects (40). It is possible that the dose-response relationship between NO_3^- ingestion and changes in exercise performance are different in elite compared with sub-elite subjects, such that larger NO_3^- doses and/or longer supplementation periods may be required to elicit improved exercise performance. The significant correlation between the change in plasma [NO_2^-] and the change in time-to-task failure indicates that the dietary NO_3^- intervention must be sufficient to increase plasma [NO_2^-] if performance is to be improved. In this regard, an important consideration may be the timing of supplementation relative to the start of exercise. The present study shows that on average, plasma [NO_2^-] takes longer to peak when larger doses of NO_3^- are imbibed. However, there are appreciable interindividual differences in the speed with which ingested NO_3^- is reduced to NO_2^-, which may preclude any more specific advice other than to consume NO_3^- some 2–3 h before the start of exercise.

It has been suggested previously that there may be "responders" and nonresponders to dietary NO_3^- supplementation (40), and there was evidence of this in the present study. Interestingly, the number of nonresponders (in terms of exercise capacity) decreased as the dose ingested increased. For example, there were three nonresponders in the 4.2-mmol condition, two in the 8.4-mmol condition, and one in the 16.8-mmol condition. Two of the subjects who did not respond at the lowest dose did respond to the larger doses, and one subject who did not respond following administration of 4.2 or 8.4 mmol did respond to the 16.8-mmol dose. This suggests that some individuals will require a larger acute dose than others to elicit any positive effects on exercise capacity from dietary NO_3^- ingestion. Unlike in our previous study (40), the increase in plasma $[NO_2^-]$ from baseline to pre-exercise for the nonresponders was not smaller than that measured in other subjects who did respond, and the nonresponders did not have particularly high baseline plasma $[NO_2^-]$. In a recent study, we found that the subjects who demonstrated improvement in high-intensity, intermittent exercise performance following dietary NO_3^- supplementation were those whose plasma $[NO_2^-]$ fell significantly during exercise (41). We did not measure plasma $[NO_2^-]$ postexercise in the present study. The explanation for the existence of responders and nonresponders to dietary NO_3^- supplementation is presently obscure.

In conclusion, dietary supplementation with NO_3^--rich BR dose dependently increased plasma $[NO_3^-]$ and $[NO_2^-]$ up to 16.8 mmol NO_3^- and caused peak reductions in systolic BP and MAP dose dependently, up to 8.4 mmol NO_3^-. These results suggest that the consumption of high NO_3^- foodstuffs may be an effective strategy for maintaining and perhaps enhancing vascular health in young adults. The present study also demonstrated that the O_2 cost of moderate-intensity exercise is reduced dose dependently, up to 16.8 mmol NO_3^-. Supplementation with 4.2 mmol NO_3^- did not enhance time-to-task failure relative to PL; however, supplementation with 8.4 mmol NO_3^- significantly improved time-to-task failure relative to PL, with no further improvement evident following supplementation with 16.8 mmol NO_3^-. Although the mechanistic bases for the reduction in the O_2 cost of submaximal exercise and enhancements in exercise tolerance following acute dietary BR remain unclear, these results provide important, practical information that may underpin the potential use of BR/NO_3^- supplementation for improving cardiovascular health in the general population and for enhancing exercise performance in athletes.

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AUTHOR CONTRIBUTIONS

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

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Dose-dependent effects of dietary nitrate on the oxygen cost of moderate-intensity exercise: Acute vs. chronic supplementation

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ABSTRACT

Purpose: To investigate whether chronic supplementation with a low or moderate dose of dietary nitrate (NO_3^-) reduces submaximal exercise oxygen uptake ($\dot{V}O_2$) and to assess whether or not this is dependent on acute NO_3^- administration prior to exercise.
Methods: Following baseline tests, 34 healthy subjects were allocated to receive 3 mmol NO_3^-, 6 mmol NO_3^- or placebo. Two hours following the first ingestion, and after 7, 28 and 30 days of supplementation, subjects completed two moderate-intensity step exercise tests. On days 28 and 30, subjects in the NO_3^- groups completed the test 2 h post consumption of a NO_3^- dose (CHR + ACU) and a placebo dose (CHR).
Results: Plasma nitrite concentration ($[NO_2^-]$) was elevated in a dose-dependent manner at 2 h, 7 days and 28–30 days on the CHR + ACU visit. Compared to pre-treatment baseline, 6 mmol NO_3^- reduced the steady-state $\dot{V}O_2$ during moderate-intensity exercise by 3% at 2 h ($P = 0.06$), 7 days and at 28–30 days (both $P < 0.05$) on the CHR + ACU visit, but was unaffected by 3 mmol NO_3^- at all measurement points. On the CHR visit in the 6 mmol group, plasma $[NO_2^-]$ had returned to pre-treatment baseline, but the steady-state $\dot{V}O_2$ remained reduced.
Conclusion: Up to ~4 weeks supplementation with 6 but not 3 mmol NO_3^- can reduce submaximal exercise $\dot{V}O_2$. A comparable reduction in submaximal exercise $\dot{V}O_2$ following chronic supplementation with 6 mmol NO_3^- can be achieved both with and without the acute ingestion of NO_3^- and associated elevation of plasma $[NO_2^-]$.

1. Introduction

Dietary nitrate (NO_3^-) has been reported to influence several physiological processes via its reduction to nitrite (NO_2^-), and subsequently to the physiological signaling molecule nitric oxide (NO) [1–3]. Indeed, acute ingestion (1–3 h) and chronic supplementation (3–15 days) with dietary NO_3^- has been found to increase plasma NO_2^- concentration $[NO_2^-]$, and, in some studies, to reduce the oxygen (O_2) cost of submaximal exercise [4–6] and improve exercise tolerance [5–7] in healthy, young adults (see Ref. [8] for review). However, the influence of different supplementation strategies on the physiological effects of NO_3^- ingestion is still not fully understood.

Whilst a reduction in the O_2 cost of submaximal exercise has been reported 1–3 h post consumption of 5.2–16.8 mmol NO_3^- in some studies [6,9,10], reports on the effect of ingesting a lower dose (i.e. 4.2 mmol NO_3^- or below) are ambiguous [6,11]. It has been suggested that chronic supplementation with NO_3^- may represent a more effective supplementation strategy [8,10]. Indeed, 3–7 days of NO_3^- supplementation has been reported to improve mitochondrial efficiency [12] and contractile function [13] by altering the expression of mitochondrial [12] and contractile [13] proteins in skeletal muscle. These structural adaptations are likely to be responsible, in part, for the reduction in submaximal O_2 uptake ($\dot{V}O_2$) after chronic NO_3^- supplementation, but would unlikely be manifest within 1–3 h after the ingestion of a single NO_3^- bolus.

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; CHR, effect of chronic nitrate supplementation alone assessed; CHR + ACU, combined effect of chronic and acute nitrate supplementation assessed; GET, gas exchange threshold; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; O_2, oxygen; PLA, placebo; RPM, revolutions per minute; $\dot{V}O_2$, oxygen uptake; $\dot{V}O_{2peak}$, peak oxygen uptake; W, watts; Δ, change.

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Given this potential benefit of a chronic NO_3^- supplementation period, it may be reasoned that a significant and consistent reduction in submaximal exercise $\dot{V}O_2$ could be achieved with a low dose of NO_3^- if consumed daily for an extended period of time. However, the effect of chronic supplementation with a low dose of NO_3^- (i.e. 4.2 mmol or lower) on the O_2 cost of submaximal exercise has not yet been investigated. Every nutritional supplement has an associated risk-benefit relationship and financial cost. Determining if a low dose of NO_3^- consumed chronically can elicit beneficial physiological effects is therefore important to guide supplementation procedures and ensure individuals do not consume more NO_3^- than is required.

To fully understand the physiological effects of chronic and acute supplementation strategies, it is important to examine the effects of each strategy independently. In all previous studies assessing the influence of chronic NO_3^- supplementation on the physiological responses to exercise, subjects have been instructed to consume their final dose of NO_3^- 1–3 h prior to final exercise testing, to ensure a significant elevation in plasma $[NO_2^-]$ [e.g. 4,5,7,9]. However, this experimental design only allows for the combined chronic and acute effect of NO_3^- ingestion to be investigated. Indeed, it is possible that: a) any chronic adaptions to daily NO_3^- intake would be detectable in the absence of an acute NO_3^- dose and thus, no significant elevation in plasma $[NO_2^-]$; and b) any chronic effects of NO_3^- supplementation may be augmented if an acute NO_3^- dose is consumed. The adoption of an experimental approach in which the chronic effects of NO_3^- exposure can be isolated from those of the acute effects is therefore warranted. This will provide important practical information to guide athletes on optimal supplementation strategies before competition after a period of chronic NO_3^- supplementation.

The purpose of this study was therefore twofold: firstly, to compare the effects of acute (2 h), 7 d and ~30 d supplementation, with a low dose (3 mmol) and a moderate dose (6 mmol; positive control) of dietary NO_3^- on the O_2 cost of moderate-intensity exercise; and, secondly, to compare the effect of ~30 day supplementation on the O_2 cost of moderate-intensity exercise with and without the acute consumption of NO_3^- 2 h prior to assessment. To achieve the latter, subjects completed an experimental visit on day 28 and 30 of supplementation: on one visit subjects consumed an acute dose of NO_3^- 2 h prior to testing (CHR + ACU); and on the other visit, exercise was initiated 24 h post consumption of the most recent NO_3^- dose (CHR), allowing plasma $[NO_2^-]$ to return to pre-NO_3^- treatment baseline [6]. It was hypothesized that: a) supplementation with 3 mmol NO_3^- and 6 mmol NO_3^- would result in a lower O_2 cost of submaximal exercise at 7 d and 28–30 d on the CHR + ACU visit; and, b) any effects observed with CHR + ACU would still be present, albeit to a lesser extent, with CHR.

2. Methods

2.1. Subjects

Thirty-four healthy, recreationally-active subjects (19 male, mean ± SD age = 21 ± 3 yr, stature = 1.74 ± 0.09 m, body mass = 73.5 ± 14.1 kg) volunteered to participate, gave written informed consent, and completed this study that was approved by the Institutional Research Ethics Committee. All subjects were nonsmokers and none were taking any nutritional supplements in the four months preceding the start of the study. Throughout the experimentation, subjects were asked to adhere to their normal exercise routine and diet. However, subjects were instructed to avoid foods rich in NO_3^- (such as green leafy vegetables and beetroot) and asked to record their diet and exercise in the 48 h preceding the first laboratory visit and to repeat this prior to all subsequent visits. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 2 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Each subject was also asked to avoid caffeine and alcohol 6 and 24 h before each test, respectively. In addition, subjects were asked to abstain from using antibacterial mouthwash and chewing gum for the duration of the study since this inhibits the reduction of NO_3^- to NO_2^- in the oral cavity [14]. All laboratory visits were scheduled at the same time of day (±1 h) for each subject.

2.2. Pre-treatment tests

Subjects were required to report to the laboratory on two separate occasions prior to beginning 30 days of supplementation with either 3 mmol NO_3^-, 6 mmol NO_3^-, or placebo (PLA). All exercise tests were performed on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During *visit 1*, subjects completed a ramp incremental exercise test to the limit of tolerance for the determination of peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$) and gas exchange threshold (GET). The subjects cycled at a constant self-selected pedal rate (80–90 rpm), and this pedal rate, along with saddle and handle bar height configuration, was recorded and reproduced in subsequent tests. Initially, subjects performed 3 min of baseline cycling at 20 W, after which the work rate increased at a rate of 30 W min^{-1} in a linear fashion until the limit of tolerance. The test was terminated when the pedal rate fell by > 10 rpm below the chosen pedal rate, despite strong verbal encouragement. The power output achieved at the point of exhaustion was recorded as the peak power output (PPO). Pulmonary gas exchange was continuously collected breath-by-breath during the incremental tests and averaged over consecutive 10-s periods. The $\dot{V}O_{2peak}$ was calculated as the highest 30-s mean value attained before the subject's volitional exhaustion. The GET was determined as described previously [15,16]. The work rate that would require 80% of the GET (moderate exercise) was then 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 GET; [17]).

During *visit 2*, subjects completed a series of PLA-controlled, baseline measurements. All subjects were asked to consume a 50 mL dose of PLA containing negligible NO_3^- (see description under *Supplementation procedures* for further details), 2 h prior to arrival at the laboratory. Upon arrival a venous blood sample was obtained for the measurement of plasma $[NO_3^-]$ and $[NO_2^-]$. Subjects then completed two step transitions to moderate-intensity cycling at 80% GET, with each bout separated by 5-min of passive recovery. Each step transition was preceded by 3-min of baseline cycling at 20 W and each bout lasted 5 min. Pulmonary $\dot{V}O_2$ was measured breath-by-breath throughout the test and averaged over 10-s periods. The data from the two moderate-intensity bouts were time aligned and averaged to improve signal-to-noise ratio [18]. The O_2 cost of moderate-intensity exercise was then calculated. All measurements made during this visit were used as pre-treatment baseline data.

2.3. Supplementation procedures

After these pre-treatment visits, subjects were assigned in an independent group-matched fashion for moderate-intensity end-exercise $\dot{V}O_2$, PPO, GET and $\dot{V}O_{2peak}$, to receive either ~3 mmol NO_3^- (administered as 9.75 g of dry beetroot extract [PepsiCo, USA] diluted in 50 mL of water), ~6 mmol NO_3^- (administered as 19.5 g of dry beetroot extract [PepsiCo, USA] diluted in 50 mL of water), or a PLA (containing ~0.01 mmol of NO_3^-, administered as 9.5 g of sucrose and red shade coloring [PepsiCo, USA] diluted in 50 mL of

water) per day, for 30 days. Subject characteristics are provided in Table 1.

Subjects reported to the laboratory on *days 1, 7, 28 and 30* of supplementation to complete the full experimental protocol from *visit 2* (Fig. 1). On supplementation *days 1* and *7*, subjects were asked to consume their 1st and 7th dose of supplement, respectively, 2 h prior to arrival at the laboratory. This pre-test consumption time was selected in order to coincide with the peak plasma [NO_2^-]. While we have previously established that peak plasma [NO_2^-] is attained 2 h post beetroot juice consumption [6], it could not be assumed that this time-to-peak is consistent after consumption of other NO_3^- containing supplements because the kinetics of plasma [NO_2^-] may be altered by the form in which the NO_3^- is administered [19]. We therefore undertook a pilot study which indicated that peak plasma [NO_2^-] occurred at 2 h post consumption of the dry beetroot extract (*unpublished observation*). On non-experimental days, each subject was instructed to consume their allocated supplement at their test time from *visit 2* (±10 min), such that the subject would consume one dose of supplement every 24 h.

To assess the effect of chronic NO_3^- supplementation on the O_2 cost of submaximal exercise with and without the acute consumption of NO_3^- 2 h prior to exercise, a randomised counterbalanced design was adopted from day 28 of supplementation for subjects in the 3 mmol and 6 mmol groups (Fig. 1). Two hours prior to arrival on *day 28* of supplementation, subjects in the 3 mmol and 6 mmol groups were asked to consume either a PLA beverage [i.e. effect of chronic NO_3^- supplementation alone (CHR) was assessed] (3 mmol: n = 6; 6 mmol: n = 6) or their allocated NO_3^--rich beverage [i.e. combined effect of chronic and acute NO_3^- supplementation (CHR + ACU) was assessed] (3 mmol: n = 5; 6 mmol: n = 6). Ten-minutes post completion of the test procedures on *day 28*, subjects in the 3 mmol and 6 mmol group who consumed PLA prior to the test, consumed a dose of their allocated NO_3^--rich beverage, and those that consumed their NO_3^--rich beverage consumed a PLA beverage. Two hours prior to arrival at the laboratory on *day 30*, subjects consumed the opposite beverage to that consumed prior to their laboratory visit on *day 28* [e.g. if PLA was consumed prior to arrival on *day 28*, then their allocated NO_3^--rich beverage was consumed prior to arrival on *day 30*]. Therefore, all subjects were assessed for the CHR + ACU and CHR effects of NO_3^- supplementation. In all cases, subjects in the 3 mmol and 6 mmol groups consumed 3 mmol NO_3^- or 6 mmol NO_3^-, respectively, per day. Subjects in the PLA group were instructed to continue their normal supplementation routine and consume one dose of PLA 2 h prior to arrival at the laboratory on both *day 28* and *30* of supplementation.

Subjects in the 3 mmol and 6 mmol groups were deliberately misinformed that the effects of two different NO_3^- supplements on the physiological responses of exercise would be tested after different supplementation periods. Subjects in the PLA group were informed that the effect of a NO_3^- supplement would be tested over different supplementation periods. All subjects were asked to not comment on the taste or appearance of the supplements to the study investigators. Follow up verbal interviews after completion of the study confirmed that subjects were unaware of the actual research hypothesis.

Table 1
Subject characteristics. Data expressed as mean ± SD.

	PLA	3 mmol NO_3^-	6 mmol NO_3^-
n	11 (7 male)	11 (6 male)	12 (6 male)
Age (yr)	21 ± 4	21 ± 2	22 ± 3
Weight (kg)	75.9 ± 20.4	74.4 ± 9.8	70.5 ± 10.4
Height (m)	1.74 ± 0.11	1.73 ± 0.08	1.75 ± 0.09

2.4. Measurements and data analysis

During all exercise tests, pulmonary gas exchange and ventilation were measured breath-by-breath, with subjects wearing a nose clip and breathing through a low dead space, low-resistance 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 (Jaeger Oxycon Pro, 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 with a 3-L syringe (Hans Rudolph, Kansas City, MO). Pulmonary gas exchange and ventilation were calculated and displayed breath-by-breath.

The breath-by-breath gas exchange data collected during the two identical, moderate-intensity step tests performed at pre-treatment baseline on *visit 2* and on *days 1, 7, 28 and 30* of supplementation, were averaged over 10-s periods, and time aligned to the start of exercise and ensemble-averaged. The end-exercise $\dot{V}O_2$ was defined as the mean value measured over the final 60 s of exercise.

Venous blood samples were drawn into lithium-heparin vacutainers (7.5 mL Monovette lithium heparin; Sarstedt, Leicester, UK). Within 1 min of collection, samples were centrifuged at 4000 rpm and 4 °C for 8 min. Plasma was extracted and immediately frozen at −80 °C for later analysis of [NO_3^-] and [NO_2^-] as previously described [6].

Capillary blood samples were collected from a fingertip into a capillary tube during the 20 s preceding each step transition in work rate and within the final 20 s of each moderate-intensity bout. These samples were subsequently stored on ice and analyzed to determine blood [lactate] (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH), within 5 min of collection.

2.5. Statistical analysis

A mixed-model ANOVA was used to assess differences in change from pre-treatment baseline, in all variables across treatment (PLA, 3 mmol NO_3^- and 6 mmol NO_3^-) and time (2 h and 7 d, and the CHR + ACU visit on day 28 or 30 of supplementation). Differences over time within each group were analyzed by a single factor repeated-measures ANOVA. Before these analyses, all data collected on *days 28* and *30* of supplementation in the PLA group were averaged together to form one data point at *days 28* and *30* of supplementation. The effect of CHR on all variables in 3 mmol and 6 mmol was analyzed separately using a single factor repeated measures (pre-treatment baseline, CHR and CHR + ACU) ANOVA. In all cases, significant effects were further explored using simple contrasts. All data analyses were performed using the SPSS (version 22; SPSS, Chicago, IL) statistical package, with statistical significance accepted at $P < 0.05$. Results are presented as mean ± SD unless stated otherwise.

3. Results

Self-reported compliance to the supplementation regime was 100% in all treatment groups. Subjects reported that their diet and exercise habits prior to each experimental test were consistent.

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

Plasma [NO_3^-] and [NO_2^-] responses in PLA, 3 mmol and 6 mmol

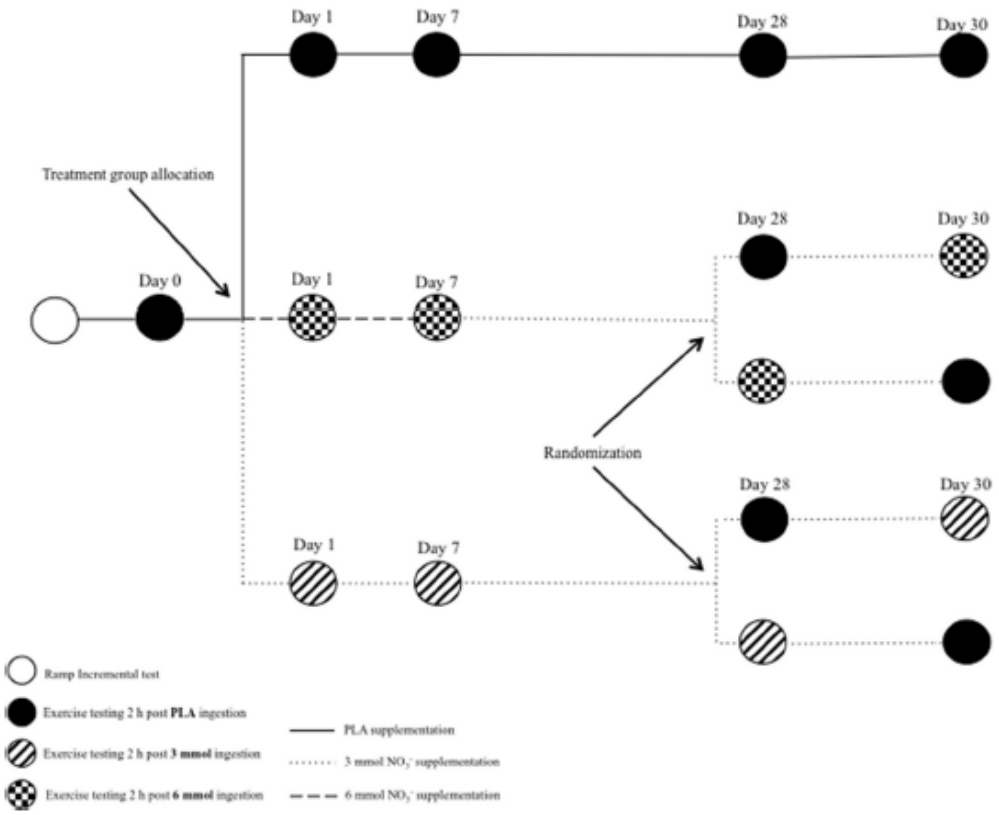

Fig. 1. Schematic illustration of the study design. Following pre-treatment tests, all subjects were allocated to receive 6 mmol NO_3^-, 3 mmol NO_3^- or placebo for 28–30 days. All subjects completed two moderate-intensity bouts of cycle, 2 h post-ingestion (day 1; acute), and after 7, 28 and 30 days of supplementation. In the 3 mmol and 6 mmol NO_3^- groups, subjects were randomised to receive a dose of placebo or NO_3^- 2 h prior to exercise testing in a counterbalanced order on days 28 and 30 of supplementation. This design enabled the efficacy of chronic NO_3^- supplementation in the absence and presence of an acute NO_3^- dose on the day of exercise testing to be determined. *See main text for more details.*

groups are presented in Figs. 2 and 3.

3.1.1. 2 h, 7 d and 28–30 day (CHR + ACU)

At pre-treatment baseline, plasma [NO_3^-] was not significantly different between treatment groups (PLA: 33 ± 11 μM; 3 mmol: 46 ± 24 μM; 6 mmol: 36 ± 11 μM: $P > 0.05$). The change in plasma [NO_3^-] from pre-treatment baseline at 2 h, 7 d and on the CHR + ACU visit after 28–30 d is shown in Fig. 2A. Plasma [NO_3^-] was significantly elevated above pre-treatment baseline and PLA at all time points in the 3 mmol and 6 mmol groups (all $P < 0.05$; Fig. 2A). The elevation in plasma [NO_3^-] above pre-treatment baseline in the 6 mmol group was greater than in the 3 mmol group at all time points (all $P < 0.05$; Fig. 2A). The mean increase in plasma [NO_3^-] from pre-treatment baseline, in the 3 mmol and 6 mmol groups across the three sample points was 313% and 867%, respectively (3 mmol: 144 ± 23 μM; 6 mmol: 312 ± 68 μM; both $P < 0.05$; Fig. 2A). No changes in plasma [NO_3^-] were observed in the PLA group at any time point (all $P > 0.05$). The rise in plasma [NO_3^-] from pre-treatment baseline was not significantly different between the three sampling time points within any treatment group (all $P > 0.05$).

At pre-treatment baseline, plasma [NO_2^-] was not significantly different between treatment groups (PLA: 43 ± 19 nM; 3 mmol: 80 ± 69 nM; 6 mmol: 48 ± 22 nM: $P > 0.05$). The change in plasma [NO_2^-] from pre-treatment baseline at 2 h, 7 d and on the CHR + ACU after 28–30 d is shown in Fig. 2B. In the 3 mmol and 6 mmol groups, plasma [NO_2^-] was significantly elevated at all time points (all $P < 0.05$; Fig. 2B), relative to pre-treatment baseline and PLA. In addition, the rise in plasma [NO_2^-] was greater in the 6 mmol group than in the 3 mmol group at all time points (all $P < 0.05$; Fig. 2B). Across the three sample points, plasma [NO_2^-] rose by 165% and 579%, above pre-treatment baseline, in the 3 mmol (132 ± 49 nM) and 6 mmol (278 ± 155 nM; all $P < 0.05$; Fig. 2B) groups, respectively. No changes in plasma [NO_2^-] were observed in the PLA group at any time point (all $P > 0.05$). The rise in plasma [NO_2^-] from pre-treatment baseline was not significantly different between the three sampling time points within any treatment group (all $P > 0.05$).

3.1.2. Comparison between chronic and chronic + acute supplementation

The change in plasma [NO_3^-] from pre-treatment baseline was significantly lower on the CHR visit compared to the CHR + ACU visit in both the 3 mmol and 6 mmol groups ($P < 0.05$; Fig. 3A). In the 3 mmol group, plasma [NO_3^-] had returned to pre-treatment baseline on the CHR visit ($P > 0.05$; Fig. 3A). In the 6 mmol group, the plasma [NO_3^-] remained elevated above pre-treatment baseline by 34 ± 36 μM ($P < 0.05$; Fig. 3A) on the CHR visit.

The change in plasma [NO_2^-] from pre-treatment baseline was significantly lower on the CHR visit compared to CHR + ACU visit in the 3 mmol and 6 mmol groups ($P < 0.05$; Fig. 3B). Plasma [NO_2^-] had returned to pre-treatment baseline on the CHR visit in both the 3 mmol and 6 mmol groups ($P > 0.05$; Fig. 3B).

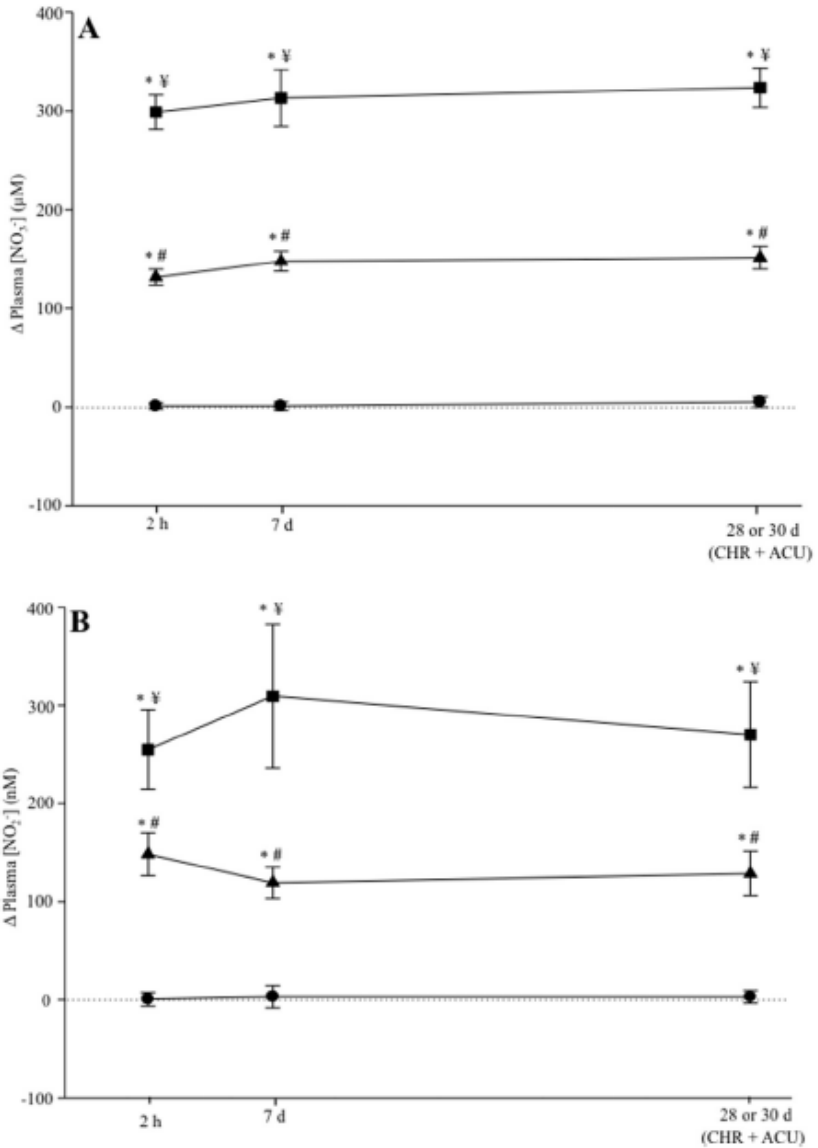

Fig. 2. Change (Δ) relative to pre-treatment baseline in plasma [NO_3^-] (**panel A**) and [NO_2^-] (**panel B**) following acute (2 h), 7 d and 28–30 d (*CHR + ACU*) supplementation with 6 mmol NO_3^- (squares), 3 mmol NO_3^- (triangles) and placebo (circles). Values are mean ± SE. * significant difference from pre-treatment baseline ($P < 0.05$); # significant difference from placebo group ($P < 0.05$); ¥ significant difference from placebo and 3 mmol NO_3^- group ($P < 0.05$).

3.2. Moderate-intensity exercise

At pre-treatment baseline, the $\dot{V}O_2$ measured over the final 60-s of moderate intensity exercise was not significantly different between treatment groups (PLA: 1.46 ± 0.32 L min^{-1}; 3 mmol: 1.44 ± 0.23 L min^{-1}; 6 mmol: 1.44 ± 0.30 L min^{-1}: $P > 0.05$).

3.2.1. 2 h, 7 d and 28–30 day (CHR + ACU)

The change in end-exercise $\dot{V}O_2$ from pre-treatment baseline at 2 h, 7 d and on the CHR + ACU visit after 28–30 d is shown in Fig. 4A. In the 3 mmol and PLA groups, end-exercise $\dot{V}O_2$ was not significantly different from pre-treatment baseline at any time point (all $P > 0.05$; Fig. 4A). Compared to pre-treatment baseline, end-exercise $\dot{V}O_2$ was significantly reduced in the 6 mmol NO_3^- group at 7 d (by ~3%; 0.04 ± 0.05 L min^{-1}) and 28–30 d on the CHR + ACU visit (by ~3%; 0.04 ± 0.04 L min^{-1}; both $P < 0.05$; Fig. 4A), and tended to be reduced at 2 h (by ~3%; 0.04 ± 0.02 L min^{-1}; $P = 0.06$; Fig. 4A). Compared to PLA, end-exercise $\dot{V}O_2$ was significantly reduced in the 6 mmol group at 28–30 d on the CHR + ACU visit ($P < 0.05$; Fig. 4A) and tended to be reduced at 7 d ($P = 0.08$; Fig. 4A). Compared to pre-treatment baseline and PLA, baseline, end-exercise and change in blood [lactate] were not altered by 3 mmol or 6 mmol NO_3^- at 2 h, 7 d or 28–30 d on the CHR + ACU visit ($P > 0.05$).

3.2.2. Comparison between chronic and chronic + acute supplementation

In the 3 mmol group, end-exercise $\dot{V}O_2$ was not different from pre-treatment baseline at 28–30 d on either the CHR or CHR + ACU visits ($P > 0.05$). In the 6 mmol group, end-exercise $\dot{V}O_2$ was

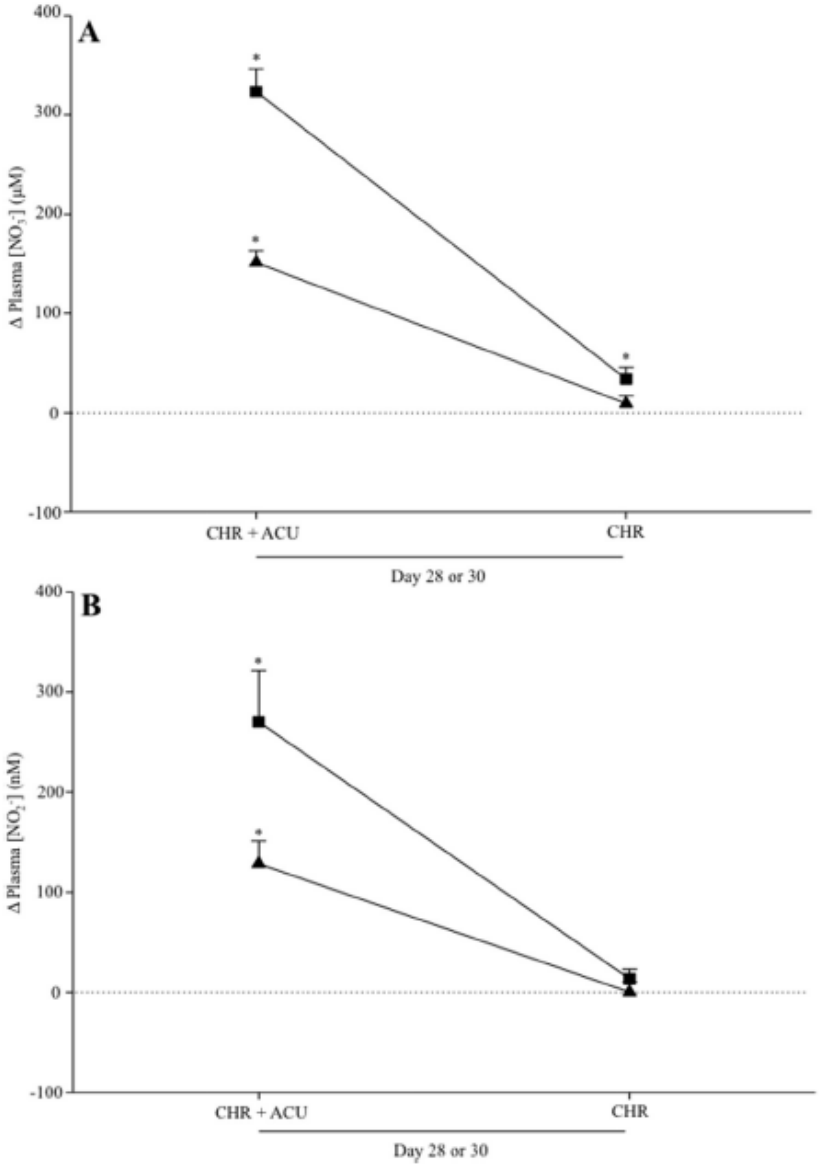

Fig. 3. Change (Δ) relative to pre-treatment baseline in plasma [NO_3^-] (**panel A**) and [NO_2^-] (**panel B**) following 28–30 d supplementation with 6 mmol NO_3^- (squares) and 3 mmol NO_3^- (triangles) when subjects consumed an acute dose of NO_3^- (*CHR + ACU*) or placebo (*CHR*) 2 h prior to assessment. Values are mean ± SE. * significant difference from pre-treatment baseline ($P < 0.05$).

significantly lower than pre-treatment baseline at 28–30 days on the CHR visit (by ~3.5%; 0.05 ± 0.06 L min^{-1}; $P < 0.05$; Fig. 4B). This reduction in end-exercise $\dot{V}O_2$ was not significantly different from that observed on the CHR + ACU visit ($P > 0.05$; Fig. 4B).

4. Discussion

The principal novel finding of the present study was that neither 7 days nor ~4 weeks of supplementation with 3 mmol NO_3^-, per day, reduced the O_2 cost of moderate-intensity cycle exercise, despite an increase in plasma [NO_3^-] and [NO_2^-] throughout the supplementation period. In contrast, the greater rise in plasma [NO_3^-] and [NO_2^-] with 6 mmol NO_3^- was associated with a significant reduction in the O_2 cost of moderate-intensity cycle exercise after 7 days and ~4 weeks of supplementation, and a trend ($P = 0.06$) for a reduction after acute (2 h) ingestion. Interestingly, this reduction in moderate-intensity $\dot{V}O_2$ after ~4 weeks of supplementation was still evident when assessed on a separate occasion without the ingestion of NO_3^- 2 h prior to the exercise test and thus, no elevation in plasma [NO_2^-]. This study is the first to demonstrate a lowering of submaximal $\dot{V}O_2$ following chronic NO_3^- supplementation without a significant concomitant increase in systemic [NO_2^-].

4.1. The influence of 2 h, 7 d and 28–30 d (chronic + acute) NO_3^- supplementation

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

The administration of beetroot extract containing 3 mmol and 6 mmol NO_3^- was successful in dose-dependently elevating plasma [NO_3^-] and [NO_2^-], a finding consistent with that observed after the

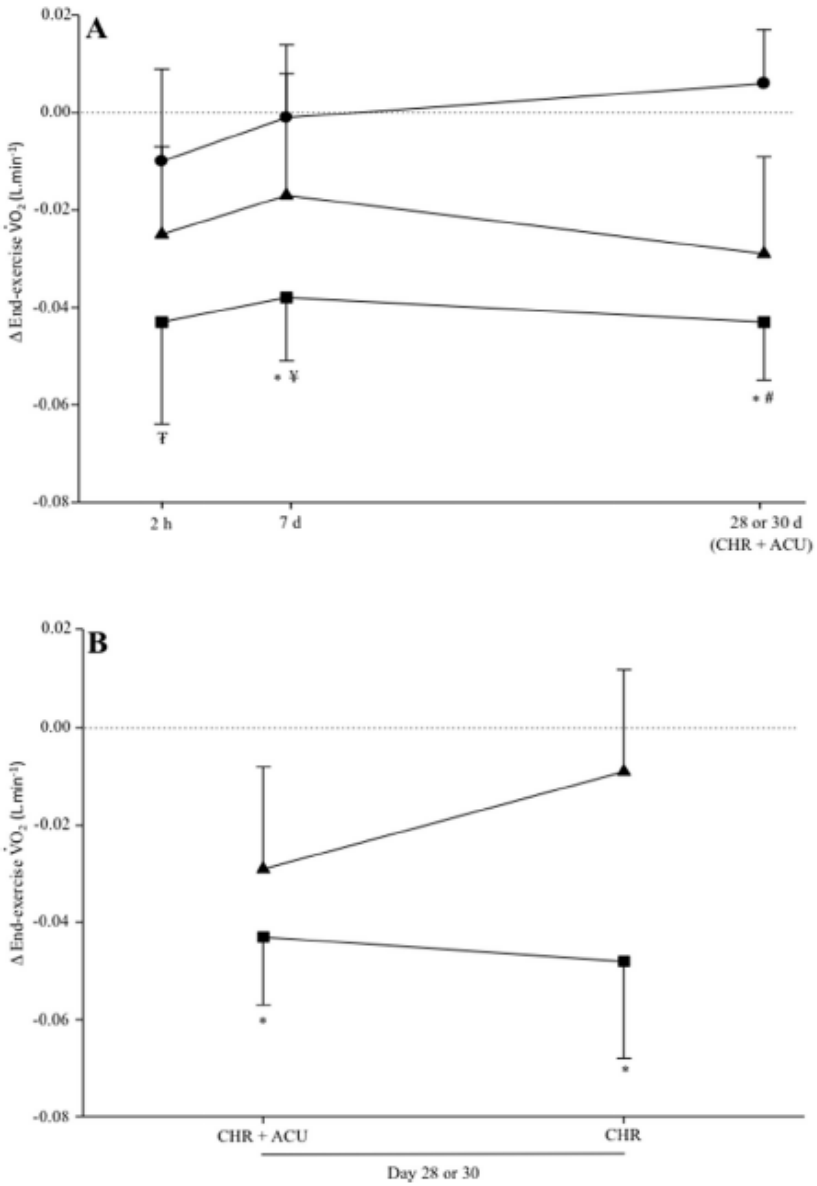

Fig. 4. The effect of NO_3^- supplementation on end-exercise $\dot{V}O_2$ during moderate-intensity cycle. **Panel A**: Change (Δ) relative to pre-treatment baseline in end-exercise $\dot{V}O_2$ following acute (2 h), 7 d and 28–30 d (*chronic + acute*) supplementation with 6 mmol NO_3^- (squares), 3 mmol NO_3^- (triangles) and placebo (circles). **Panel B**: Change (Δ) relative to pre-treatment baseline in end-exercise $\dot{V}O_2$ following 28–30-days supplementation with 6 mmol NO_3^- (squares) and 3 mmol NO_3^- (triangles) when subjects consumed a dose of NO_3^- (*CHR + ACU*) or placebo (*CHR*) 2 h prior to assessment. Values are mean ± SE. $\dot{V}O_2$, oxygen uptake. * Significant difference from pre-treatment baseline ($P < 0.05$); ₮ trend towards difference from pre-treatment baseline ($P < 0.10$); # significant difference from placebo group ($P < 0.05$); ¥ trend towards difference from placebo group ($P < 0.10$).

ingestion of concentrated NO_3^--rich beetroot juice [6] and pharmaceutical NO_3^- salt [20]. In both the 3 mmol and 6 mmol groups, there was no further increase in plasma $[NO_3^-]$ or $[NO_2^-]$ after 7 days or 28−30 days supplementation (when an acute bolus of NO_3^- was consumed 2 h prior to assessment), compared to the acute ingestion of a single bolus of NO_3^- on day 1. This finding is also in line with previous observations [9,21].

4.1.2. O_2 cost of submaximal exercise

In the present study we found that the steady-state $\dot{V}O_2$ measured over the final 60 s of moderate-intensity cycle exercise was unaffected by the acute ingestion of 3 mmol NO_3^- but tended ($P = 0.06$) to be lower by 3% following acute ingestion of 6 mmol NO_3^-. Several previous studies have also reported a dose-response relationship, with higher but not lower doses of dietary NO_3^- eliciting physiological effects [6,22,23]. In particular, we have previously reported that the O_2 cost of submaximal cycling was unaffected by the acute administration of 4.2 mmol NO_3^-, tended to be reduced (by 1.7%) following administration of 8.4 mmol NO_3^-, and was significantly reduced (by 3%) following administration of 16.8 mmol NO_3^-, administered in the form of concentrated beetroot juice [6]. The reason for the greater relative reduction in submaximal exercise $\dot{V}O_2$ for a given NO_3^- dose in the present study (i.e. 3% reduction with 6 mmol NO_3^-) compared to that reported by us

previously (i.e. 1.7% reduction with 8.4 mmol NO_3^-) [6] is unclear. However, despite this, the results presented herein and by us previously [6] support a dose-dependent effect of acute NO_3^- ingestion on submaximal exercise $\dot{V}O_2$, and suggest that higher doses of NO_3^- (≥6 mmol NO_3^-) are required to acutely lower the O_2 cost of submaximal exercise.

This is the first study to investigate the effect of chronic supplementation with a low dose of NO_3^- on the O_2 cost of moderate-intensity exercise. Contrary to our experimental hypothesis, we found that prolonging the supplementation period with 3 mmol NO_3^- to 7 days, and further to ~4 weeks, did not lower the O_2 cost of submaximal cycle exercise. These results suggest that a reduction in submaximal exercise $\dot{V}O_2$ cannot be achieved with a low dose of NO_3^- even with an extension of the supplementation period. In contrast, we found that the O_2 cost of submaximal exercise was significantly lower than pre-treatment baseline after both 7 days and ~4 weeks of supplementation with 6 mmol NO_3^-. These findings are in agreement with the 3–6% reduction in the O_2 cost of submaximal exercise reported in untrained subjects following 3–15 days of supplementation with 5–8 mmol NO_3^- [4,5,9,12,24]. Therefore, these results indicate that the daily ingestion of 6 mmol NO_3^- reduces the O_2 cost of submaximal exercise after short duration (7 day) supplementation, and that this effect is maintained over ~4 weeks of continued supplementation with no evidence of a reduced sensitivity to supplementation.

The lowering of submaximal exercise $\dot{V}O_2$ may be a result of improved mitochondrial efficiency [12,25,cf. 26] and/or a reduction in the ATP cost of skeletal muscle force production [27]. Alterations in the expression, and therefore content, of mitochondrial [12] and contractile proteins [13] have been proposed as the mechanistic bases for these effects. However, alterations in protein expression are unlikely to occur within 1–3 h of acute NO_3^- ingestion, and can therefore only contribute to a reduction in submaximal exercise $\dot{V}O_2$ after a period of chronic NO_3^- supplementation. Instead, a reduction in $\dot{V}O_2$ following acute NO_3^- ingestion may be related to an acute and reversible change in protein function through post-translational protein modifications [28]. Given the potential for the protein expression changes to occur after chronic supplementation but not acute ingestion, we hypothesized that the prolonged exposure to elevated concentrations of NO_2^- provided by a chronic supplementation regime may reduce the dose of NO_3^- required to lower submaximal exercise $\dot{V}O_2$. In contrast to this hypothesis we found that extending the duration of supplementation on a low dose of NO_3^- to ~4 weeks did not result in a reduction in submaximal exercise $\dot{V}O_2$. This observation implies that these structural modifications are not only dependent on the duration of exposure but also the magnitude of the exposure. The influence of different NO_3^- doses and durations of supplementation on structural modifications to skeletal muscle warrants further investigation.

4.2. The effect of chronic NO_3^- supplementation in the absence of acute NO_3^- ingestion

In all previous studies examining the effect of chronic NO_3^- supplementation on the physiological responses to exercise, subjects have been instructed to consume their final dose of NO_3^- 1–3 h prior to testing [4,5,9,12,27,29]. This experimental approach has been adopted to ensure a significant elevation in plasma $[NO_2^-]$ (and therefore the potential for O_2-independent NO synthesis) at the time of assessment. However, considering that: 1) for a given dose, the rise in plasma $[NO_2^-]$ afforded by this chronic supplementation strategy is not different to that afforded by a single acute bolus of NO_3^- [9,21]; 2) acute NO_3^- supplementation may reduce submaximal exercise $\dot{V}O_2$ and improve exercise tolerance [6]; and 3) the acute effects of NO_3^- are believed to be mediated via an elevation in plasma $[NO_2^-]$ [6,9]; it is not possible to fully ascertain if the effects observed after chronic NO_3^- supplementation would still be evident when an acute NO_3^- dose is not administered. Therefore, in the present study, we examined the effects of chronic NO_3^- supplementation both with and without administration of an acute NO_3^- dose. Specifically, in the 'without' condition, subjects were asked to consume their final dose of NO_3^- 24 h prior to exercise testing. Importantly, 24 h provided sufficient time for plasma $[NO_2^-]$ to return to pre-treatment baseline, a finding that is in agreement with the pharmacokinetics of plasma $[NO_2^-]$ following acute ingestion of similar NO_3^- doses [6,20].

An original finding of the present study was that moderate-intensity steady-state $\dot{V}O_2$ was significantly lowered following ~4 weeks of supplementation with 6 mmol NO_3^-, even when 24 h had elapsed since the ingestion of the last NO_3^- dose. To our knowledge, these results are the first to demonstrate a reduction in submaximal exercise $\dot{V}O_2$ following NO_3^- supplementation in the absence of an increase in plasma $[NO_2^-]$. However, this observation is consistent with a previous report that the physiological effects of NO_3^- supplementation can be achieved without an accompanying rise in plasma $[NO_2^-]$ [30]. Specifically, Ferguson et al. [30], showed that despite no increase in plasma $[NO_2^-]$ after 5 days of NO_3^- supplementation in rats, microvascular O_2 partial pressure was elevated during contraction in fast-twitch skeletal muscle. Interestingly, the magnitude of reduction in $\dot{V}O_2$ was not significantly different to that observed after ~4 weeks of supplementation when an acute bolus of NO_3^- was consumed prior to testing. These results therefore suggest that following chronic supplementation with 6 mmol NO_3^-, a reduction in the O_2 cost of submaximal exercise may be preserved up to at least 24 h after the final dose of NO_3^- is ingested, and that the addition of an acute NO_3^- dose, and the resulting elevation in plasma $[NO_2^-]$, does not augment the reduction in submaximal exercise $\dot{V}O_2$. The mechanistic basis for this preserved reduction in $\dot{V}O_2$ is currently unclear. However, as briefly discussed earlier, the reduction in submaximal $\dot{V}O_2$ following chronic NO_3^- supplementation may be mediated, in part, by a change in the content of mitochondrial [12,26] and contractile [13] proteins, and an associated improvement in mitochondrial [12,cf. 26] and contractile [13,27] efficiency. It is likely that such structural adaptations are maintained for some period of time after NO_3^- supplementation is stopped, resulting in the preserved reduction in steady-state $\dot{V}O_2$. Indeed, the increased expression of mitochondrial proteins following sprint interval training have been observed up to 6 weeks after training was ceased [31].

Another possible explanation for the preserved reduction in steady-state $\dot{V}O_2$ is that other reactive nitrogen intermediates and/or NO bioavailability remained elevated post NO_3^- supplementation, despite plasma $[NO_2^-]$ returning to pre-treatment baseline. Previous research in rodents has shown that skeletal muscle tissue acts as an endogenous NO_2^- and NO_3^- reservoir [32] and that NO_2^- infusion increases the concentration of NO_2^- in heart, liver and kidney tissue [33]. If this uptake is also true for human skeletal muscle following NO_3^- supplementation, it is possible that the pharmacokinetics of these changes are different to that of plasma $[NO_2^-]$ and $[NO_3^-]$, and as a result, tissue NO_2^- may accumulate and remain elevated beyond 24 h after the cessation of NO_3^- supplementation. An increase in tissue NO bioavailability may contribute to the reduction in $\dot{V}O_2$ via acutely and reversibly impacting mitochondrial and/or contractile protein function through post-translational protein modifications [28]. The preserved lowering of $\dot{V}O_2$ in the present study may therefore reflect a structural adaptation and/or elevated NO bioavailability in skeletal muscle tissue following ~4 weeks of supplementation with 6 mmol NO_3^-. Further research is required to determine how long the reduction in submaximal exercise $\dot{V}O_2$ may be preserved following cessation of NO_3^- supplementation.

It should be noted that although the acute elevation of plasma $[NO_2^-]$ following chronic NO_3^- supplementation did not augment the reduction in moderate-intensity $\dot{V}O_2$ in the present study, there are other potential benefits of the systemic rise in plasma $[NO_2^-]$ resulting from the ingestion of an acute bolus of NO_3^-. Recent evidence suggests that NO_3^- ingestion significantly improves the perfusion and oxygenation of skeletal muscle, particularly type II muscle fibers, during exercise [30,34]. The vascular effects of NO_3^- consumption are believed to be mediated acutely by NO_2^- or its reduction to the potent vasodilator NO [35,36]. Indeed, an increase in blood flow to exercising forearm muscle has been reported following acute NO_2^- infusion [37]. Moreover, the acute increase in plasma $[NO_2^-]$ following acute NO_3^- ingestion has been closely coupled to a reduction in blood pressure, with both returning back to pre-treatment baseline values simultaneously at 24 h [6,20]. Collectively, these results suggest that improvements in perfusion and oxygenation following NO_3^- intake would not be preserved once NO_3^- supplementation is ceased and plasma $[NO_2^-]$ returns back to baseline. If this is the case, an acute dose of dietary NO_3^- following chronic supplementation may be beneficial before exercise in which improved oxygenation and perfusion in type II muscle fibers may be advantageous, i.e. high-intensity continuous and intermittent exercise. However, it must be acknowledged that a preserved improvement in vascular function could be possible if chronic NO_3^- supplementation results in a greater storage of tissue NO_2^- (as discussed above) and this tissue NO_2^- is released into the circulation during exercise [32]. Therefore, further research is required to determine if any improvement in high-intensity exercise performance following chronic NO_3^- supplementation is preserved after supplementation is stopped and plasma $[NO_2^-]$ returns to baseline. In the present study, we focused on the effects of NO_3^- on submaximal exercise $\dot{V}O_2$ and did not address possible changes in exercise performance.

In conclusion, low dose (3 mmol) NO_3^- supplementation does not significantly reduce the O_2 cost of submaximal cycle exercise acutely, or when the supplementation period is extended to ~4 weeks, despite a significant elevation in plasma $[NO_3^-]$ and $[NO_2^-]$ throughout the supplementation period. In contrast, the greater elevation in plasma $[NO_3^-]$ and $[NO_2^-]$ following 6 mmol NO_3^- supplementation was accompanied by a reduction in the O_2 cost of submaximal cycle exercise after 2 h ($P = 0.06$), 7 days and ~4 weeks (both $P < 0.05$) of supplementation. The present study also demonstrated that the reduction in submaximal exercise $\dot{V}O_2$ after ~4 weeks supplementation with 6 mmol NO_3^- is preserved up to 24 h after the latest dose of NO_3^- is ingested and thus, in the absence of elevated plasma $[NO_2^-]$. To our knowledge, this study is the first to dissociate the reduction in submaximal exercise $\dot{V}O_2$ from an increase in plasma $[NO_2^-]$ following NO_3^- supplementation. This novel observation implies that beneficial structural skeletal muscle adaptations had occurred and were preserved, and/or muscle $[NO_2^-]$ remained elevated following chronic NO_3^- supplementation.

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ORIGINAL ARTICLE

Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance

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Abstract Recent studies have suggested that dietary inorganic nitrate (NO_3^-) supplementation may improve muscle efficiency and endurance exercise tolerance but possible effects during team sport-specific intense intermittent exercise have not been examined. We hypothesized that NO_3^- supplementation would enhance high-intensity intermittent exercise performance. Fourteen male recreational team-sport players were assigned in a double-blind, randomized, crossover design to consume 490 mL of concentrated, nitrate-rich beetroot juice (BR) and nitrate-depleted placebo juice (PL) over ~30 h preceding the completion of a Yo–Yo intermittent recovery level 1 test (Yo–Yo IR1). Resting plasma nitrite concentration ($[NO_2^-]$) was ~400 % greater in BR compared to PL. Plasma $[NO_2^-]$ declined by 20 % in PL ($P < 0.05$) and by 54 % in BR ($P < 0.05$) from pre-exercise to end-exercise. Performance in the Yo–Yo IR1 was 4.2 % greater ($P < 0.05$) with BR (1,704 ± 304 m) compared to PL (1,636 ± 288 m). Blood [lactate] was not different between BR and PL, but the mean blood [glucose] was lower (3.8 ± 0.8 vs. 4.2 ± 1.1 mM, $P < 0.05$) and the rise in plasma $[K^+]$ tended to be reduced in BR compared to PL ($P = 0.08$). These findings suggest that NO_3^- supplementation may promote NO production via the nitrate-nitrite-NO pathway and enhance Yo–Yo IR1 test performance, perhaps by facilitating greater muscle glucose uptake or by better maintaining muscle excitability. Dietary NO_3^- supplementation improves performance during intense intermittent exercise and may be a useful ergogenic aid for team sports players.

Keywords Repeated high-intensity exercise · Nitric oxide · Team sports · Ergogenic aids

Introduction

Emerging research suggests that dietary inorganic nitrate (NO_3^-) supplementation may improve muscle efficiency and fatigue resistance (Bailey et al. 2009, 2010; Hernández et al. 2012; Larsen et al. 2007, 2011). It has been shown that nitrate ingestion results in 15–25 % increase in time-to-exhaustion during severe-intensity, constant work-rate exercise (Bailey et al. 2009, 2010; Lansley et al. 2011b). In addition, some studies have reported a 1–2 % improvement in time-trial performance during high-intensity endurance exercise (Cermak et al. 2012a; Lansley et al. 2011a; Murphy et al. 2012), although these findings have not been confirmed in well-trained athletes (Bescós et al. 2012; Christensen et al. 2013; Wilkerson et al. 2012). Consequently, nitrate supplementation, in the form of nitrate-rich beetroot products, has rapidly gained popularity as an ergogenic aid for athletic performance.

Research to date has primarily focused on the use of nitrate to improve performance in continuous, endurance-type exercise ranging from ~6 min to ~2 h. This neglects team sport players or other athletes whose aim is to maximize performance in intermittent high-intensity exercise. The rapid, repeated transitions from a low to a high metabolic rate in team sport exercise place divergent demands on the energy transfer system compared to continuous

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endurance exercise (Bangsbo et al. 2007; Krustrup et al. 2003, 2006; Mohr et al. 2007). It is presently not known whether the performance-enhancing effects of dietary nitrate that have been observed during continuous performance tests may also be present during team sport-specific intense intermittent exercise.

Phosphocreatine and glycogen degradation is greater in type II than type I muscle fibres during maximal exercise (Greenhaff et al. 1994). Type II muscle fibres are heavily recruited during a transition from low to high metabolic rate (Krustrup et al. 2004, 2009). As this pattern is repeated during multiple sprint sports, the rate at which fatigue develops in type II fibres plays a key role in determining intermittent exercise performance (Colliander et al. 1988; Krustrup et al. 2003, 2006). High-intensity intermittent exercise also results in a high O_2 demand relative to O_2 delivery which may result in the development of muscle hypoxia, as well as low muscle pH and disturbances of ionic balance, factors which likely also contribute to the fatigue process (Krustrup et al. 2003; Mohr et al. 2004).

The physiological effects of nitrate supplementation are likely mediated by the reduction of inorganic NO_3^- to nitrite (NO_2^-) and nitric oxide (NO) (Lundberg and Weitzberg 2010; Lundberg et al. 2011). NO has numerous physiological signalling functions including the regulation of blood flow, muscle contractility, glucose and calcium homeostasis, and mitochondrial O_2 consumption (Stamler and Meissner 2001), factors which may impact on muscle fatigue and performance (Bailey et al. 2011). The reduction of NO_2^- to NO is facilitated when O_2 availability is limited (Lundberg and Weitzberg 2010; Lundberg et al. 2011) and nitrate supplementation appears to be particularly effective in enhancing performance in hypoxia and ischaemia (Kenjale et al. 2011; Vanhatalo et al. 2011; Masschelein et al. 2012). Recent studies suggest that nitrate supplementation may specifically improve calcium handling and force production in type II muscle fibres (Hernández et al. 2012) and result in preferential distribution of blood flow to type II muscles (Ferguson et al. 2013). Given the importance of type II fibre recruitment and metabolism to performance in high-intensity intermittent exercise, and the likelihood that tissue hypoxia develops to a greater extent during such exercise compared to continuous low-intensity exercise, these recent studies therefore provide a clear rationale for nitrate supplementation to enhance performance during multiple sprint sports.

It is important that the influence of nitrate supplementation on intermittent exercise performance is examined using a valid and reliable test. The Yo–Yo Intermittent Recovery, level 1 (IR1) test has been specifically developed to mimic the high-intensity running bouts in football match-play (Bangsbo et al. 2008) and to assess an athlete's fatigue resistance during intermittent exercise taxing both aerobic and anaerobic energy systems (Krustrup et al. 2003). This test has a high reproducibility and is sensitive to changes in fitness status and the competitive level of team sport players (Krustrup et al. 2003; Mohr et al. 2003; Ingebrigtsen et al. 2012) and to training interventions (Iaia et al. 2008; Mohr et al. 2007).

The purpose of this study was to assess the physiological and performance effects of dietary nitrate supplementation on submaximal and exhaustive intermittent exercise using a well-established and ecologically valid field performance test. We hypothesized that dietary nitrate supplementation would increase the distance covered in the Yo–Yo IR1 test.

Methods

Subjects

Fourteen male, recreational team-sport players (mean ± SD: age 22 ± 2 years, body mass 83 ± 10 kg, height 1.80 ± 0.10 m, $\dot{V}O_{2\,max}$: 52 ± 7 mL kg^{-1} min^{-1}) familiar with intense intermittent exercise volunteered to participate in this study. Subjects gave their written informed consent to participate after the experimental procedures, associated risks, and potential benefits of participation had been explained in detail. The study was approved by the Institutional Research Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki.

Experimental design

The subjects reported to the laboratory on three separate occasions over a 12-day period. On visit 1, subjects were familiarized to the Yo–Yo intermittent recovery level 1 test (Yo–Yo IR1; see Krustrup et al. 2003). Subjects were then assigned in a double-blind, randomized, crossover design to receive either NO_3^--rich beetroot juice (BR) or NO_3^--depleted BR (PL). The PL was created by passing standard beetroot juice through an ion-exchange resin specific for NO_3^- (Lansley et al. 2011b). On each experimental visit (2 and 3), the first 5 min of the Yo–Yo IR1 test was first completed to familiarize the subjects with the experimental procedures (including blood drawing from an intravenous cannula) to be employed during the main test. This also served to assess the reproducibility of the physiological responses to repeated intermittent exercise. Then, after a 45 min passive rest period, the full Yo–Yo IR1 test was completed. Blood samples were obtained before, during and after the tests as previously described (Krustrup et al. 2003) and heart rate (HR) was recorded continuously throughout the experiment (Polar RS400, Polar Electro Oy, Kempele, Finland).

The experimental trials were carried out at the same time of the day (±1 h). Subjects were asked to record their food intake in the 24 h preceding the first experimental trial and to replicate this same diet in the 24 h preceding the subsequent trial. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, ≥3 h post-prandial, and to avoid strenuous exercise in the 24 h preceding each experimental trial. Subjects were also asked to refrain from caffeine and alcohol in the preceding 24 h and to abstain from using antibacterial mouthwash and chewing gum throughout the study because this is known to prevent the reduction of nitrate to nitrite in the oral cavity (Govoni et al. 2008).

Experimental protocol

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 test. The Yo–Yo IR1 test was performed indoors on a wooden surface on running lanes with a width of 2 m and a length of 20 m. The Yo–Yo IR1 test has been described and evaluated previously (Bangsbo et al. 2008; Krustrup et al. 2003). In brief, it consists of repeated 20 m runs at a progressively increased speed controlled by audio bleeps from a CD player. Each running bout is interspersed by a 10-s active recovery period where the subject jogs around a marker placed 5 m behind the starting line (for details see Bangsbo and Mohr 2012). When a subject twice fails to reach the finishing line in time, the distance covered is recorded and this represents the test result.

Blood was sampled at rest (baseline) prior to both the submaximal and exhaustive Yo–Yo IR1 tests, as well as after 160, 440 and 600 m during the submaximal test, and at 160, 440, 600, 760, 920, 1,080, 1,240, 1,400 and 1,560 m during the exhaustive test. In addition, blood was collected at 1, 3, 5 and 45 min post the submaximal test, and at exhaustion and at 1, 3, 5, 10 and 15 min post the exhaustive test. Blood lactate and glucose concentrations were analyzed in all samples. Plasma [NO_2^-] and [NO_3^-] were analyzed in samples collected at baseline before both tests; at 600 m during and at 5 and 45 min post the submaximal test; and at 600 m, exhaustion, and at 15 min post the exhaustive test. Plasma [K^+] and [Na^+] were measured in a subsample of the subjects ($n = 7$). Plasma [K^+] and [Na^+] were analyzed in samples collected at baseline; at 600 m during and at 5 and 45 min post the submaximal test; and at 600 m, 1,080 m, exhaustion, and at 5 min post the exhaustive test.

Supplementation

After completion of the familiarization test, the subjects were assigned in a double-blind, randomized, crossover design to consume concentrated NO_3^--rich beetroot juice (BR; organic beetroot juice containing ~4.1 mmol of NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK) and NO_3^--depleted beetroot juice (PL; organic beetroot juice containing ~0.04 mmol NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK). On the day before each experimental trial, subjects were instructed to consume 2 × 70 mL of the beverage in the morning (~10 a.m.) and 2 × 70 mL in the evening (~7 p.m.). On each experimental day, subjects consumed a further 2 × 70 mL 2.5 h prior to and 1 × 70 mL 1.5 h prior to the start of the exercise protocol. A minimum of 72-h washout period separated the supplementation periods.

Blood analyses

Blood samples were drawn into 5-mL heparin syringes (Terumo Corporation, Leuven, Belgium). 200 µL of blood was immediately haemolyzed in 200 µL of ice-cold Triton X-100 buffer solution (Triton X-100, Amresco, Salon, OH) and analyzed to determine blood [lactate] and [glucose] within ~5 min of collection (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining whole blood was then centrifuged at 4,000 rpm for 3 min (Hettich EBA 20, Germany) before plasma was extracted and stored on ice for ~30 min prior to being frozen at −80 °C for later analysis of plasma [K^+] and [Na^+] (9180 Electrolyte Analyzer, F. Hoffmann-La Roche, Basel, Switzerland). Blood samples for the determination of plasma [NO_2^-] and [NO_3^-] were collected into lithium-heparin tubes (Vacutainer, Becton–Dickinson, New Jersey, USA) and centrifuged at 4,000 rpm and 4 °C for 5 min within 3 min of collection. Plasma was extracted and immediately frozen at −80 °C for later analysis of [NO_2^-] and [NO_3^-].

All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO prior to [NO_2^-] and [NO_3^-] analysis. The [NO_2^-] of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and 4 % (w/v) aqueous NaI. The spectral emission of electronically excited nitrogen dioxide product, from the NO reaction with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers NOA 280i. Analytix Ltd, Durham, UK). The [NO_2^-] was determined by plotting signal (mV) area against a calibration plot of 100 nM to 1 µM sodium nitrite. Prior to determination of [NO_3^-], samples were deproteinized using zinc sulphate/sodium hydroxide precipitation. 400 µL of 10 % (w/v) aqueous $ZnSO_4$ and 400 µL of 0.5 M NaOH were added to 200 µL of sample and vortexed for 30 s before being left to stand at room temperature for 15 min. Thereafter, samples were centrifuged at 4,000 rpm for 5 min, and the supernatant was

removed for subsequent analysis. The $[NO_3^-]$ of the deproteinized plasma sample was determined by its reduction to NO in the presence of 0.8 % (w/v) VCl_3 in 1 M HCl. The production of NO was detected using the chemiluminescence nitric oxide analyzer, as described above. The $[NO_3^-]$ was determined by plotting signal (mV) area against a calibration plot of 500 nM to 15 nM sodium nitrate.

Statistical analyses

Differences between PL and BR in distance covered and HR during the Yo–Yo IR1 test were analyzed using a paired-samples *t* test. Differences between PL and BR in blood and plasma variables were analyzed using a two-way repeated measures ANOVA (supplement x time). Significant effects were further analyzed using simple contrasts with the α-level adjusted via Fisher's LSD. Relationships between (changes in) plasma $[NO_2^-]$ and (changes in) performance were analyzed using Pearson product moment correlation coefficients. Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± SD unless stated otherwise.

Results

There were no significant differences between the initial 600 m submaximal Yo–Yo IR1 test and the initial 600 m portion of the subsequent exhaustive Yo–Yo IR1 test for plasma $[NO_3^-]$, $[NO_2^-]$, $[Na^+]$ or $[K^+]$ or blood [glucose] or [lactate].

Plasma $[NO_2^-]$ and $[NO_3^-]$

There were significant main effects by supplement and time, and an interaction effect on the plasma $[NO_2^-]$ (all $P < 0.001$) and $[NO_3^-]$ (all $P < 0.05$). At resting baseline the $[NO_2^-]$ was 118 ± 44 nM in PL and 584 ± 343 nM in BR ($P < 0.05$). Overall, $[NO_2^-]$ was greater in BR than PL at each measurement time point (Fig. 1), and was ~377 % greater, on average, across the entire protocol. At resting baseline the $[NO_3^-]$ was 25 ± 9 µM in PL and 768 ± 180 µM in BR ($P < 0.05$). $[NO_3^-]$ was greater in BR than PL at each measurement time point (Fig. 2) and was ~2,833 % greater, on average, across the entire protocol. During the submaximal test, the $[NO_2^-]$ and $[NO_3^-]$ were not significantly altered relative to baseline in either the PL or BR conditions. During the exhaustive test, however, the $[NO_2^-]$ declined by 20 ± 26 nM (~20 %) in PL ($P < 0.05$) and by 288 ± 221 nM (~54 %) in BR ($P < 0.05$) relative to the pre-exercise baseline (Fig. 1). Concurrently, $[NO_3^-]$ rose by 4.4 ± 3.8 µM (~17 %) in PL ($P < 0.05$) and by 71.8 ± 82.3 µM (~10 %) in BR ($P < 0.05$; Fig. 2). The reduction in $[NO_2^-]$ and the rise in $[NO_3^-]$ were significantly greater in BR than PL (both $P < 0.05$).

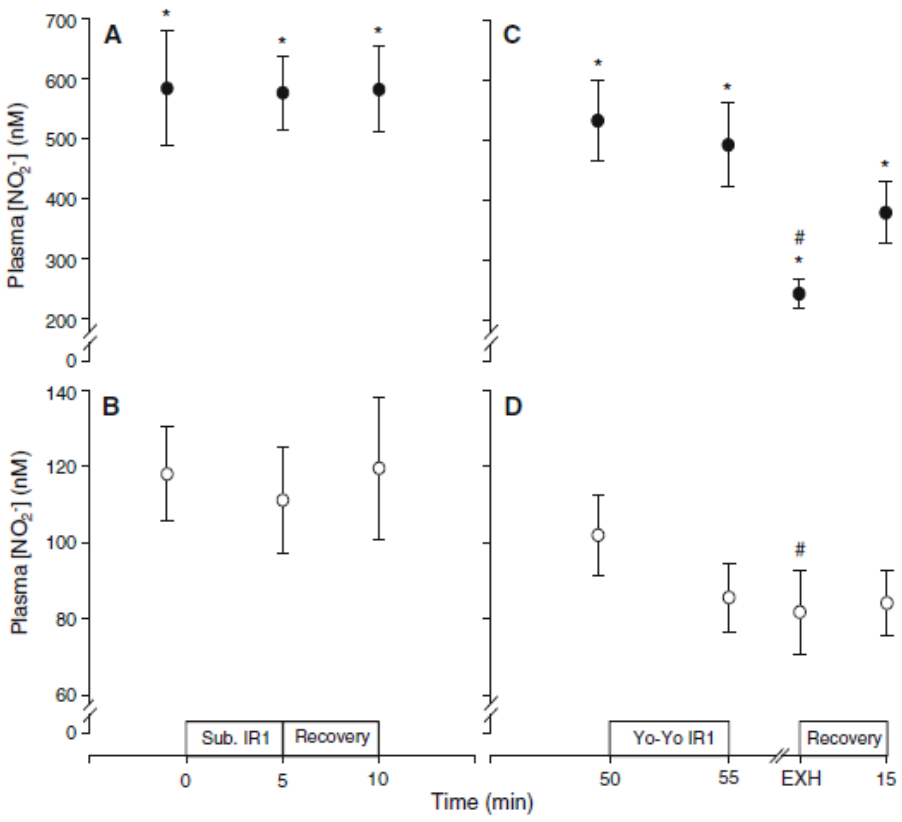

Fig. 1 Plasma $[NO_2^-]$ did not change during the submaximal test in either condition (panels **a** and **b**), but decreased significantly during the exhaustive test in both BR (*filled circle*) and PL (*open circle*) (panels **c** and **d**) with the decline being greater in BR. *Error bars* indicate the SE. $^*P < 0.05$ compared to PL; $^\#P < 0.05$ compared to baseline

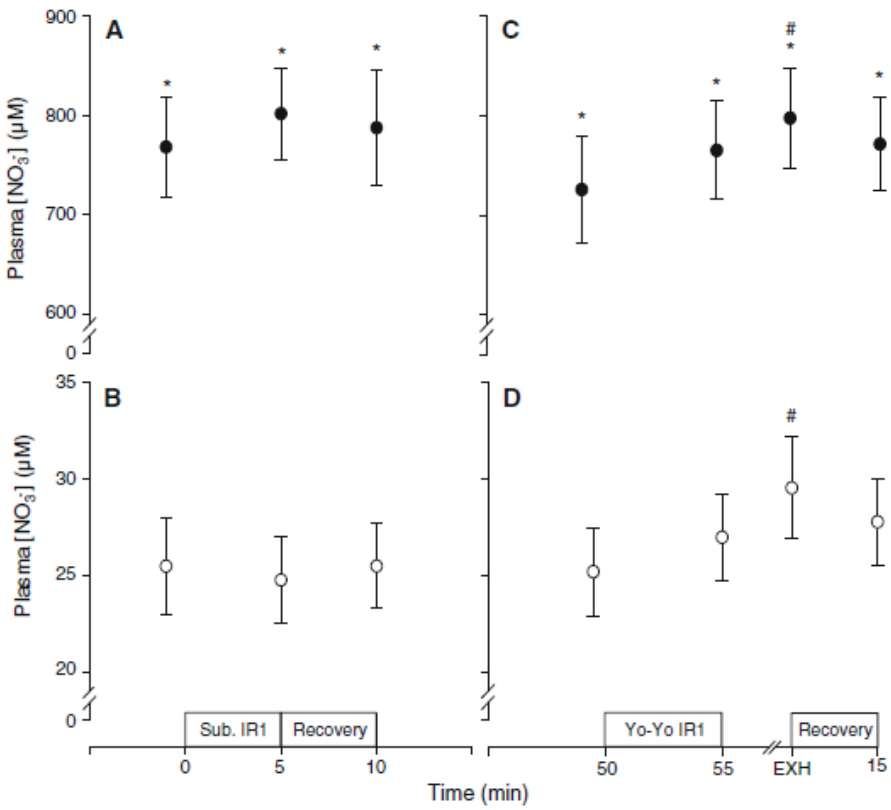

Fig. 2 Plasma [NO_3^-] did not change during the submaximal test (panels **a** and **b**), but increased during the exhaustive Yo–Yo IR1 test in both BR (*filled circle*) and PL (*open circle*) (panels **c** and **d**) with the increase being greater in BR. *Error bars* indicate the SE. $^*P < 0.05$ compared to PL; $^\#P < 0.05$ compared to baseline

Yo–Yo IR1 performance

The distance covered in the Yo–Yo IR1 test was 4.2 % greater ($P < 0.05$) in BR (1,704 ± 304 m) compared to PL (1,636 ± 288 m) (Fig. 3). There were no differences between conditions in HR at the end of either the submaximal test (PL: 170 ± 7 vs. BR: 168 ± 4 b min^{-1}; $P > 0.05$) or the exhaustive test (PL: 198 ± 5 vs. BR 194 ± 11 b min^{-1}; $P > 0.05$). Following BR ingestion, the baseline plasma [NO_2^-] tended to be correlated with Yo–Yo IR1 test performance ($r = 0.48$; $P = 0.08$) and the change in plasma [NO_2^-] from baseline to end-exercise also tended to be correlated with Yo–Yo IR1 test performance ($r = 0.46$; $P = 0.09$).

Of the 14 subjects tested, ten improved their Yo–Yo IR1 performance (by between 40 and 280 m), one was unchanged, and three became slightly worse (by 40–80 m). In the three subjects whose Yo–Yo IR1 test performance became worse, baseline plasma [NO_2^-] was substantially elevated by BR compared to PL (by 131, 205 and 668 %). However, in two of these subjects, the decline in plasma [NO_2^-] from baseline to exhaustion was similar in BR and PL (difference of −30 and −12 nM) compared to a substantially greater fall of plasma [NO_2^-] in BR compared to PL in those subjects whose performance improved (−31 ± 49 nM in PL vs. −388 ± 358 nM in BR, $n = 10$).

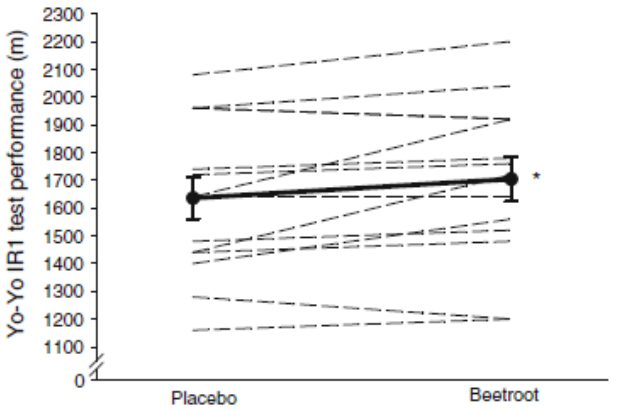

Fig. 3 The distance covered in the Yo–Yo IR1 test was 4.2 % greater with BR compared to PL. The *dashed lines* indicate the responses of individual subjects. The *solid line* indicates the group mean (±SE). $^*P < 0.05$

Blood [lactate] and [glucose]

Blood [lactate] increased significantly during both the submaximal and exhaustive Yo–Yo IR1 tests ($P < 0.05$)

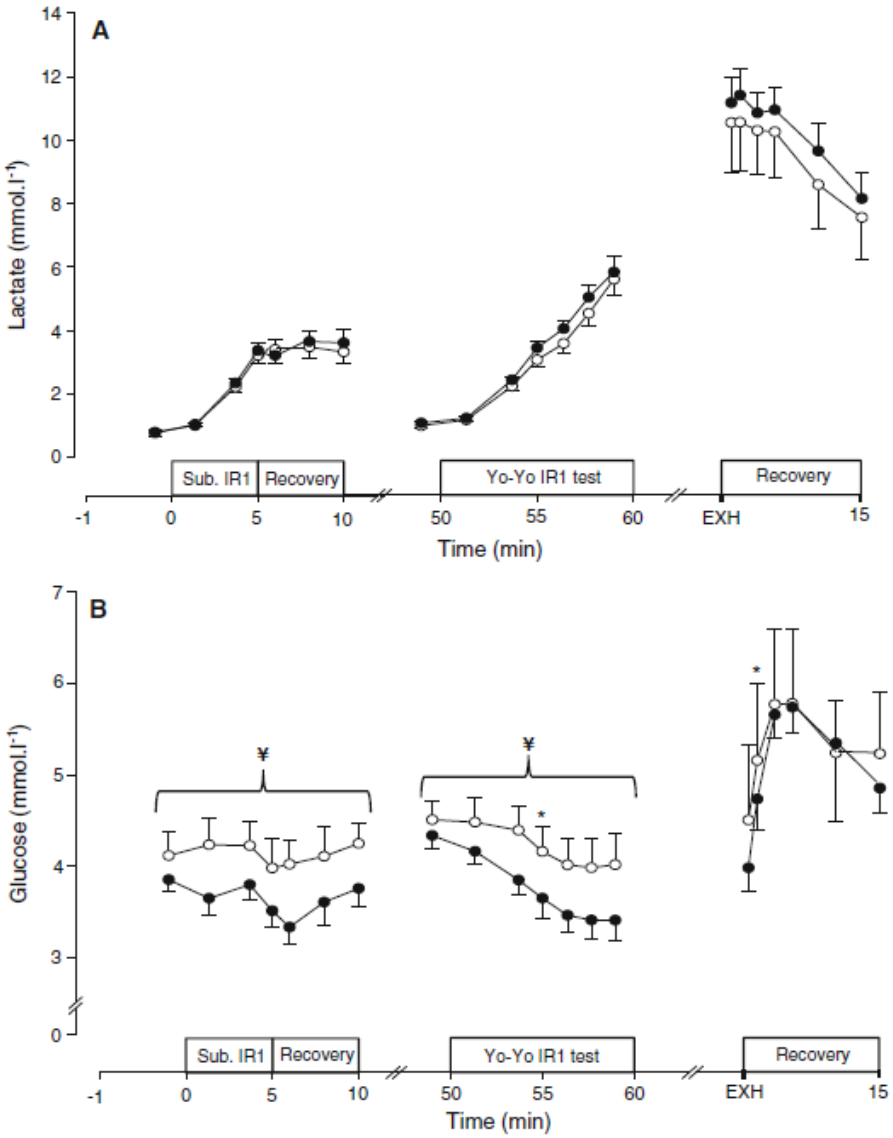

Fig. 4 Blood [lactate] during the submaximal and exhaustive Yo–Yo IR1 test was not different between BR (*filled circle*) and PL (*open circle*) (panel a). There was a greater decline in blood [glucose] in BR than PL during the submaximal test and the mean blood [glucose] was lower in BR than PL during both the submaximal and the exhaustive Yo–Yo IR1 tests (panel B). *Error bars* indicate the SE. $^{*}P < 0.05$ compared to PL; $^{¥}P < 0.05$ compared to PL (mean of all samples)

but there were no differences between supplements (Fig. 4a). There was a trend towards a significant interaction effect on blood [glucose] during the submaximal test ($P = 0.06$), suggesting that blood [glucose] decreased to a greater extent during the BR trial compared to PL (Fig. 4b). The follow-up tests indicated that blood [glucose] was lower in BR than PL after 600 m and at 1 min into recovery ($P < 0.05$ for both). The mean blood [glucose] during the submaximal test was greater in PL (4.1 ± 1.1 mM) compared to BR (3.7 ± 0.7 mM) ($P < 0.01$, paired samples t test including all measurements). Blood [glucose] declined with time during the exhaustive Yo–Yo IR1 test in both BR and PL condition ($P < 0.05$). Although there was no significant interaction or main effect by supplement during the exhaustive test, the mean blood [glucose] was significantly greater in PL (4.2 ± 1.1 mM) compared to BR (3.8 ± 0.8 mM) ($P < 0.001$, paired samples t test including all measurements) (Fig. 4b).

Plasma [Na^+] and [K^+]

The plasma [Na^+] was not significantly different between PL and BR during the submaximal (Fig. 5c) or exhaustive Yo–Yo IR1 tests (Fig. 5d). The plasma [K^+] was not significantly different between BR and PL during the submaximal Yo–Yo IR1 test ($P > 0.05$; Fig. 5a). However, there was a trend for a significant interaction effect on plasma [K^+] during the exhaustive Yo–Yo IR1 test ($P = 0.08$; Fig. 5b) suggesting that the rise in plasma [K^+] was blunted with BR compared to PL. Post hoc analysis

indicated that plasma [K^+] was lower in BR than PL after 600 m ($P < 0.05$).

Discussion

The principal original finding of this study was that dietary nitrate supplementation, in the form of beetroot juice, significantly improved intense intermittent exercise performance as measured by an increased distance covered in the Yo–Yo IR1 test. This finding is consistent with our experimental hypothesis and suggests that increasing dietary nitrate intake might be an effective nutritional strategy to improve performance in intermittent sports such as association football. Plasma [NO_2^-] declined over the course of the Yo–Yo IR1 test in both the PL and BR trials; however, plasma [NO_2^-] was greater with BR prior to the onset of the test and the magnitude of the decline in plasma [NO_2^-] was greater in this condition. Concomitantly, plasma [NO_3^-] increased during the Yo–Yo IR1 test in both PL and BR, but plasma [NO_3^-] was greater with BR prior to the onset of the test and the magnitude of the increase in plasma [NO_3^-] was greater with BR. Blood [lactate] and plasma [Na^+] were not significantly impacted by dietary NO_3^- supplementation across the Yo–Yo IR1, but there were trends for blood [glucose] and plasma [K^+] to be lower with BR compared to PL.

Influence of dietary NO_3^- supplementation on intermittent exercise performance

In recreationally active and trained but sub-elite subjects, short-term dietary nitrate supplementation has been shown to increase exercise tolerance at a fixed sub-maximal work rate (Bailey et al. 2009, 2010; Lansley et al. 2011b) and to increase power output during self-paced endurance exercise leading to improved cycle time-trial performance (Cermak et al. 2012a; Lansley et al. 2011a). It has recently been reported that dietary nitrate supplementation might also improve performance during repeated high-intensity intervals on a rowing ergometer (Bond et al. 2012). Specifically, these authors asked trained rowers to complete 6 × 500 m rowing ergometer repetitions (~90 s completion time per repetition) with 90-s recovery and observed a higher mean power output with nitrate supplementation. These results might have implications for improving performance in high-intensity interval training sessions. However, no study to date has investigated the influence of nitrate supplementation on team sport-specific, intense intermittent exercise performance.

To assess the potential ergogenic effects of dietary nitrate supplementation on intermittent exercise performance where the interval and recovery durations are characteristic of those encountered during team sports, we measured the distance covered during the Yo–Yo IR1 test after supplementation with BR and PL. Performance (distance covered) in the Yo–Yo IR1 test has been shown to closely correlate with high-intensity running during soccer games (Krustrup et al. 2003), which is a key determinant of soccer performance (Bradley et al. 2011; Mohr et al. 2003). Moreover, since performance in the Yo–Yo IR1 test has been shown to discriminate between players of different ability levels in different team sports (Atkins, 2006; Bangsbo et al. 2008; Veale et al. 2010; Vernillo et al. 2012), improved Yo–Yo IR1 test performance would be expected to contribute towards improved performance in invasion games. In this study, we observed a 4.2 % increase in the total distance covered in the Yo–Yo IR1 test with BR compared to PL. This suggests that dietary nitrate supplementation might enhance performance in intermittent team sports, at least in recreationally active subjects. Further research is required to establish whether nitrate supplementation can enhance intermittent exercise performance in highly trained team sport athletes. Nitrate supplementation has been reported be less effective in enhancing endurance exercise performance in highly trained endurance athletes in some (Bescós et al. 2012; Cermak et al. 2012b; Christensen et al. 2013; Peacock et al. 2012; Wilkerson et al. 2012) but not all (Bond et al. 2012; Cermak et al. 2012a) studies.

Although dietary nitrate supplementation produced a statistically significant improvement in Yo–Yo IR1 test performance that is likely to be practically meaningful during team sports which rely on intermittent high-intensity exercise (Bangsbo et al. 2008), the size of the improvement was less than has been reported following other interventions. Specifically, caffeine ingestion (6 mg/kg body weight) resulted in a 16 % mean improvement in performance in the Yo–Yo IR2 test (Mohr et al. 2011) whereas sprint and speed endurance training resulted in 10 and 29 % improvements in Yo–Yo IR2 performance, respectively (Mohr et al. 2007). There is evidence that the improvements in Yo–Yo test performance following these interventions are related, in part, to better control of muscle K^+ homeostasis (Iaia et al. 2008; Mohr et al. 2007, 2011). In contrast, short-term dietary supplementation with L-arginine, a substrate for NO production via nitric oxide synthase, altered neither plasma [NO_3^-] and [NO_2^-] nor high-intensity intermittent exercise performance in judo players (Liu et al. 2009).

Influence of dietary NO_3^- supplementation on NO metabolism

In this study dietary nitrate supplementation increased the resting baseline plasma [NO_3^-] and [NO_2^-] by ~3,000

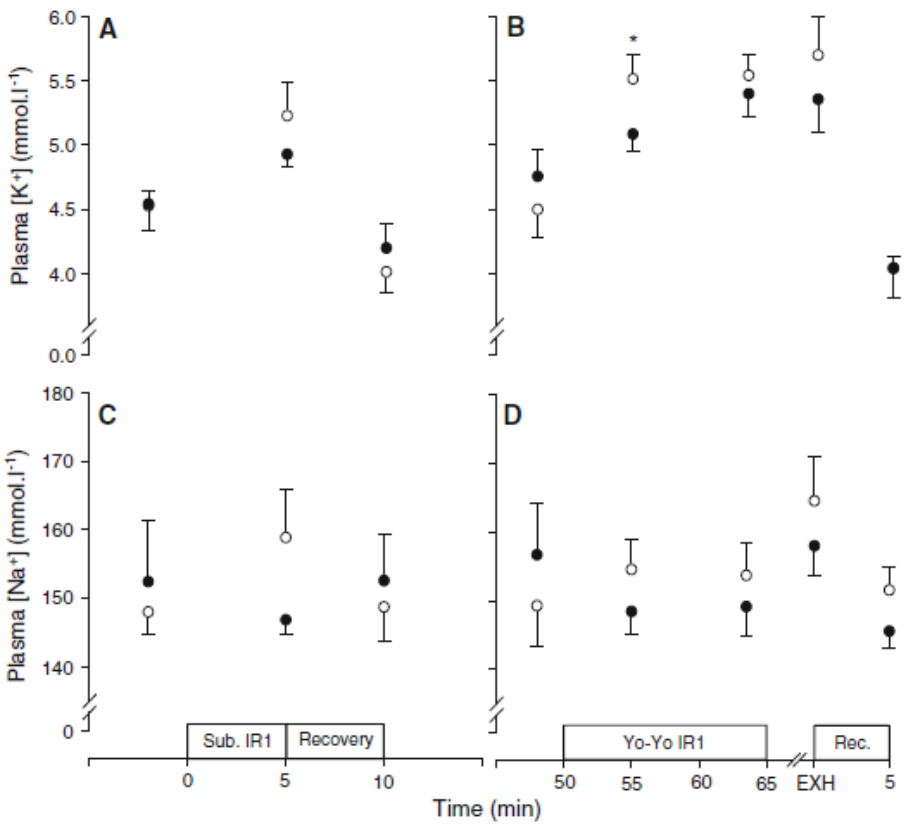

Fig. 5 Plasma [K^+] during the submaximal test was not different between BR (*filled circle*) and PL (*open circle*) (panel **a**; note that the data points for BR and PL at rest are superimposed), but there was a trend towards an attenuated rise in plasma [K^+] in BR (*filled circle*) relative to PL (*open circle*) during the exhaustive Yo–Yo IR1 test (panel **b**; note that the data points for BR and PL at 5-min recovery are superimposed). Plasma [Na^+] during the submaximal (panel **c**) and exhaustive (panel **d**) Yo–Yo IR1 test was not different between BR (*filled circle*) and PL (*open circle*). *Error bars* indicate the SE. *$P < 0.05$ compared to PL ($n = 7$)

and ~400 %, respectively. While several previous reports have shown that dietary nitrate supplementation can increase resting plasma [NO_3^-] and/or [NO_2^-] (Bailey et al. 2009, 2010; Bescós et al. 2012; Larsen et al. 2007, 2010; Lansley et al. 2011a; Vanhatalo et al. 2010a, 2011), the magnitude of the increase has typically been smaller than the values attained in the present study. Indeed, in previous studies, plasma [NO_3^-] has been reported to increase by ~400–600 % (Bescós et al. 2012; Larsen et al. 2007, 2010) and plasma [NO_2^-] by ~50–150 % (Bailey et al. 2009, 2010; Bescós et al. 2012; Larsen et al. 2007, 2010; Lansley et al. 2011a; Vanhatalo et al. 2010a) after dietary nitrate supplementation. The greater increases in plasma [NO_3^-] and [NO_2^-] in the present study is most likely a consequence of the higher nitrate dose (~29 mmol in ~36 h) administered compared to our previous studies using beetroot juice (~5–6 mmol/day; Bailey et al. 2009, 2010; Lansley et al. 2011b; Vanhatalo et al. 2010a).

It should be noted that while the daily nitrate dose used in this study was approximately four times greater than the recommended dietary allowance in the UK, it is similar to that used in the study of Kapil et al. (2010) who investigated the dose–response relationship between nitrate ingestion and the reduction of resting blood pressure. The nitrate supplementation regimen we used also resulted in a similar nitrate intake to the influential 'Dietary Approaches to Stop Hypertension' (DASH) diet (Hord et al. 2009) and traditional Mediterranean and Japanese diets (Sobko et al. 2010) which are promoted for their cardiovascular health benefits. We used a relatively high nitrate dose and selected the nitrate ingestion regimen in this study to ensure that plasma [NO_2^-] remained elevated throughout the relatively lengthy testing protocol, which included an initial submaximal Yo–Yo IR1 test and a subsequent exhaustive Yo–Yo IR1 test. We have previously reported improvements in endurance exercise tolerance using a nitrate dose of ~5–6 mmol/day for 4–6 days (Bailey et al. 2009, 2010; Lansley et al. 2011b). It is unclear whether the smaller nitrate dose used in our previous studies might be ergogenic during intermittent exercise or whether the nitrate dose administered herein was optimal for enhancing intermittent exercise performance. Moreover, since we have shown that performance in certain exercise tests is improved with more chronic (15 days) nitrate supplementation (Vanhatalo et al. 2010a), further research is required to determine whether intermittent exercise performance can be increased to a greater extent, safely, with longer term nitrate supplementation.

It is now known that NO_3^-, which was once considered to be an inert product of NO oxidation, can be reduced in vivo to bioactive NO_2^- and further to NO and other reactive nitrogen species (Lundberg and Weitzberg 2010;

Lundberg et al. 2011). The reduction of NO_2^- to NO is potentiated by acidosis and hypoxia (Lundberg and Weitzberg 2010; Lundberg et al. 2011). NO_2^- reduction to NO would be anticipated during the Yo–Yo IR1 test because the test results in blood (Rampinini et al. 2010) and muscle (Krustrup et al. 2003) acidosis, and it is known that high-intensity exercise reduces muscle PO_2 (Richardson et al. 1999). It has been reported that exercise may both increase (Allen et al. 2010; Gladwin et al. 2000) and decrease (Cosby et al. 2003; Dreissigacker et al. 2010; Larsen et al. 2010) plasma [NO_2^-]. In the present study we found that plasma [NO_2^-] declined and that plasma [NO_3^-] increased during exhaustive intermittent exercise. Importantly, the decline in plasma [NO_2^-] and the increase in plasma [NO_3^-] were significantly greater with BR compared to PL. Given that plasma [NO_2^-] can serve as a circulating reservoir for hypoxic NO production (Lundberg and Weitzberg 2010), a decline in venous plasma [NO_2^-] may be interpreted to represent a reduction of NO_2^- to NO within the muscle or in the surrounding microvasculature. On this basis, it is possible that increasing plasma [NO_2^-] via dietary nitrate supplementation might augment the synthesis of NO during high-intensity intermittent exercise. This interpretation is not straightforward, however. Because NO_2^- is both an oxidation product of NO generated via the 'conventional' nitric oxide synthase pathway and a substrate for NO production in hypoxia, the net NO_3^-–NO_2^-–NO 'flux' during submaximal and high-intensity exercise is unclear.

In the present study we found that, following BR ingestion, the baseline plasma [NO_2^-] and the change in plasma [NO_2^-] from baseline to end-exercise tended to be correlated with Yo–Yo IR1 test performance. Other studies have also suggested that baseline plasma [NO_2^-] and/or the change in plasma [NO_2^-] during exercise may be related to exercise performance. It has been shown that plasma [NO_3^-] and [NO_2^-] are significantly higher in trained athletes compared to untrained controls (Poveda et al. 1997). Dreissigacker et al. (2010) reported that plasma [NO_2^-] decreased from the start to the end of exercise and that the magnitude of this reduction was correlated with exercise capacity at 80 % of maximal work rate. Totzeck et al. (2012) also showed that baseline plasma [NO_2^-] was correlated with lactate threshold and predicted exercise capacity during an incremental cycle test in highly trained athletes. Collectively, these results suggest that both a high baseline plasma [NO_2^-] and the capacity to 'utilize' [NO_2^-] during exercise may be related to high-intensity exercise performance.

The possibility that there may be 'responders' and 'non-responders' to nitrate supplementation has been suggested previously (Christensen et al. 2013; Wilkerson et al. 2012). Wilkerson et al. (2012) reported that, while group mean 50-mile cycle time-trial performance was not significantly improved by acute nitrate supplementation, subjects who evidenced a >30 % increase in plasma [NO_2^-] ('responders') improved their performance whereas those whose plasma [NO_2^-] changed by <30 % did not improve their performance. In the present study, we found that nitrate supplementation resulted in a substantial elevation of plasma [NO_2^-] in all subjects (range 131–746 % increase with BR compared to PL), presumably because the amount of nitrate ingested was relatively high. However, we also found that while the majority of subjects exhibited a substantially greater decline of plasma [NO_2^-] with BR compared to PL during the exhaustive Yo–Yo IR1 test, this was not the case in two of the three subjects whose test performance did not improve with BR. The explanation for this difference is not clear but might relate to inter-individual differences in muscle oxygenation and acidosis during exercise, factors which might, in turn, be linked to differences in effort, anaerobic capacity or muscle fibre-type distribution.

Mechanisms for enhanced Yo–Yo IR1 test performance after NO_3^- supplementation

Fatigue in the Yo–Yo IR1 test is accompanied by a significant reduction in muscle [phosphocreatine] ([PCr]), [glycogen] and pH, and a significant increase in muscle [lactate] (Krustrup et al. 2003). Therefore, delaying the attainment of this limiting intramuscular milieu might be expected to improve performance in the Yo–Yo IR1 test. Existing evidence suggests that dietary nitrate supplementation can attenuate the decline in muscle PCr and the accumulation of muscle adenosine diphosphate and inorganic phosphate (Bailey et al. 2010), metabolites linked to the process of muscle fatigue (Allen et al. 2008). Blunting the rate of change of these substrates and metabolites would be expected to delay the attainment of a 'critical' intramuscular environment and to extend the tolerable duration of high-intensity constant-work-rate exercise (Burnley and Jones 2007; Vanhatalo et al. 2010b). In the present study, there were no significant differences in blood lactate accumulation with BR compared to PL. However, blood [glucose] was lower over the Yo–Yo IR1 test with BR compared to PL. To our knowledge, this is the first study to show that dietary nitrate supplementation lowers blood [glucose] during exercise. Among its various signalling properties, NO is known to play an important role in skeletal muscle glucose uptake (Merry et al. 2010). Therefore, it is possible that a greater increase in NO synthesis following nitrate supplementation enhanced skeletal muscle glucose uptake during the Yo–Yo IR1 test. If so, it is possible that this might have contributed to the enhanced intermittent exercise performance with BR by sparing muscle glycogen utilization (Tsintzas and Williams

1998) in individual (especially type II) fibres or fibre compartments. Further research is required to determine whether dietary nitrate supplementation can increase skeletal muscle glucose uptake during exercise using more specific experimental techniques.

Fatigue during intense exercise has been linked to depolarized muscle membrane potential induced by disturbances in muscle ion homeostasis where interstitial K^+ accumulation is the main component (McKenna et al. 2008; Mohr et al. 2011; Nielsen et al. 2003). In the present study, there was a trend ($P = 0.08$) for the rise in plasma [K^+] during the exhaustive Yo–Yo IR1 test to be attenuated with BR compared to PL, with post hoc analysis showing that plasma [K^+] was significantly lower with BR compared to PL after 600 m of the test. Therefore, assuming that fatigue during intermittent high-intensity exercise is related, in part, to reduced muscle excitability due to a net loss of muscle K^+ (McKenna et al. 2008; Nielsen and de Paoli, 2007), these results suggest that performance in the Yo–Yo IR1 test may have been enhanced by BR due to a reduced muscle K^+ efflux and accumulation in the extracellular fluids. The muscle K^+ efflux appears to be accelerated by acidosis (Mohr et al. 2004; Nordsborg et al. 2003). While there was no difference in blood [lactate] between BR and PL during the exhaustive Yo–Yo IR1 test, it is recognized that measurements of blood [lactate] are not sufficiently sensitive to reflect possible differences in muscle anaerobic energy turnover and H^+ accumulation between BR and PL.

It has been reported that glycogen content is reduced in a high proportion of type II muscle fibres during the Yo–Yo IR1 test (Krustrup et al. 2003) and that most type II fibres are depleted or almost depleted of glycogen after a football game (Krustrup et al. 2006). This suggests that type II fibres are heavily recruited and that a significant metabolic perturbation in these fibres may coincide with exhaustion during the Yo–Yo IR1 test. Recent research indicates that type II muscle fibres might be specifically impacted by dietary nitrate supplementation (Ferguson et al. 2013; Hernández et al. 2012). Ferguson et al. (2013) reported that dietary nitrate supplementation increased hind limb skeletal muscle blood flow in rats, with this additional blood flow being preferentially distributed to type II muscle fibres. An increase in muscle O_2 delivery might be expected to increase the proportional energy contribution from oxidative metabolism (Bangsbo et al. 2001), particularly in type II muscle fibres where O_2 requirements may outstrip O_2 supply (Behnke et al. 2003; McDonough et al. 2005) and to blunt the rate of PCr decline and accumulation of fatigue-related metabolites (Hogan et al. 1999; Vanhatalo et al. 2010b). Increased muscle O_2 delivery may also hasten the restoration of homeostasis in type II muscle fibres in the recovery periods between exercise bouts during the Yo–Yo IR1 test. For example, greater muscle O_2 availability would be expected to speed PCr resynthesis in the recovery phase of intermittent exercise (Paganini et al. 1997; Vanhatalo et al. 2011). It has also been reported that dietary nitrate supplementation increases sarcoplasmic reticulum calcium release and force production, specifically in type II fibres (Hernández et al. 2012). Assuming these findings are applicable to humans, an enhanced force production in type II muscle fibres might lower energy turnover by reducing the number of muscle fibres recruited to generate a given submaximal force production during the Yo–Yo IR1 test. Therefore, changes in skeletal muscle contractile properties and motor unit recruitment, as well as changes in bulk blood flow and its distribution, might account, at least in part, for the improved intermittent exercise performance with BR compared to PL in this study.

In conclusion, this study has shown for the first time that short-term dietary nitrate supplementation can improve intermittent exercise performance in recreationally active adults. Plasma [NO_2^-] was elevated prior to exercise with BR compared to PL and declined to a greater extent with BR compared to PL during the exhaustive Yo–Yo IR1 test, suggesting that NO_2^- may have served as a substrate for NO production during high-intensity exercise. Nitrate supplementation resulted in strong trends for reductions in blood [glucose] and plasma [K^+] during the Yo–Yo IR1 test, suggesting that changes in muscle glucose uptake and muscle excitability may have contributed to the increased fatigue resistance. The results of this study suggest that nitrate may be an effective ergogenic aid for intermittent high-intensity exercise performance in recreational team sport players.

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ORIGINAL ARTICLE

Influence of beetroot juice supplementation on intermittent exercise performance

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Abstract
Purpose This study tested the hypothesis that nitrate (NO_3^-) supplementation would improve performance during high-intensity intermittent exercise featuring different work and recovery intervals.
Method Ten male team-sport players completed high-intensity intermittent cycling tests during separate 5-day supplementation periods with NO_3^--rich beetroot juice (BR; 8.2 mmol NO_3^- day^{-1}) and NO_3^--depleted beetroot juice (PL; 0.08 mmol NO_3^- day^{-1}). Subjects completed: twenty-four 6-s all-out sprints interspersed with 24 s of recovery (24 × 6-s); seven 30-s all-out sprints interspersed with 240 s of recovery (7 × 30-s); and six 60-s self-paced maximal efforts interspersed with 60 s of recovery (6 × 60-s); on days 3, 4, and 5 of supplementation, respectively.
Result Plasma [NO_2^-] was 237 % greater in the BR trials. Mean power output was significantly greater with BR relative to PL in the 24 × 6-s protocol (568 ± 136 vs. 539 ± 136 W; $P < 0.05$), but not during the 7 × 30-s (558 ± 95 vs. 562 ± 94 W) or 6 × 60-s (374 ± 57 vs. 375 ± 59 W) protocols ($P > 0.05$). The increase in blood [lactate] across the 24 × 6-s and 7 × 30-s protocols was greater with BR ($P < 0.05$), but was not different in the 6 × 60-s protocol ($P > 0.05$).
Conclusion BR might be ergogenic during repeated bouts of short-duration maximal-intensity exercise interspersed with short recovery periods, but not necessarily during longer duration intervals or when a longer recovery duration is applied. These findings suggest that BR might have implications for performance enhancement during some types of intermittent exercise.

Keywords Nitric oxide · Beetroot juice · Repeated sprint exercise · Exercise performance · Team sports

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BR	NO_3^--rich beetroot juice
LSD	Least significant difference
MPO	Mean power output
MPO_{mean}	Mean of mean power output
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
O_2	Oxygen
PCr	Phosphocreatine
PL	NO_3^--depleted beetroot juice
PO_2	Partial pressure of oxygen
PPO	Peak power output
PPO_{mean}	Mean of peak power output
Rpm	Revolutions per minute
$\dot{V}O_2$	Oxygen uptake
$\dot{V}O_{2peak}$	Peak oxygen uptake
Δ	Change

Introduction

Nitric oxide (NO) is a multi-functional physiological signaling molecule that can be endogenously derived from

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the oxygen (O_2)-dependent catabolism of L-arginine in a reaction catalyzed by the NO synthase (NOS) enzymes (Stamler and Meissner 2001), or from the O_2-independent reduction of nitrite (NO_2^-) by numerous NO_2^- reductases (Lundberg and Weitzberg 2009). In recreationally active and moderately trained subjects, increasing the circulating plasma [NO_2^-] via inorganic nitrate (NO_3^-) supplementation has been reported to improve performance during sub-maximal endurance exercise in most (e.g., Bailey et al. 2009; Cermak et al. 2012; Porcelli et al. 2015; Wylie et al. 2013a; see Jones 2014 for review), but not all (e.g., Kelly et al. 2014), previous studies. However, the reduction of NO_2^- to NO, and therefore the potential for NO-mediated physiological signaling following NO_3^- supplementation, is potentiated as O_2 tension (Castello et al. 2006) and pH (Modin et al. 2001) decline. Given that muscle PO_2 and pH decline to a greater extent (Richardson et al. 1995) at higher exercise intensities, NO_3^- supplementation may be more likely to increase NO synthesis, and perhaps improve performance, at higher exercise intensities.

Fatigue development during high-intensity intermittent exercise is linked, in part, to the decline in muscle phosphocreatine concentration [PCr] (Fulford et al. 2013; Gaitanos et al. 1993), whereas recovery of intermittent exercise performance is linked to muscle PCr resynthesis (Bogdanis et al. 1995, 1996; Mendez-Villanueva et al. 2012). Importantly, NO_3^- supplementation has been shown to lower the PCr cost of force production during high-intensity intermittent exercise (Fulford et al. 2013), which might delay the attainment of a critically low muscle [PCr] during intermittent exercise (Chidnok et al. 2013). Moreover, the increased perfusion and oxygenation of type II muscle that has been reported following NO_3^- supplementation (Ferguson et al. 2013, 2015) might facilitate the O_2-dependent recovery of PCr (Trump et al. 1996; Vanhatalo et al. 2011) in the type II muscle fibers that are preferentially recruited during high-intensity intermittent exercise (Essén 1978; Green 1978; Krustrup et al. 2004, 2009; Thomson et al. 1979). Supplementation with NO_3^- has also been reported to: enhance calcium handling and augment the rate of force development in type II muscle fibers (Hernández et al. 2012); and to increase force production (Coggan et al. 2015; Haider and Folland 2014) and attenuate fatigue development at high muscle contraction frequencies (Bailey et al. 2015) and when the proportional contribution of type II muscle to force production is expected to be increased (Breese et al. 2013). These findings suggest that NO_3^- supplementation might improve performance during high-intensity intermittent exercise. Moreover, given that the recovery of muscle PCr and force production during intermittent exercise is incomplete when shorter recovery durations are applied (Bogdanis et al. 1995), it is possible that NO_3^- supplementation might be most effective during high-intensity intermittent exercise when the recovery duration between work intervals is relatively short.

Given that NO_3^- supplementation has been shown to improve (Aucouturier et al. 2015; Bond et al. 2012; Thompson et al. 2015; Wylie et al. 2013a), compromise (Martin et al. 2014) or have no effect (Christensen et al. 2013; Muggeridge et al. 2013) on high-intensity intermittent exercise performance, the potential for this supplement to be ergogenic in intermittent exercise is controversial. These equivocal findings might be attributed to inter-study differences in the training status of the participants, the exercise modality employed, the NO_3^- supplementation procedures, and differences in the intermittent exercise protocols (work and rest intensities, work and rest durations, work-to-rest ratio, and number of work intervals). Further research is required to elucidate the relative efficacy of NO_3^- supplementation in enhancing performance in different intermittent exercise performance protocols.

The purpose of this investigation was to assess the effects of NO_3^- supplementation on performance during a variety of high-intensity intermittent exercise tests using the same subject population, exercise modality, and NO_3^- dosing procedures. We hypothesized that: (1) NO_3^- supplementation would improve performance during all high-intensity intermittent protocols administered and (2) performance would be enhanced to the greatest extent when maximal-intensity intermittent exercise was accompanied by the shortest recovery duration.

Methods

Subjects

Ten male recreational team-sport players (mean ± SD: age 21 ± 1 years, body mass 87.5 ± 9.5 kg, height 1.82 ± 0.01 m, $\dot{V}O_{2peak}$, 58 ± 8 mL kg^{-1} min^{-1}) familiar with intense intermittent exercise volunteered to participate in this study. Prior to testing, subjects were informed of the protocol and the possible risks and benefits of participation before written informed consent was obtained. All procedures were approved by the Institutional Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki.

Experimental design

The subjects reported to the laboratory on eight separate occasions over a ~4-week period. During the first visit to the laboratory, subjects initially performed a ramp incremental test for assessment of ramp test peak power and peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$). After 30 min of passive recovery, subjects completed 5 × 6-s all-out cycle sprints interspersed

by 24 s of recovery and, following an additional 10 min of passive recovery, 2 × 30-s all-out cycle efforts interspersed by 4 min of recovery. After a minimum of 24 h recovery, subjects returned to the laboratory to complete 6 × 60-s self-paced maximal efforts separated by 60 s of active recovery. These sprints and self-paced efforts served as a familiarization to the three experimental protocols that are outlined in detail below.

Following completion of the preliminary testing, subjects were assigned in a randomised, double-blind, cross-over experimental design to receive either NO_3^--rich beetroot juice (BR) or NO_3^--depleted beetroot juice (PL) for 5 days. Subjects completed 24 × 6-s all-out sprints, 7 × 30-s all-out sprints, and 6 × 60-s self-paced maximal efforts on days 3, 4, and 5 of supplementation, respectively. A washout period of at least 7 days separated each supplementation period. Subjects were asked to record their food intake in the 24 h preceding the first experimental trial and to replicate this same diet in the 24 h preceding all subsequent trials. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h post-prandial, 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 laboratory visit, respectively, and to abstain from antibacterial mouthwash and chewing gum use throughout the study as these products blunt the reduction of nitrate to nitrite in the oral cavity (Govoni et al. 2008). All tests were performed at the same time of day (±1 h).

Determination of peak oxygen uptake and ramp test peak power

All exercise tests were performed on an electrically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The ergometer saddle and handle bar height configuration was recorded and reproduced in all subsequent tests. The ramp incremental test protocol consisted of 3 min of "unloaded" baseline cycling at 20 W followed by a linear 30 W min^{-1} increase in power output until volitional exhaustion. Subjects were instructed to maintain a cadence of 80 rpm and, when cadence fell below 70 rpm despite strong verbal encouragement, the test was terminated and peak power was recorded. Pulmonary gas exchange was measured throughout the test on a breath-by-breath basis and subsequently averaged into 10-s bins for analysis. $\dot{V}O_{2peak}$ was calculated as the mean value over the final 30 s of exercise.

Intermittent exercise tests

Before the onset of all intermittent exercise tests, subjects completed a standardized warm-up that comprised 5 min of cycling at 80 W (80 rpm), followed immediately by 3 × 3-s sprints, each interspersed by 20 s of passive recovery. Five minutes after completing the warm-up protocol, subjects performed one of the individual intermittent protocols as described below.

24 × 6-s protocol

After undergoing the standardized warm-up, subjects completed a single 6-s all-out sprint. The mean power output (MPO) recorded in this 6-s sprint was used as a criterion score during the subsequent 24 × 6-s cycle test. Upon completion of this sprint, subjects rested for 5 min before completing the 24 × 6-s test, which consisted of 24 6-s all-out sprints departing every 30 s. To mitigate the potential confounding influence of different pacing strategies between supplements (Waldron and Highton 2014), the MPO during sprint 1 of the 24 × 6-s protocol was required to equal or exceed 95 % of the MPO during the benchmark sprint (Fitzsimons et al. 1993). Participants met this criterion in all tests. All 6-s sprints were performed in a standing position and interspersed by 24 s of passive seated recovery.

7 × 30-s protocol

The 7 × 30-s protocol consisted of seven 30-s all-out cycle sprints interspersed with 4 min of recovery. Each 4 min recovery period consisted of 210 s of active recovery at 20 W followed by 30 s of passive recovery. Subjects remained seated during all 30-s sprints and recovery periods. During all 6- and 30-s sprints, subjects were verbally encouraged to perform with maximum effort, but were not informed of the sprint number to prevent pacing.

6 × 60-s self-paced protocol

The 6 × 60-s protocol consisted of six 60-s self-paced cycle efforts interspersed with 60 s of recovery. Subjects were instructed to achieve the highest MPO across all 60-s efforts. Each 60-s recovery period consisted of 40 s of active recovery at 20 W ('unloaded') and 20 s of seated passive recovery. Subjects remained seated during all 60-s self-paced work intervals and recovery periods. Subjects were not provided with any verbal encouragement or information pertaining to the interval number until the final 60-s effort. At this point, subjects were instructed to maximize MPO.

Five seconds before the onset of each work interval, subjects were asked to position the right crank 45° down from the vertical axis. Subsequently, all work intervals were preceded by a 3-s countdown followed by a clear "GO" command. The resistance during each sprint and self-paced exercise bout was applied using the cadence-dependent

linear function (linear factor = power/cadence squared) of the Lode ergometer. The fixed resistance for the 6- and 30-s sprints was set so that upon attaining a cadence of 120 rpm, subjects would achieve a power output equivalent to 270 and 220 % of their ramp test peak power, respectively. These resistances were selected, based on findings from pilot experiments, as they allowed subjects to achieve peak power output (PPO) at ~120 rpm, which is the optimal cadence for attainment of PPO during all-out cycling exercise (McCartney et al. 1985). The fixed resistance during the 60-s self-paced exercise bouts was set so that subjects would achieve 80 % of their ramp peak power at 80 rpm. Subjects were blinded to the elapsed exercise time in the 24 × 6-s and 7 × 30-s intermittent exercise performance tests.

Supplementation

Following completion of the pre-supplementation tests, subjects were assigned using a randomised, balanced, cross-over design to receive concentrated NO_3^--rich beetroot juice (BR; containing ~4.1 mmol of NO_3^- per 70 mL; Beet It, James White Drinks Ltd., Ipswich, UK) and NO_3^--depleted beetroot juice (PL; containing ~0.04 mmol NO_3^- per 70 mL; Beet It, James White Drinks Ltd., Ipswich, UK) for 5 days. On non-experimental days (days 1 and 2) of each supplementation period, subjects consumed 1 × 70 mL in the morning (~10 a.m.) and 1 × 70 mL in the evening (~7 p.m.). On experimental days (days 3, 4, and 5 of supplementation), subjects consumed 2 × 70 mL 2.5 h prior to the onset of testing procedures. This dose of BR and the timing of ingestion was selected based on our previous research which suggested that 8.4 mmol of NO_3^- (administered as 140 mL BR) resulted in a peak increase in plasma [nitrite] 2–3 h later that coincided with improved exercise tolerance (Wylie et al. 2013a). On experimental days 1 and 2 for both BR and PL (days 3 and 4 of supplementation), subjects consumed a further 1 × 70 mL dose 3 h post-completion of testing procedures.

Measurements and data analysis procedures

Performance variables

Power output was recorded continuously at 1 Hz throughout each exercise test using customized software (created through Labview) and exported for subsequent analysis. For the 24 × 6-s and 7 × 30-s tests, MPO and PPO were calculated for each sprint. In addition, the mean of MPO (MPO_{mean}) was calculated for each protocol and mean of PPO (PPO_{mean}) was calculated for the 24 × 6-s and 7 × 30-s protocols. To assess performance at different stages of the 24 × 6-s protocol, MPO and PPO data were pooled into bins of six sprints (i.e. sprints 1–6, 7–12, 13–18, 19–24) prior to statistical analysis.

Pulmonary gas exchange and ventilation

Pulmonary gas exchange and ventilation were collected breath-by-breath in all exercise tests. Subjects wore a nose clip and breathed through a low-dead space (90 mL), low-resistance (0.75 mmHg L^{-1} s^{-1} at 15 L/s^{-1}) mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas concentration signals were continuously sampled using paramagnetic (O_2) and infrared (CO_2) analyzers (Oxycon Pro; Jaeger, Hoechberg, Germany) via a capillary line connected to the mouthpiece. These 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). Breath-by-breath $\dot{V}O_2$ data from each test were linearly interpolated to provide second-by-second values. Subsequently, mean $\dot{V}O_2$ was assessed during each work and recovery period and averaged to provide the overall mean $\dot{V}O_2$ during the work and recovery periods for each intermittent exercise test. The mean $\dot{V}O_2$ across all interval and recovery periods for each intermittent exercise test was also calculated.

Venous and capillary blood sampling

Upon arrival at the laboratory for each intermittent exercise test, a venous blood sample was taken for the determination of plasma [NO_2^-]. Venous blood samples were drawn into 7.5 mL lithium–heparin tubes (Monovette lithium heparin; Sarstedt, Leicester, UK). Within 1 min of collection, samples were centrifuged at 4000 rpm and 4 °C for 7 min. Plasma was subsequently aliquoted and immediately frozen at −80 °C for later analysis of [NO_2^-] as described previously (Wylie et al. 2013a, b).

Capillary blood samples were collected from a fingertip into a capillary tube prior to the warm-up procedure and 20 s prior to the onset of each exercise test. Additionally, capillary blood samples were collected after every two sprints in the 24 × 6-s protocol and after every exercise interval in the 7 × 30-s and 6 × 60-s protocols. These samples were stored on ice and analyzed within 5 min of collection to determine blood lactate concentration [lactate] using an automated blood [lactate] analyzer (YSI 1500; Yellow Springs Instrument, Yellow Springs, OH).

Statistical analysis

Between-supplement differences in MPO_{mean}, PPO_{mean}, and the overall changes in pulmonary $\dot{V}O_2$ were analyzed using a paired-sample *t* test for each intermittent performance

test. Changes in plasma [NO_2^-] recorded on days 3, 4, and 5 of supplementation were determined via a two-way (supplement × test) repeated-measures ANOVA. Likewise, alterations in power output, blood [lactate], and pulmonary $\dot{V}O_2$ for work and recovery intervals in the 24 × 6-s, 7 × 30-s and 6 × 60-s protocols were assessed via two-way (supplement × interval) repeated-measures ANOVAs. Significant effects were further explored using Fisher's LSD. Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± SD unless otherwise stated.

Results

Subjects reported that they consumed all servings of each supplement at the required times and that their diet and exercise habits prior to each experimental test were consistent.

Plasma [NO_2^-]

The group mean plasma [NO_2^-] values obtained on days 3, 4, and 5 of the BR and PL supplementation periods are shown in Fig. 1. Plasma [NO_2^-] was elevated during the BR supplementation compared to PL at all sample points ($P < 0.05$; Fig. 1a). The mean increase in plasma [NO_2^-] with BR ingestion across the three sample points was 237 % (BR 358 ± 119 vs. PL 106 ± 32 nM; $P < 0.05$; Fig. 1b). Plasma [NO_2^-] was not significantly different across days 3–5 in PL or BR ($P > 0.05$; Fig. 1a).

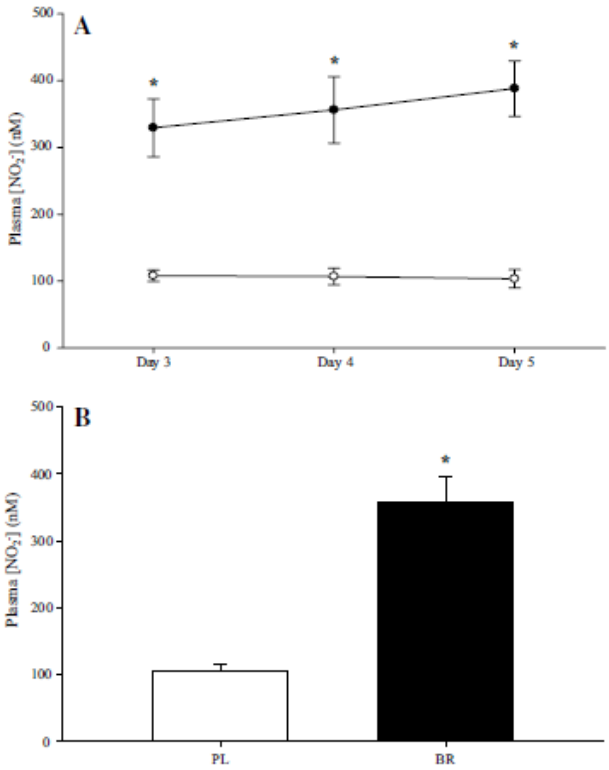

Fig. 1 Plasma nitrite concentration ([NO_2^-]) was elevated in BR (*closed circles*) compared to PL (*open circles*) on days 3, 4 and 5 of supplementation (**a**). On average across the three sample points, plasma [NO_2^-] was 237 % higher in BR (*filled bar*) compared to PL (*open bar*) (**b**). *Error bar* indicates the SE. *$P < 0.05$ compared to PL

Power output

The power output data during the 24 × 6-s, 7 × 30-s and 6 × 60-s protocols are illustrated in Figs. 2, 3, and 4, respectively.

24 × 6-s protocol

There was no significant difference in PPO_{mean} between BR (792 ± 159 W) and PL (782 ± 154 W; $P > 0.05$). However, compared to PL, MPO_{mean} was significantly greater following BR supplementation (BR 568 ± 136 vs. PL 539 ± 136 W; $P < 0.05$; Fig. 2a). Further analyses revealed that BR supplementation did not significantly increase MPO in any individual sprints ($P > 0.05$). However, when the 24 × 6-s sprints were pooled into four groups of six sprints, MPO was greater with BR in sprints 1–6 (BR 694 ± 125 vs. PL 647 ± 122 W; $P < 0.05$), but not in sprints 7–12 (BR 560 ± 100 vs. PL 539 ± 112 W; $P > 0.05$), 13–18 (BR 518 ± 111 vs. PL 492 ± 121 W; $P > 0.05$), and 19–24 (BR 500 ± 114 vs. PL 477 ± 119 W; $P > 0.05$; Fig. 2b).

7 × 30-s protocol

There were no significant differences between BR and PL in PPO_{mean} (BR 768 ± 157 vs. PL 776 ± 142 W; $P > 0.05$) or MPO_{mean} (BR 558 ± 95 vs. PL 562 ± 94 W; $P > 0.05$; Fig. 3a) during the 7 × 30-s sprint exercise tests. There were also no differences between PL and BR in MP (Fig. 3b) and PP across individual sprints ($P > 0.05$).

6 × 60-s protocol

The MPO_{mean} was not significantly different between PL (375 ± 59 W) and BR (374 ± 57 W; $P > 0.05$; Fig. 4a) in the 6 × 60-s exercise test. There were no differences in MP between PL and BR for any of the individual intervals ($P > 0.05$; Fig. 4b).

Pulmonary gas exchange

Pulmonary gas exchange data collected during the three intermittent performance tests in BR and PL are

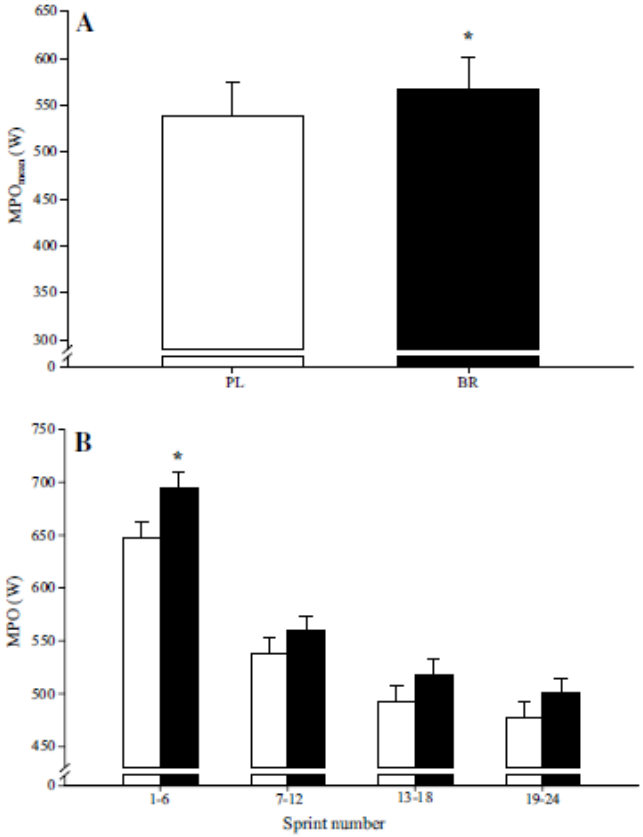

Fig. 2 The mean power output (MPO_{mean}) across the 24 × 6-s sprint protocol was 5 % greater with BR (*filled bars*) relative to PL (*open bars*) (**a**). Specifically, mean power output (MPO) was greater with BR in sprints 1–6, but not in sprints 7–12, 13–18 or 19–24, when compared to PL (**b**). *Error bars* indicate the SE. *$P < 0.05$ compared to PL

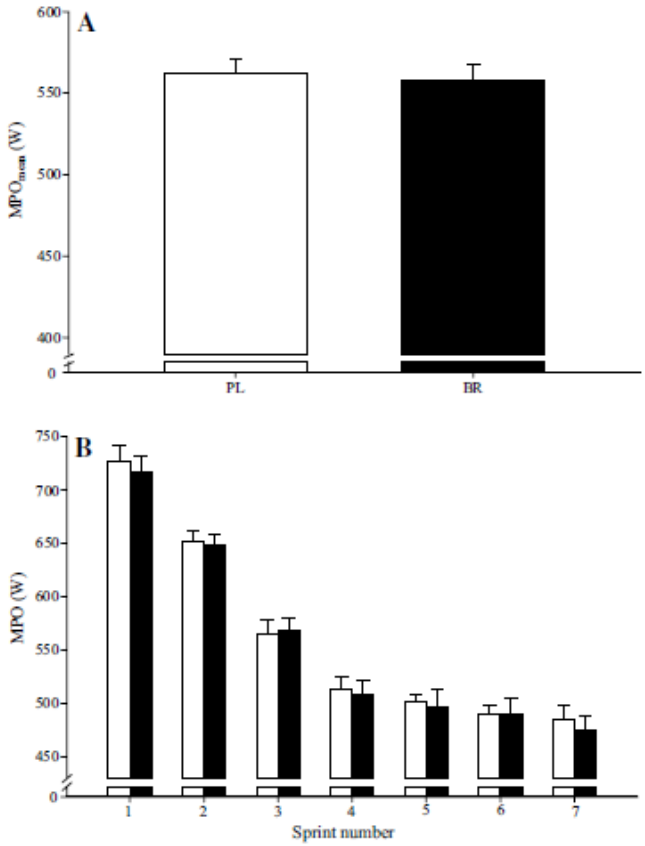

Fig. 3 The mean power output (MPO_{mean}) across the 7 × 30-s protocol (**a**) and mean power output (MPO) during each individual sprint (**b**) were not different between BR (*filled bars*) and PL (*open bars*). *Error bars* indicate the SE

displayed in Table 1. While pulmonary $\dot{V}O_2$ and $\dot{V}CO_2$ increased with time across the three intermittent exercise protocols ($P < 0.05$), mean pulmonary $\dot{V}O_2$ and $\dot{V}CO_2$ was not different between PL and BR during the work intervals, recovery periods or across the overall protocol in any of the exercise test protocols (all $P > 0.05$; Table 1). RER was not different between PL and BR during the work intervals, recovery periods or across the overall protocol in the 7 × 30-s and 6 × 60-s protocols (all $P > 0.05$; Table 1). However, compared to PL, RER was increased during the work intervals, recovery periods, and across the overall protocol in the 24 × 6-s protocol (all $P < 0.05$; Table 1).

Blood [lactate]

Blood [lactate] was not significantly different between BR and PL at baseline (before completion of the warm-up procedure) in any test protocol (all $P > 0.05$). The change in blood [lactate] during the intermittent exercise tests with BR and PL is shown in Fig. 5.

24 × 6-s protocol

The change in blood [lactate] from pre-exercise to the completion of sprint 24 was significantly greater in BR compared to PL (BR 7.3 ± 2.2 vs. PL 6.2 ± 2.1 mM; $P < 0.05$; Fig. 5a). The rise in blood [lactate] above the pre-exercise value was also significantly greater with BR after sprint 22 ($P < 0.05$), but not in the earlier sprints ($P > 0.05$; Fig. 5a).

7 × 30-s protocol

The change in blood [lactate], from pre-exercise to the completion of sprint 7, was significantly greater in BR compared to PL (BR 10.5 ± 1.8 vs. PL 9.5 ± 1.9 mM; $P < 0.05$; Fig. 5b). BR supplementation also resulted in a significantly greater Δ blood [lactate] post-sprint 1 (BR 3.8 ± 1.3 vs. PL 2.0 ± 0.9 mM; $P < 0.05$), but not post-sprints 2–6 ($P > 0.05$; Fig. 5b).

6 × 60-s protocol

The change in blood [lactate], from baseline, to post completion of interval 6, was not significantly different

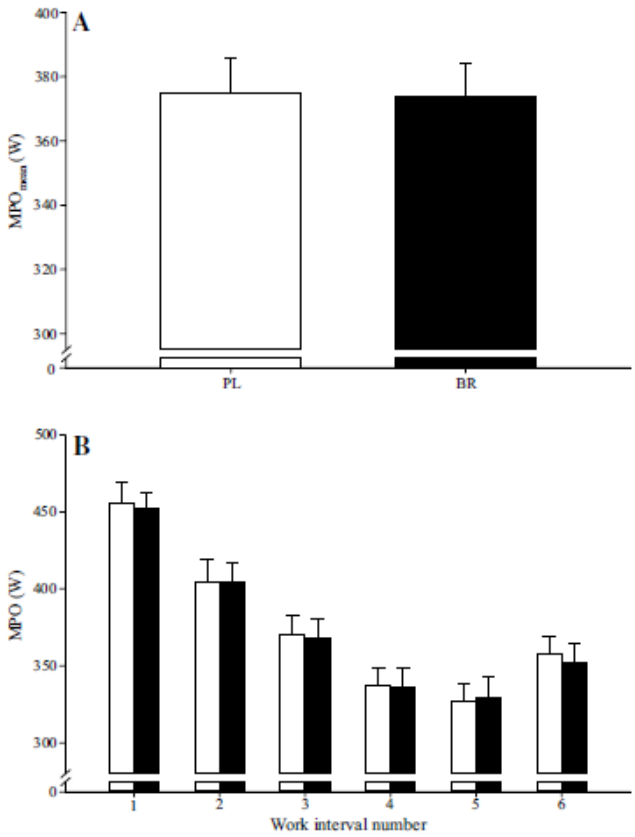

Fig. 4 The mean power output (MPO_{mean}) across the 6 × 60-s protocol (**a**) and mean power output (MPO) during each individual exercise interval (**b**) were not different between BR (*filled bars*) and PL (*open bars*). *Error bars* indicate the SE

between supplements (BR 9.5 ± 1.8 vs. PL 8.6 ± 1.6 mM; $P = 0.07$; Fig. 5c). Furthermore, Δ blood [lactate] was not significantly impacted by BR post-intervals 1–5 ($P > 0.05$; Fig. 5c).

Discussion

The principal original finding of this study was that short-term supplementation with BR significantly improved mean power output during 24 6-s all-out sprints interspersed with 24 s of recovery but not during protocols comprising seven 30-s all-out sprint efforts interspersed with 4 min of recovery or six 60-s self-paced maximal efforts interspersed with 60 s of recovery. BR was especially effective in improving MPO_{mean} in the early part of the 24 × 6-s protocol, with the difference between conditions being significantly different in the first 6 but not the subsequent 18 sprints. These findings suggest that BR supplementation may improve performance during high-intensity intermittent exercise when short-duration, maximal-intensity intervals are repeated with a short recovery duration, but not when interval and recovery durations are longer.

Consistent with previous studies, the circulating plasma [NO_2^-] was significantly increased following the short-term BR supplementation regimen employed in this study (Bailey et al. 2009, 2010, 2015; Breese et al. 2013; Thompson et al. 2015; Vanhatalo et al. 2011; Wylie et al. 2012, 2013a, b). Importantly, plasma [NO_2^-] was elevated above the corresponding PL trials by a similar magnitude on each

Table 1 Mean (±SD) pulmonary gas exchange variables during the work intervals, recovery periods, and across the overall protocol in all three exercise test protocols following PL and BR supplementation

	24 × 6-s protocol		7 × 30-s protocol		6 × 60-s protocol	
	PL	BR	PL	BR	PL	BR
$\dot{V}O_2$						
Work interval (L min^{-1})	3.66 ± 0.29	3.64 ± 0.31	2.57 ± 0.22	2.55 ± 0.26	3.33 ± 0.23	3.27 ± 0.32
Recovery period (L min^{-1})	3.11 ± 0.24	3.12 ± 0.28	1.64 ± 0.15	1.66 ± 0.16	2.73 ± 0.17	2.70 ± 0.28
Overall (L min^{-1})	3.22 ± 0.24	3.23 ± 0.28	1.74 ± 0.15	1.76 ± 0.16	3.02 ± 0.18	2.99 ± 0.29
$\dot{V}CO_2$						
Work interval (L min^{-1})	4.11 ± 0.32	4.27 ± 0.41	2.50 ± 0.23	2.49 ± 0.30	3.61 ± 0.31	3.57 ± 0.36
Recovery period (L min^{-1})	3.39 ± 0.28	3.52 ± 0.32	2.00 ± 0.16	2.02 ± 0.18	3.50 ± 0.27	3.50 ± 0.28
Overall (L min^{-1})	3.53 ± 0.28	3.67 ± 0.33	2.05 ± 0.16	2.07 ± 0.18	3.55 ± 0.27	3.53 ± 0.32
RER						
Work interval	1.13 ± 0.01	1.18 ± 0.04*	1.04 ± 0.04	1.04 ± 0.06	1.12 ± 0.03	1.13 ± 0.03
Recovery period	1.09 ± 0.02	1.13 ± 0.05*	1.26 ± 0.06	1.26 ± 0.04	1.33 ± 0.10	1.35 ± 0.10
Overall	1.10 ± 0.02	1.14 ± 0.05*	1.24 ± 0.05	1.23 ± 0.04	1.23 ± 0.06	1.24 ± 0.06

* Significantly different from PL ($P < 0.05$)

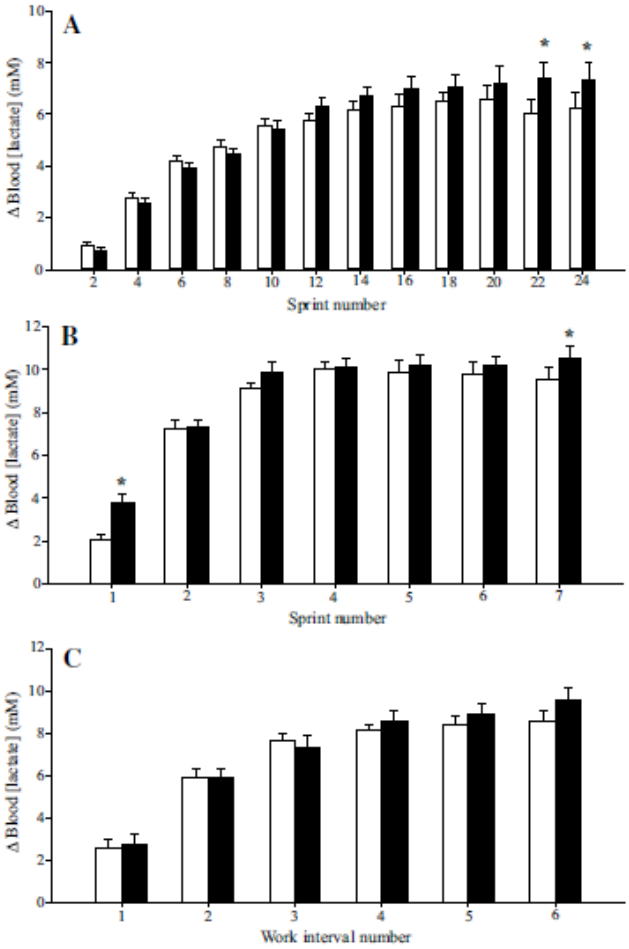

Fig. 5 Change (**Δ**) relative to pre-exercise baseline in blood lactate concentration ([lactate]) during the 24 × 6-s (**a**), 7 × 30-s (**b**) and 6 × 60-s (**c**) exercise protocols, following PL (*open bars*) and BR (*filled bars*) supplementation. Note the greater **Δ** in blood [lactate] with BR after sprints 22 and 24 in the 24 × 6-s protocol, and after sprints 1 and 7 in the 7 × 30-s protocol. *Error bars* indicate the SE. *$P < 0.05$ compared to PL

of the BR trials. Therefore, the relative efficacy of BR to improve performance in the intermittent tests cannot be attributed to differences in plasma [NO_2^-] between tests. An increase in plasma [NO_2^-] represents an increased 'substrate' for NO synthesis through the O_2-independent reduction of NO_2^- to NO (Lundberg and Weitzberg 2009). A comparable BR-induced increase in plasma [NO_2^-] has previously been reported to enhance performance in continuous sub-maximal endurance exercise, at least in recreationally active/moderately trained subjects (e.g., Bailey et al. 2015; Breese et al. 2013; Vanhatalo et al. 2011; Wylie et al. 2013a). However, it is important to note that the reduction of NO_2^- to NO is enhanced as PO_2 (Castello et al. 2006) and pH (Modin et al. 2001) decline, and recent reports suggest that the physiological and performance effects of NO_3^- supplementation are enhanced in type II muscle compared to type I muscle or when type II fiber recruitment is expected to be greater (Bailey et al. 2015; Breese et al. 2013; Coggan et al. 2015; Ferguson et al. 2013, 2015; Hernández et al. 2012). Therefore, given that muscle PO_2 and pH decline (Richardson et al. 1995) and the recruitment of type II muscle is greater (Essén 1978; Green 1978; Krustrup et al. 2004, 2009; Thomson et al. 1979) as exercise intensity increases, we reasoned that BR supplementation would be effective in enhancing performance during the intermittent high-intensity exercise protocols employed in this study.

Consistent with our experimental hypothesis and some previous reports of improved intermittent exercise performance after BR supplementation (Aucouturier et al. 2015; Bond et al. 2012; Thompson et al. 2015; Wylie et al. 2013a), performance (MPO) in the 24 × 6-s protocol was 5 % greater with BR relative to PL. When the 24 × 6-s protocol was divided into 25 % completion segments, MPO was significantly improved with BR in sprints 1–6 (+7 %), but not in sprints 7–12 (+4 %; $P = 0.18$), 13–18 (+5 %; $P = 0.12$) or 19–24 (+5 %; $P = 0.14$). These findings are consistent with recent observations that BR supplementation increased total work done during the first of two 40-min halves comprising repeated 2-min blocks of a 6-s all-out sprint, 100-s active recovery, and 20 s of rest (Thompson et al. 2015). However, in contrast to our experimental hypothesis, performance was not significantly impacted by BR supplementation in the 7 × 30-s or 6 × 60-s intermittent exercise protocols. Some previous studies have also reported no improvement in high-intensity intermittent exercise performance following BR supplementation (Christensen et al. 2013; Martin et al. 2014; Muggeridge et al. 2013). It should be acknowledged that, since subjects were asked to complete the three intermittent exercise protocols on consecutive days within each supplementation period, it is possible that basal fatigue increased across the series of tests and that this impacted performance during the second and/or third test days. However, since the participants were accustomed to high-intensity intermittent exercise, it is unlikely that changes in basal fatigue resistance would have substantially impacted our results.

Inconsistency in the efficacy of BR supplementation to improve high-intensity intermittent exercise performance in previous studies can be attributed, in part, to inter-study differences in participant training status, exercise modality, exercise protocol, and NO_3^- supplementation procedures. Indeed, studies that have reported no improvement in intermittent exercise performance after BR supplementation have tested highly trained endurance athletes (Christensen et al. 2013; Muggeridge et al. 2013) and/or administered a low (<5 mmol NO_3^-) acute dose of BR (Martin et al. 2014; Muggeridge et al. 2013). In contrast, studies that have reported improved intermittent exercise performance

following BR supplementation have involved recreationally active or moderately trained participants (Aucouturier et al. 2015; Thompson et al. 2015; Wylie et al. 2013b) and/or administered more chronic (≥3 days) BR supplementation (Aucouturier et al. 2015; Bond et al. 2012; Thompson et al. 2015) or a large (~29 mmol NO_3^-) dose of BR administered over 24 h (Wylie et al. 2013b). Another complication when interpreting the existing literature with regard to the efficacy of BR supplementation is that previous studies employed single intermittent exercise performance tests that have differed considerably with regard to work and rest intensities, work and rest durations, work-to-rest ratio, and number of work intervals (Aucouturier et al. 2015; Bond et al. 2012; Christensen et al. 2013; Martin et al. 2014; Muggeridge et al. 2013; Thompson et al. 2015; Wylie et al. 2013a). The findings of the present study therefore make an important contribution to our understanding of the effectiveness of BR supplementation to improve performance in different intermittent exercise protocols.

In contrast to the reduced steady-state $\dot{V}O_2$ that has previously been observed during submaximal constant work rate exercise following nitrate supplementation (e.g. Bailey et al. 2009; Larsen et al. 2007; Wylie et al. 2013a; see Jones 2014 for review), $\dot{V}O_2$ was not altered during any of the intermittent exercise protocols in the present study, which is consistent with our recent observations (Thompson et al. 2015). Therefore, the improved intermittent exercise performance in the 24 × 6-s protocol was not a function of changes in whole-body O_2 consumption. Given reports that NO_3^- supplementation is more effective at enhancing physiological responses and performance in type II compared to type I muscle (Ferguson et al. 2013, 2015; Hernández et al. 2012) or in situations where type II muscle fiber recruitment is expected to be greater (Bailey et al. 2015; Breese et al. 2013; Coggan et al. 2015), the differing effects of BR supplementation on performance in the different intermittent exercise protocols in the present study might be linked to differences in the muscle fiber recruitment patterns, and contribution to force production, across the different protocols tested. Studies assessing muscle fiber recruitment patterns from single-fiber high-energy phosphate depletion suggest that the recruitment of type II muscle is greater in 6 s sprints than 30 s sprints (Casey et al. 1996; Esbjörnsson-Liljedahl et al. 1999; Gray et al. 2008; Karatzaferi et al. 2001). The improved performance in the 24 × 6-s protocol, but not the 7 × 30-s or 6 × 60-s protocols, might therefore be linked to improved force production of type II muscle (Coggan et al. 2015; Hernández et al. 2012) as a consequence of increased sarcoplasmic reticulum calcium release (Hernández et al. 2012) and/or improved perfusion/oxygenation (Ferguson et al. 2013, 2015) in type II muscle. Increased perfusion of type II muscle would be particularly important given that the decline in muscle PCr is an important determinant of fatigue development during maximal-intensity intermittent exercise (Fulford et al. 2013; Gaitanos et al. 1993) and that muscle PCr resynthesis in recovery is an O_2-dependent process (Trump et al. 1996; Vanhatalo et al. 2011). However, given that type II fibers are also heavily recruited during a 30-s all-out cycling sprint (Esbjörnsson-Liljedahl et al. 1999), it is unclear why dietary nitrate supplementation did not improve performance, at least during the first 30-s sprint, in the 7 × 30-s protocol.

A novel observation was that the increase in blood [lactate] from baseline to the end of exercise was greater after BR supplementation in the 24 × 6-s and 7 × 30-s protocols, although this was not the case in the 6 × 60-s protocol. Interestingly, RER was also significantly elevated after BR supplementation in the 24 × 6-s protocol. It is uncertain to what extent these changes contributed to enhanced performance with BR. In the 7 × 30-s protocol, performance was not enhanced despite the greater blood [lactate]; in the 24 × 6-s protocol, blood [lactate] was only greater with BR after sprints 22 and 24 where performance was not significantly enhanced (although it should be noted that there will be a temporal lag between muscle lactate production and the appearance of lactate in the blood; Jorfeldt et al. 1978). It has previously been reported that BR supplementation does not influence muscle pH during maximal-intensity intermittent exercise (Fulford et al. 2013) or alter glycolytic ATP turnover during continuous sub-maximal exercise (Bailey et al. 2010). Therefore, the increased blood lactate accumulation during the 24 × 6-s and 7 × 30-s protocols after BR supplementation may not necessarily reflect increased ATP flux through anaerobic glycolysis, but additional research is required before this possibility can be excluded. Instead, the increased blood lactate accumulation during these tests might be a function of increased perfusion of type II muscle following BR supplementation (Ferguson et al. 2013, 2015) since lactate production is greater in type II muscle (Esbjörnsson-Liljedahl et al. 1999) and lactate efflux can be increased with a greater muscle perfusion (Juel 1997). Further research is required to investigate the underlying mechanisms for the improved performance during short-duration maximal-intensity intermittent exercise following BR supplementation.

The present study provides important new insights into the efficacy of BR supplementation to improve performance in different types of intermittent exercise, and specifically indicates that BR supplementation enhanced performance during the 24 × 6-s protocol in which 6-s sprints were separated by 24 s of recovery. Repeated bouts of short-duration, high-intensity exercise interspersed with brief recovery intervals is a hallmark of many invasion games such as Association football, rugby union/league, and field hockey (King et al. 2009; Mohr et al. 2003; Spencer et al. 2004). It is interesting,

therefore, that BR supplementation was most effective at enhancing performance in the intermittent exercise test that most closely resembled the exercise patterns manifest during many team sports, findings which are consistent with Thompson et al. (2015). Although performance was only significantly improved (by 7 %) after BR supplementation over the first six sprints of the 24 × 6-s protocol, it is possible that the non-significant 4–5 % increase in power output over the remaining 18 sprints might represent a practically meaningful improvement in performance in intermittent team sports. However, further research using validated field tests is required to assess the potential of dietary nitrate supplementation to improve team sport performance. Moreover, it should be acknowledged that a larger sample size than was used in the present study might be required to detect changes that are small but potentially practically meaningful in short-duration high-intensity intermittent exercise performance after BR supplementation.

In conclusion, the results of this study suggest that short-term BR supplementation, which increased the circulating plasma [NO_2^-], can improve performance during 24 6-s all-out sprints interspersed with 24 s of recovery. This improvement arose chiefly as a result of a specific performance enhancement over the first six sprints of this test protocol. However, despite a comparable increase in plasma [NO_2^-], BR supplementation did not significantly improve performance during seven 30-s all-out sprints interspersed with 4 min of recovery or six 60-s self-paced maximal efforts interspersed with 60 s of recovery. These findings elucidate the conditions under which BR supplementation may be ergogenic during high-intensity intermittent exercise and invite further mechanistic and practical exposition.

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Chapter 8: General Discussion

NO is a crucial signalling molecule that is known to modulate a number of physiological responses (Moncada and Higgs 1993; Stamler and Meissner 2001; Clerc et al. 2007; Garthwaite 2008; Merry et al. 2010). For a long time, NO was thought to be synthesised solely by the O_2-dependent oxidation of L-arginine via a family of NOS enzymes located in various tissues throughout the body (Moncada and Higgs 1993; Stamler and Meissner 2001). However, from research performed over the past two decades, it is now clear that an alternative, complementary and O_2-independent pathway for NO production exists where NO_3^- is reduced to NO_2^- and further to NO (Benjamin et al. 1994; Lundberg et al. 1994). Interestingly, the ingestion of dietary NO_3^- has been shown to significantly elevate plasma [NO_2^-] (e.g. Larsen et al. 2007; Webb et al. 2008; Bailey et al. 2009; Kapil et al. 2010) (and therefore the potential for O_2-independent NO generation) and have a number of beneficial physiological effects (Larsen et al. 2007; Webb et al. 2008; Bailey et al. 2009; Lundberg et al. 2010; Bailey et al. 2012). In particular, supplementation with dietary NO_3^- has been reported to reduce the O_2 cost of submaximal exercise (Larsen et al. 2007; Bailey et al. 2009), improve exercise tolerance (Bailey et al. 2009; Bailey et al. 2010; Lansley et al. 2011b) and enhance continuous endurance exercise performance (Lansley et al. 2011a; Cermak et al. 2012a). Both scientific interest and the use of dietary NO_3^- as an ergogenic aid has increased considerably in recent years. This thesis has contributed novel information regarding: 1) optimal dosing regimens and associated effects on O_2 cost of exercise; and 2) the ergogenic effects of dietary NO_3^- in intermittent exercise. These contributions to knowledge and their practical implications are discussed below.

Research questions addressed

The aim of this thesis was to further understanding on the optimal supplementation procedures for dietary NO_3^- to enhance exercise economy and performance and to examine the ergogenic potential of dietary NO_3^- in intermittent exercise.

Specifically, we set about answering the following research questions:

1) What are the pharmacokinetic responses of plasma [NO_3^-] and [NO_2^-] following the ingestion of different doses of BR and do the acute physiological and performance benefits of BR/NO_3^- intake occur dose-dependently?
2) How does the duration of supplementation influence the dose-response relationship of NO_3^- supplementation on the physiological response to exercise, and what are the effects of chronic NO_3^- supplementation without the acute ingestion NO_3^- pre-exercise?
3) What are the physiological and performance effects of dietary NO_3^- supplementation on team-sport specific intermittent exercise?
4) In what type of intermittent exercise is dietary NO_3^- supplementation most likely to improve performance?

Summary of main findings

Influence of dietary NO_3^- : pharmacokinetics and acute dose-response relationships

Chapter 4, for the first time, characterised the pharmacokinetic and pharmacodynamic effects of different doses of NO_3^--rich BR and investigated the dose-dependent effects of acute BR ingestion on the physiological response to exercise. In short, we investigated how the acute ingestion of three different NO_3^- doses (4.2, 8.4 and 16.8 mmol NO_3^-), administered as three different BR volumes (70, 140 and 280 mL, respectively), influenced plasma [NO_3^-] and [NO_2^-], resting BP, the physiological response to moderate- and severe-intensity exercise, and exercise tolerance. Results demonstrated that plasma [NO_3^-] and [NO_2^-] were dose-dependently increased, with peak increases occurring 1 h and 2-3 h post consumption, respectively. Both systolic blood pressure and mean arterial pressure were also dose-dependently reduced, up to an intake of 8.4 mmol NO_3^-, with peak reductions of 10 mmHg and 5 mmHg, respectively.Relative to PL, a NO_3^- dose of 16.8 mmol was required to cause a significant reduction in submaximal exercise $\dot{V}_{O2}$, although there was a trend ($P = 0.06$) for a reduction with 8.4 mmol NO_3^-. In contrast, 8.4 mmol NO_3^- improved exercise tolerance, with 16.8 mmol NO_3^- not providing any further benefit. An acute dose of 4.2 mmol NO_3^- did not significantly influence either the O_2 cost of submaximal exercise or exercise tolerance. Together, these data suggest that a low dose ($\leq$ 4.2 mmol) of NO_3^- is insufficient to acutely lower the O_2 cost of submaximal exercise and improve exercise

performance. These results provide important practical information to guide the use of acute BR supplementation as a nutritional aid to enhance exercise performance.

Having established that the acute ingestion of a low dose of dietary NO_3^- is ineffective at lowering submaximal exercise $\dot{V}O_2$, Chapter 5 sought to determine if a significant and consistent reduction in submaximal exercise $\dot{V}O_2$ can be achieved with a low dose of dietary NO_3^- if it is consumed daily for a prolonged period of time. Hypothetically, this was possible due to favourable changes in the expression of mitochondrial and skeletal muscle Ca^{2+} handling proteins that may accompany chronic, but not acute, NO_3^- supplementation.

Dose-dependent effect of dietary NO_3^-: acute vs. chronic supplementation

Chapter 5 investigated the influence of 3 mmol and 6 mmol NO_3^- on the O_2 cost of submaximal exercise after 2 h, 7 days and ~4 weeks of supplementation. As expected, the O_2 cost of submaximal exercise was not acutely affected by 3 mmol NO_3^-; however, the O_2 cost of submaximal exercise was also unaffected when the supplementation period was extended to ~4 weeks, despite plasma [NO_3^-] and [NO_2^-] being significantly elevated throughout the supplementation period. These data therefore suggest that a reduction in submaximal exercise $\dot{V}O_2$ cannot be achieved with a low dose of dietary NO_3^- even when consumed daily for up to ~4 weeks. In contrast, the greater rise in plasma [NO_3^-] and [NO_2^-] following 6 mmol NO_3^- tended to lower submaximal exercise $\dot{V}O_2$ after 2 h, and significantly lowered submaximal exercise $\dot{V}O_2$ at both 7 days and ~4 weeks of supplementation. A secondary aim of this experiment was to determine the importance of acute NO_3^- ingestion, and the accompanying pre-exercise rise in plasma [NO_2^-], for lowering submaximal exercise $\dot{V}O_2$ after a period of chronic supplementation. To achieve this, the influence of chronic NO_3^- supplementation on the O_2 cost of submaximal exercise was also assessed without the acute ingestion of dietary NO_3^- 2 h prior to exercise. An interesting novel finding here was that the reduction in submaximal $\dot{V}O_2$ after ~4 weeks supplementation with 6 mmol NO_3^- was preserved up to 24 h after the final dose of NO_3^- was ingested, and thus, in the absence of an elevated plasma [NO_2^-]. Together these data provide further important, practical information to inform the supplementation guidelines for individuals wishing to use dietary NO_3^- as

an ergogenic aid. They also lend insight into the potential mechanism by which NO_3^- supplementation improved exercise economy.

Influence of dietary NO_3^- in team-sport specific intermittent exercise

The remaining two experimental chapters of this thesis were focused on exploring the potential ergogenic effects of dietary NO_3^- supplementation on high-intensity intermittent exercise performance. Chapter 6 aimed to determine if dietary NO_3^- could improve performance during the Yo-Yo IR1 test, which is designed to mimic the interval and recovery durations that are characteristic of team sports game play. In this Chapter we observed a 4.2% increase in the total distance covered in the Yo-Yo IR1 test with BR compared to PL. Further measurements indicated that plasma [NO_2^-] declined at exhaustion in both PL and BR, but that the relative magnitude of decline was greater with BR. Interestingly, the magnitude of this reduction was significantly and positively correlated with the distance covered during the Yo-Yo IR1 test, suggesting that the utilization of plasma [NO_2^-] after dietary NO_3^- supplementation may be important in determining if dietary NO_3^- is effective at improving intermittent exercise performance. Strong trends for a reduction of blood [glucose] and plasma [K^+] during the Yo-Yo IR1 test with BR were also observed, suggesting that changes in muscle glucose uptake excitability may have contributed to improvement intermittent exercise performance following NO_3^- supplementation. The results of this study suggest that NO_3^- may be an effective ergogenic aid for improving intermittent high-intensity exercise performance in team sport players.

Having established that dietary NO_3^- supplementation could improve team sport specific high-intensity intermittent exercise performance, Chapter 7 sought to establish if dietary NO_3^- could improve performance during a variety of intermittent exercise tests consisting of different work-to-rest ratios as well as exercise and recovery durations. Importantly, performance was assessed in all protocols using the same participant population, exercise modality and NO_3^- dosing procedures, to facilitate the determination of the relative efficacy of NO_3^- supplementation to improve performance in the different intermittent exercise protocols.

Influence of dietary NO_3^- on intermittent exercise featuring different work and recovery intervals

The principal novel finding of Chapter 7 was that NO_3^- supplementation increased plasma [NO_2^-] and significantly improved cycling mean power output (by ~5%) during 24 × 6-s all-out sprints interspersed with 24 s of recovery, but not during protocols comprising seven 30-s all-out sprints interspersed with 4 min of recovery or six 60-s self-paced maximal efforts interspersed with 60 s of recovery. When the 24 × 6-s protocol was divided into 25% segments, mean power output following NO_3^- supplementation was significantly improved in sprints 1-6 (+7 %), and non-significantly higher during sprints 7-12 (+ 4%; P = 0.18), 13-18 (+5%; P = 0.12) and 19-24 (+5%; P = 0.14). These findings highlight the conditions under which NO_3^- supplementation may be most likely to benefit high-intensity intermittent exercise performance. More specifically, they suggest that chronic dietary NO_3^- supplementation may be most effective at improving intermittent exercise comprising repeated bouts of brief-duration high-intensity exercise interspersed with short recovery intervals, which is consistent with the typical intermittent exercise pattern required of team sport players (e.g. association football, hockey and rugby union/league).

Optimising the dietary NO_3^- supplementation procedure – insights from the measurement of plasma [NO_3^-] and [NO_2^-]

Timing of acute supplementation

In all four experimental Chapters the results confirmed a number of previous reports (Larsen et al. 2007; Webb et al. 2008; Bailey et al. 2009; Kapil et al. 2010; Vanhatalo et al. 2010) by showing that both acute and chronic NO_3^- supplementation resulted in a significant elevation in plasma [NO_3^-] and [NO_2^-]. Prior to commencing data collection for this thesis, studies had characterized plasma [NO_3^-] and [NO_2^-] pharmacokinetics after the ingestion of different doses of KNO_3^- (Kapil et al. 2010) and a single dose of NO_3^--rich non-concentrated BR (Webb et al. 2008). The time-to-peak plasma [NO_2^-] in these studies have been used to guide the timing of dietary NO_3^- ingestion pre-experiment and to ensure that plasma [NO_2^-] peaks at the time of assessment in studies investigating the physiological effects of dietary NO_3^-. However, in the present thesis, the recent availability of concentrated BR enabled the influence of different doses of

BR on plasma [NO_3^-] and [NO_2^-] pharmacokinetics (as well as the O_2 cost of exercise and exercise tolerance; see later) to be examined. This was not previously feasible due to the large volumes of non-concentrated BR that would have been required. This novel contribution to knowledge is important because the polyphenol and antioxidant content of BR has the potential to alter plasma [NO_2^-] pharmacokinetics when compared to that observed following KNO_3^- ingestion and therefore have important implications for the timing of BR supplementation prior to an athletic event or scientific experiment. This is especially important considering the growing use of BR as an ergogenic aid and its increased use in basic scientific research.

Results from Chapter 4 demonstrated that plasma [NO_3^-] and [NO_2^-] increased in a dose-dependent manner following the consumption of 70, 140 and 280 mL of concentrated BR, containing 4.2, 8.4 and 16.8 mmol NO_3^-. While peak increases in plasma [NO_3^-] occurred 1-2 h post consumption, peak elevation in plasma [NO_2^-] occurred later, peaking at 2-3 h, which is consistent with the time required for NO_3^- to enter the enterosalivary circulation and be reduced to NO_2^- by lingual bacteria (Govoni et al. 2008; Webb et al. 2008). These pharmacokinetic responses to BR ingestion are consistent with those reported previously following acute ingestion of KNO_3^- (Kapil et al. 2010). The observed time-to-peak plasma [NO_2^-] in Chapter 4 should be used by both athletes and scientists to guide the timing of concentrated BR ingestion to optimize its physiological effects. In line with this, the pharmacokinetic data in Chapter 4 informed supplementation procedures in Chapters 5, 6 and 7, as well as the exercise dose-response sub-study in Chapter 4. Specifically, NO_3^- was consumed 2-2.5 h prior to exercise in all Chapters in order to coincide with the peak elevation in plasma [NO_2^-].

In contrast to the pharmacokinetic assessment in Chapter 4, the increases in plasma [NO_3^-] and [NO_2^-] were assessed at a single pre-determined time-point post acute ingestion of dietary NO_3^- in Chapter 5 and in the exercise dose-response sub-study of Chapter 4. These measurements were made 2-2.5 h post ingestion to coincide with peak elevation in plasma [NO_2^-] observed in Chapter 4. Plasma [NO_3^-] was increased by 334%, 778% and 1,556% while plasma [NO_2^-] was increased by 121%, 218%, and 338%, 2.5 h post-administration of 4.2, 8.4 and 16.8 mmol NO_3^- (administered as 70, 140, 280 ml concentrated BR) respectively, in Chapter 4. Note that these measurements

were made in a different set of participants than that used for the pharmacokinetic sub-study (Chapter 4). Consistent with this dose-dependent response, the ingestion of 3 mmol and 6 mmol NO_3^- (administered in the form of beetroot extract) in Chapter 5 resulted in a ~300% and 867% increase in plasma [NO_3^-] and a ~165% and 579% increase in plasma [NO_2^-] 2 h post consumption. Together the data from Chapters 4 and 5 showed that the acute ingestion of different doses of dietary NO_3^- does result in a dose-dependent increase in plasma [NO_3^-] and [NO_2^-].

Interestingly, comparison of the plasma [NO_2^-] pharmacokinetics in Chapter 4, and, and the acute increases in plasma [NO_2^-] 2-2.5 h post consumption in Chapter 5 and the dose-response study in Chapter 4, provide some important novel insights into the variability of plasma [NO_2^-] responses between individuals and studies.

Variability in plasma [NO_2^-] responses to acute dietary NO_3^- supplementation between individuals and studies

An important observation in Chapter 4 was that the group mean rise in plasma [NO_2^-] in the exercise dose-response study was somewhat lower than those observed at 2-4 h postingestion of the same BR doses in the pharmacokinetic assessment. Because a different participant cohort was recruited for each sub-study, this discrepancy may have arisen from between-participant variations in the pharmacokinetic response of plasma [NO_3^-] and [NO_2^-] to BR ingestion. Evidence for this variability was provided by kinetic analysis of the plasma [NO_2^-] responses following the ingestion of 16.8 mmol NO_3^- in the pharmacokinetic sub-study. Importantly, these analyses found that peak NO_2^- concentrations ranged from 493 to 1,523 nM, and the time-to-peak concentration ranged from 130 to 367 min (see Figure 8.1). This large inter-participant variation may be related to between-participant differences in the presence and/or activity of the oral bacteria responsible for the reduction of NO_3^- to NO_2^- (Govoni et al. 2008; Webb et al. 2008), and/or other factors associated with the enterosalivary circulation such as saliva flow rate and the uptake of NO_3^- by the protein sialin in salivary glands (Qin et al. 2012). A further possibility is that the variability is in part related to the somewhat different NO_3^- doses administered when expressed relative to BM (i.e. 4.2, 8.4 and 16.8 mmol NO_3^- = 0.05-0.07, 0.09-0.13, and 0.19-0.25 mmol NO_3^-/kg BM, respectively). Although further research is required to understand the cause of the pharmacokinetic

variability, these findings do suggest that by setting a fixed time of supplementation before assessment or an athletic event, the potential peak increase in plasma [NO_2^-] may not be achieved in all individuals when the same absolute dose of NO_3^- is administered (see Figure 8.1 for visual illustration of this). This interesting issue has recently been discussed in detail elsewhere (James et al. 2015) and might account for some of the disparate effects of NO_3^- supplementation.

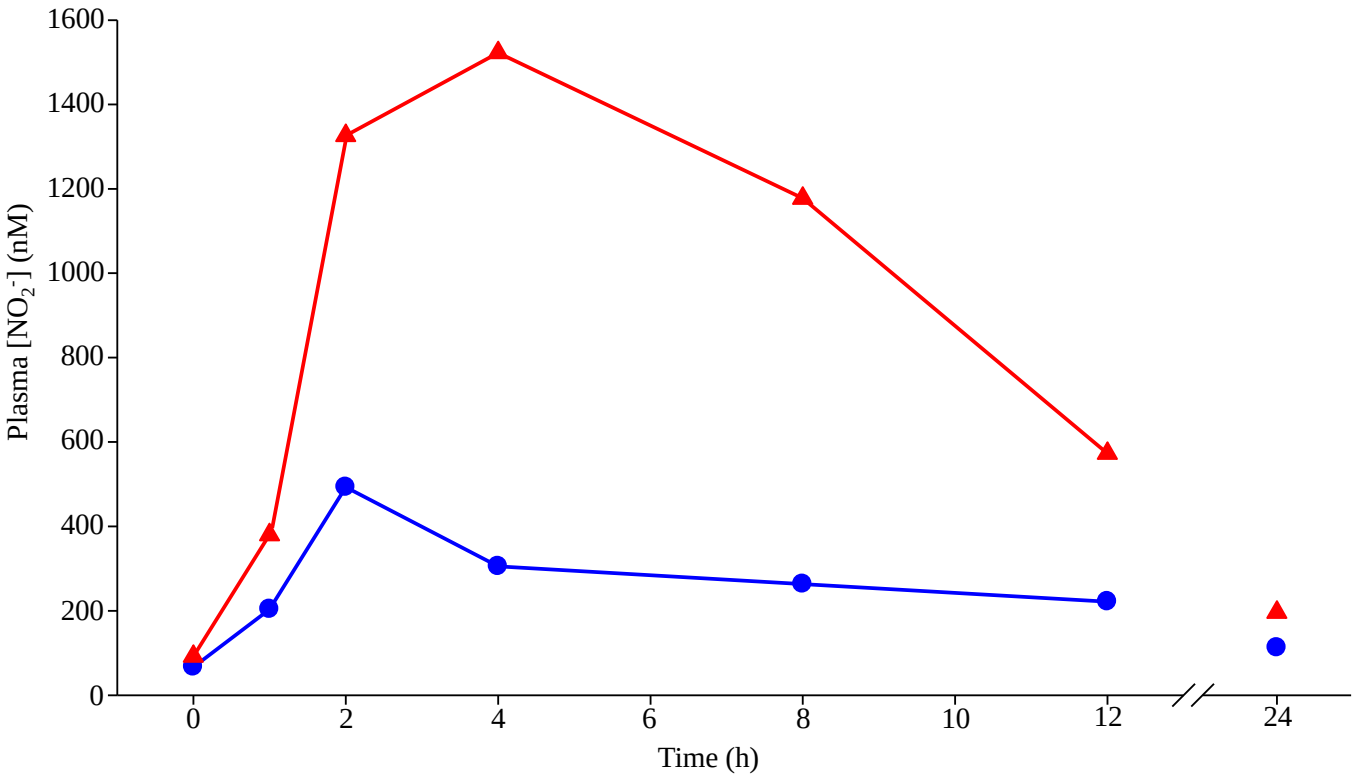

Figure 8.1. Plasma [NO_2^-] pharmacokinetic response following the ingestion of 280 mL concentrated BR containing 16.4 mmol NO_3^- in two representative participants. Note the substantially higher and later peak increase in plasma [NO_2^-] in participant 1 (▲) compared to participant 2 (●).

This inter-participant variation in pharmacokinetics may have also contributed to the greater relative percentage rise in plasma [NO_2^-] in Chapter 5 compared to Chapter 4 (e.g. Chapter 4: 4.2 mmol NO_3^- = 121%; vs. Chapter 5: 3 mmol NO_3^- = 165%) 2-2.5 h post ingestion. However, other factors may have contributed, including between-study differences in: 1) group mean baseline plasma [NO_2^-] (Chapter 4: ~120 nM vs. Chapter 5: ~40 nM); 2) the timing of ingestion (Chapter 4: 2.5 h vs. Chapter 5: 2 h); and/or, 3) the NO_3^- supplement administered (Chapter 4: concentrated BR vs. Chapter 5: beetroot extract diluted in water) which may have an influence on the uptake and metabolism of

NO_3^- (James et al. 2015). Also note that another potential source of variability between studies is the chemiluminescence technique used to measure plasma [NO_3^-] and [NO_2^-]. In the present thesis this was minimised by the use of the same analysis procedures in all experimental Chapters. The factors above, in addition to a variation in the analysis technique used may, have contributed to the significantly lower rise reported previously following the acute ingestion of 4-6 mmol NO_3^- (Larsen et al. 2010; Vanhatalo et al. 2010; Bescós et al. 2011; Lansley et al. 2011a).

Chapters 5, 6 and 7 demonstrated that both short-term (36 h - 7 d) and longer term (~4 weeks) NO_3^- supplementation procedures significantly elevated plasma [NO_3^-] and [NO_2^-], a finding consistent with previous reports (Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011b; Cermak et al. 2012a). In Chapter 5, the daily ingestion of 3 mmol and 6 mmol NO_3^- significantly elevated plasma [NO_3^-] and [NO_2^-] at 7 days and ~4 weeks of supplementation. Importantly, the increases in plasma [NO_3^-] and [NO_2^-] from baseline after 7 days and ~4 weeks of supplementation were not different to those observed 2 h following the first ingestion of the same NO_3^- dose. These findings are consistent with with previous reports (Vanhatalo et al. 2010; Fulford et al. 2013) and suggest that prolonging the supplementation period does not affect (i.e. neither increases or decreases) the bioavailability of plasma [NO_2^-]. However, it must be acknowledged that skeletal muscle tissue [NO_2^-] was not measured in these studies and therefore it is not feasible to exclude the possibility that prolonging the supplementation period may result in the accumulation of tissue [NO_2^-].

In Chapters 6 and 7, a short-term supplementation period was used (i.e. < 6 days). In Chapter 6, the consumption of ~29 mmol NO_3^- (administered as 490 mL of concentrated BR) over a 36 h period resulted in a ~3,000% and ~400% increase in plasma [NO_3^-] and [NO_2^-] whereas in Chapters 7, 5 days supplementation with 8.4 mmol NO_3^- (administered as 140 mL concentrated BR) per day significantly elevated plasma [NO_2^-] by 237% relative to PL on days 3-5. Importantly, and consistent with findings from Chapter 5, plasma [NO_2^-] did not continue to rise on days 3, 4 and 5 of supplementation in Chapter 7. The magnitude of increase in plasma [NO_2^-] in Chapters 6 and 7 are similar to those observed in some previous studies (Kelly et al. 2013; Kelly et al. 2014). In comparison, the large ~3000% increase in plasma [NO_3^-] in Chapter 6

is somewhat higher than these previous studies, probably due to the large dose (29 mmol) administered across a short period of time (36 h).

Despite the variations in the magnitude of increase between studies, plasma [NO_3^-] and [NO_2^-] are clearly elevated by NO_3^- supplementation in all Chapters. Interestingly, Chapter 6 presented novel information to suggest that the 'utilization' of this elevated plasma [NO_2^-] during exercise is an important determinant of the effectiveness of dietary NO_3^- supplementation, as described below.

Utilisation of NO_3^- and NO_2^- as substrate for NO production during exercise?

Chapter 6 characterised the changes in plasma [NO_3^-] and [NO_2^-] during high-intensity intermittent exercise for the first time. Results indicated that plasma [NO_2^-] declined and plasma [NO_3^-] increased at exhaustion during the Yo-Yo IR1 test in both the PL and BR conditions. The majority of previous research has also shown a significant decrease in plasma [NO_2^-] during exercise (Cosby et al. 2003; Larsen et al. 2010; Dreißigacker et al. 2010; Kelly et al. 2013; Vanhatalo et al. 2013; Kelly et al. 2014); however, others have also observed an increase (Gladwin et al. 2000; Rassaf et al. 2004; Allen et al. 2010). The increase in plasma [NO_3^-] at exhaustion in the Yo-Yo IR1 test is in contrast to a previous reports of no change (Kelly et al. 2014). Interestingly, we found that the decline in plasma [NO_2^-] and the increase in plasma [NO_3^-] were significantly greater with BR compared to PL. Given that circulating NO_2^- can serve as a storage pool for hypoxic NO production (Lundberg et al. 2010), and that high-intensity exercise reduces muscle P_{O_2} (Richardson et al. 1999), a decline in plasma [NO_2^-] during high intensity exercise could represent a reduction of NO_2^- to NO within the muscle or surrounding areas. Therefore, it may be reasoned that increasing the amount of circulating NO_2^- by supplementation with dietary NO_3^- might augment the synthesis of NO during high-intensity intermittent exercise. However, it must be acknowledged that while NO_2^- is an end-product of NO production via the classical NOS pathway, it can also be reduced to NO, particularly in hypoxic conditions (Castello et al. 2006), as part of the NO_3^--NO_2^--NO pathway of NO generation (Ignarro et al. 1987). Therefore, changes in plasma [NO_2^-] during high-intensity exercise are likely to reflect a balance between these two pathways. Importantly, the decline in plasma [NO_2^-

] observed during intermittent exercise in Chapter 6 suggests that NO_2^- reduction to NO outweighed NO oxidation to NO_2^-.

Despite the complexities of NO_2^- metabolism, an important observation in Chapter 6 was that the reduction in venous plasma [NO_2^-] observed at exhaustion during the intermittent Yo-Yo IR1 test following BR supplementation was significantly correlated with Yo-Yo IR1 test performance. These results are consistent with Dreissigacker et al., (2010) who reported that the magnitude of reduction in plasma [NO_2^-] from the beginning to end of constant work rate exercise at 80% of maximal work rate was correlated with exercise capacity. Together these results suggest that the ability to 'utilize' plasma [NO_2^-] may be an important determinant of high intensity exercise performance, and the effectiveness of dietary NO_3^- supplementation. Interestingly, in Chapter 6, between-participant differences in the 'utilisation' of NO_2^- were also reported which may provide a novel insight into why NO_3^- supplementation does not improve exercise performance in some individuals. See further discussion under *Responders and Non-responders*.

Dietary NO_3^- and BP: acute dose-response relationship

A body of research now exists to demonstrate that acute and chronic supplementation with inorganic NO_3^-, administered in the form of BR (e.g. Bailey et al., 2009; Vanhatalo et al., 2010; Webb et al., 2008) or NO_3^- salts (e.g. Larsen et al., 2006; Kapil et al., 2010), has the potential to lower resting BP in humans. Chapter 4 added to this existing literature by assessing, for the first time, how the acute administration of three different doses of concentrated BR (i.e. 70 mL, 140 mL and 280 mL providing NO_3^- doses of 4.2, 8.4 and 16.8 mmol NO_3^-, respectively) affected BP over a 24 h period. The findings indicated a dose-dependent reduction in both systolic BP and MAP up to an intake of 8.4 mmol NO_3^-. Specifically, peak reductions in systolic BP of ~5, ~10, and ~9 mmHg and peak reductions in MAP of ~2, ~5, and ~5 mmHg, were observed after acute ingestion of 4.2, 8.4 and 16.8 mmol NO_3^-, respectively. These findings are similar to those reported by Kapil et al., (2010) who administered inorganic NO_3^- as NO_3^- salts. However, Kapil et al., (2010) reported a dose-dependent reduction up to a dose of 24 mmol NO_3^- with no evidence of a 'threshold' effect. Interestingly, despite the 'threshold' effect, the reductions in systolic BP afforded by BR in Chapter 4 (e.g. peak

reductions of 10 mmHg after 140 mL BR containing 8.4 mmol NO_3^-) were greater than those reported by Kapil et al., (2010) with higher doses of KNO_3^- (e.g. peak reductions of 6 and 9 mmHg after 12 and 24 mmol KNO_3^-, respectively). This greater potency of a NO_3^- rich vegetable juice compared with NO_3^- salt in reducing BP is consistent with recent work by Jonvik et al., (2016). Although currently unclear, this enhanced potency may be related to the NO_2^- reducing properties of polyphenols and antioxidants which are present in natural NO_3^- sources but not NO_3^- salts (Weitzberg and Lundberg 1998; Gago et al. 2007).

The reductions in BP evident in Chapter 4 suggest that BR consumption can provide an effective, natural and practical approach to maintaining and improving BP. More specifically, the results provide novel practical information to guide the optimal BR dose for lowering BP in young healthy individuals, while also contributing to the growing evidence (Jonvik et al., 2016; Flueck et al., 2016) to suggest that a natural source of NO_3^- (e.g. vegetables) may be advantageous over NO_3^- salts in eliciting physiological effects.

Influence of different supplementation regimes on the O_2 cost of submaximal exercise

Acute dose-response relationship

A high exercise efficiency (i.e. a low $\dot{V}_{O2}$ for a given power output) is an important determinant of exercise performance (Jones & Burnley, 2009). The O_2 cost of cycling at a fixed work rate below the GET (i.e. moderate-intensity exercise) has long been considered to be independent of factors such as age, training status, as well as unaffected by known physical (e.g. exercise training), nutritional and pharmaceutical agents (Jones & Poole 2005). Remarkably, however, a number of research experiments have now reported that both chronic (3-15 d; Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011b) and acute (1-2.5 h; (Larsen et al. 2010; Vanhatalo et al. 2010; Thompson et al. 2014) supplementation with dietary NO_3^- can lower the O_2 cost of submaximal exercise, at least in untrained healthy young adults. In line with one of the overreaching aims of this thesis, to further understand the optimal dietary NO_3^- supplementation procedure, Chapters 4 and 5 assessed the influence of

different dietary NO_3^- supplementation procedures on the O_2 cost of submaximal exercise, with a particular focus on the influence of the NO_3^- dose administered.

For the first time, Chapter 4 examined the acute dose-response relationship between NO_3^- ingestion and the O_2 cost of submaximal exercise. Specifically, the influence of 70, 140 and 280 ml of concentrated BR (containing 4.2, 8.4 and 16.8 mmol NO_3^-, respectively) on submaximal exercise $\dot{V}_{O2}$ was assessed 2.5 h post administration. The findings indicated a dose-dependent effect. Specifically, the O_2 cost of submaximal exercise was unaffected following the administration of 4.2 mmol NO_3^-, tended (P = 0.06) to be lower following the administration of 8.4 mmol NO_3^- and was lowered significantly following the administration of 16.8 mmol NO_3^-. The magnitude of reduction in submaximal $\dot{V}_{O2}$ following consumption of 16.8 mmol NO_3^- (~3%) was similar to that reported after acute NO_3^- supplementation (~5-5.2 mmol NO_3^-) previously (Vanhatalo et al. 2010; Muggeridge et al. 2013). Based on these results from Chapter 4, the acute ingestion of low doses (≤ 4.2 mmol) of NO_3^- should be considered insufficient to lower the O_2 cost of submaximal cycling exercise.

As outlined in the literature review, a reduction in submaximal exercise $\dot{V}_{O2}$ following NO_3^- supplementation may result from improved mitochondrial efficiency (Larsen et al. 2011; Vaughan et al. 2015; cf. Whitfield et al. 2016) and/or a reduction in the ATP cost of force production (Bailey et al. 2010), which might be mediated by enhanced Ca^{2+}-related contractility (Hernández et al. 2012). An alteration in the expression (and therefore content) of mitochondrial and contractile proteins in skeletal muscle is believed to be the mechanistic basis of these effects (Larsen et al. 2011; Hernández et al. 2012). For instance, Larsen et al., (2011) found that a reduction in submaximal exercise $\dot{V}_{O2}$ was correlated to an improvement in mitochondrial efficiency (i.e. increase in mitochondrial P/O ratio) after 3 days of NO_3^- supplementation, and that this was associated with a decreased protein expression of ANT, which is responsible for a major part of the proton leak in mitochondria (Parker et al. 2008; Bevilacqua et al. 2010). Interestingly, Larsen et al., (2011) proposed that the downregulation of ANT may have resulted from the activation of a signalling pathway triggered by the cell sensing mild hypoxia after the acute binding of NO to cytochrome-c oxidase (COX) in place of O_2 (Brown and Cooper 1994). However, structural adaptations, such as those observed by Larsen et al., (2011), are unlikely to be manifest within 1-3 h post ingestion

of a single NO_3^- bolus and are therefore unlikely to explain the observed reduction in $\dot{V}O_2$ in Chapter 4. Alternatively, the acute lowering of submaximal $\dot{V}O_2$ may be related to the NO mediated acute and reversible alteration in mitochondrial and/or contractile protein function via post-translational protein modifications (Reid 1998), possibly by nitos(yl)ation, as has been described previously (Shiva et al. 2007b). The mechanistic basis for a lowering of submaximal exercise $\dot{V}O_2$ as soon as 2.5 h after the ingestion of a single bolus of dietary NO_3^- warrants further investigation.

Influence of supplementation duration on dose-response relationship

The potential for beneficial structural adaptations post chronic but not acute NO_3^- supplementation suggested that the duration of supplementation may have influenced the acute-dose response relationship between NO_3^- intake and the reduction in submaximal exercise $\dot{V}O_2$ observed in Chapter 4. Therefore, Chapter 5 assessed the influence of 3 mmol and 6 mmol NO_3^- on the O_2 cost of submaximal exercise after 2 h, 7 days and ~4 weeks of supplementation. Of particular interest was whether a low dose of dietary NO_3^- (i.e. ≤4.2 mmol NO_3^-), that was unsuccessful at acutely lowering $\dot{V}O_2$ in Chapter 4, would elicit a significant reduction in submaximal exercise $\dot{V}O_2$ if consumed daily for an extended period of time (i.e. up to ~4 weeks). Determining if a reduction in submaximal $\dot{V}O_2$ can be achieved when a low dose of NO_3^- is consumed chronically was important for providing further information to guide the optimal chronic supplementation procedure.

In Chapter 5, the submaximal exercise $\dot{V}O_2$ was unaffected 2 h post ingestion of 3 mmol NO_3^- but tended (P = 0.06) to be lower (by 3%) following acute ingestion of 6 mmol NO_3^-. These results are consistent with the acute dose-response effect observed in Chapter 4. However, a novel finding of Chapter 5 was that that the O_2 cost of submaximal exercise was not affected with 3 mmol NO_3^- even when consumed daily for a period of up to ~4 weeks, despite a consistent elevation in plasma [NO_2^-] throughout this period. These results suggest that: a) a reduction in submaximal $\dot{V}O_2$ cannot be achieved with a low dose of NO_3^- even with an extension of the supplementation period; and, b) a threshold NO_3^- dose and/or rise in plasma [NO_2^-] may be required to elicit physiological and performance benefits after acute and chronic NO_3^- supplementation. Furthermore, the results indicate that the aforementioned

structural modifications associated with the reduction of $\dot{V}_{O2}$ following chronic NO_3^- supplementation may not only be dependent on the duration of exposure but also the magnitude of the exposure to NO_2^- and/or NO. In contrast, 6 mmol NO_3^- significantly lowered the O_2 cost of submaximal exercise by 3% after both 7 days and ~4 weeks of supplementation. This observation is consistent with the reduction in submaximal exercise $\dot{V}_{O2}$ reported in untrained participants following 3-15 days of supplementation with 5-8 mmol NO_3^- per day (Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010). Moreover, the consistent reduction in submaximal exercise $\dot{V}_{O2}$ over 30 days of supplementation indicates that sensitivity to supplementation is not reduced with a prolonged supplementation procedure, a finding consistent with previous work (Vanhatalo et al. 2010).

Together, the findings from Chapters 4 and 5 suggest that a low dose (≤4.2 mmol) of dietary NO_3^- is unlikely to be effective at lowering submaximal $\dot{V}_{O2}$ after acute ingestion or chronic supplementation of up to 30 days.

Chronic NO_3^- supplementation: importance of acute NO_3^- ingestion and plasma [NO_2^-]

It is clear from Chapters 4 and 5, and previous research (Bailey et al. 2009; Larsen et al. 2010; Vanhatalo et al. 2010; Muggeridge et al. 2013) that both an acute and chronic supplementation strategy can lower the O_2 cost of submaximal exercise. However, in all previous studies assessing the influence of chronic NO_3^- supplementation on the O_2 cost of submaximal exercise, participants have been instructed to consume their final dose of NO_3^- 1-3 hours prior to exercise testing, to ensure a significant elevation in plasma [NO_2^-] at the time of re-assessment (e.g. Larsen et al. 2007; Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011b). The use of this study design left two obvious and important unanswered questions: 1) are the effects of chronic NO_3^- supplementation evident without the acute ingestion of NO_3^- and associated rise in plasma [NO_2^-]? 2) Does the ingestion of an acute dietary NO_3^- dose augment the effects of chronic dietary NO_3^- supplementation without the consumption of the acute NO_3^- dose prior to assessment? These questions were addressed in Chapter 6 by testing participants after ~4 weeks of supplementation both with and without the administration of an acute NO_3^- dose. Using the plasma [NO_2^-] pharmacokinetic profiles in Chapter 4

as a reference, participants were asked to consume their final dose of NO_3^- 24 h prior to exercise to ensure plasma [NO_2^-] returned to pre-supplementation baseline.

A novel finding of Chapter 5 was that the O_2 cost of submaximal exercise was significantly reduced following ~4 weeks of supplementation with 6 mmol NO_3^-, even 24 h after the final ingestion of NO_3^- when plasma [NO_2^-] was not elevated. This is the first study to demonstrate a reduction in submaximal exercise $\dot{V}_{O2}$ following NO_3^- supplementation, in the absence of an elevated plasma [NO_2^-]. Interestingly, the results also suggested that the inclusion of an acute bolus NO_3^- prior to exercise after ~4 weeks of supplementation did not augment the reduction in submaximal exercise $\dot{V}_{O2}$. Therefore, these data suggest that an acute dose of NO_3^- prior to exercise (and an increase in plasma [NO_2^-]) is not required to lower submaximal exercise $\dot{V}_{O2}$ following chronic dietary NO_3^- supplementation. In this Chapter it was suggested that the mechanistic basis for this preserved reduction in submaximal $\dot{V}_{O2}$ may be related to: 1) a preserved change in the content of mitochondrial and/or contractile proteins that have been associated with a reduction in $\dot{V}_{O2}$ following chronic NO_3^- supplementation (Larsen et al. 2011; Hernández et al. 2012); or, 2) a maintained elevation in NO bioavailability in the skeletal muscle tissue, despite plasma [NO_2^-] returning to pre-treatment baseline (Piknova et al. 2015). These findings from Chapter 5 provide important novel information to inform the dietary NO_3^- supplementation strategy pre-competition for endurance athletes. Further research is, however, required to confirm the mechanistic basis of the preserved reduction in submaximal $\dot{V}_{O2}$ following chronic dietary NO_3^- supplementation, and to determine how long this effect is preserved for following cessation of NO_3^- supplementation.

In summary, Chapters 4 and 5 contribute substantial novel information to guide the dietary NO_3^- supplementation regime used to lower the O_2 cost of submaximal exercise. Specifically, results from both Chapters suggest that higher doses (>6 mmol NO_3^-) are required to acutely lower submaximal exercise $\dot{V}_{O2}$, and that the acute ingestion of lower doses (≤4.2 mmol NO_3^-) should not be considered sufficient for this purpose. Chapter 5 extends these findings by showing that a lowering of submaximal exercise $\dot{V}_{O2}$ cannot be achieved with a low dose of dietary NO_3^- even by extending the supplementation period. Chapter 5 also indicates that a reduction in submaximal

exercise $\dot{V}_{O2}$ following chronic NO_3^- supplementation can be achieved without an acute dose of NO_3^- and the associated rise in plasma [NO_2^-].

Dietary NO_3^- and exercise tolerance: acute dose-response effects

Chapter 4 was the first study to examine the acute dose-response relationship between NO_3^- ingestion and exercise tolerance. Specifically, the effect of 70, 140 and 280 ml of BR (containing 4.2, 8.4 and 16.8 mmol NO_3^-, respectively) on exercise tolerance during severe-intensity exercise was investigated. A novel finding was that 8.4 and 16.8 mmol NO_3^-, but not 4.2 mmol NO_3^-, administered 2.5 h prior to the test significantly improved exercise tolerance by 14 and 12%, respectively. Therefore, these findings indicate a dose-dependent effect of BR ingestion on exercise tolerance up to 8.4 mmol NO_3^-, and that the ingestion of more BR does not provide a further ergogenic effect. The 12-14% improvement in exercise tolerance is similar to the 12-17% improvement observed previously following 3-6 days of BR supplementation with doses of 5-8.4 mmol NO_3^- (Bailey et al. 2009; Lansley et al. 2011b; Kelly et al. 2013).

The improvement in exercise tolerance in Chapter 4 with 8.4 and 16.8 mmol NO_3^- was accompanied by no change in the $\dot{V}_{O2}$ slow component or $\dot{V}_{O2peak.}$ This is consistent with some (Kelly et al., 2013) but not all (Bailey et al. 2009; Lansley et al. 2011b) previous research. For instance, Bailey et al., (2009) reported a significant improvement in exercise tolerance and a reduction in the $\dot{V}_{O2}$ slow-component following 3 days of dietary NO_3^- supplementation. Research on the influence of dietary NO_3^- supplementation on the $\dot{V}_{O2peak}$ is also conflicting, with some studies showing an unchanged $\dot{V}_{O2peak}$ (Kelly et al., 2013; Bailey et al., 2009; Thompson et al., 2014; Bailey et al., 2010) and others reporting a lowered $\dot{V}_{O2peak}$ (Lansley et al., 20; Bescos et al., 2011; Larsen et al., 2010) or increased $\dot{V}_{O2peak}$ (Vanhatalo et al., 2010). The reason for these discrepancies is currently unclear but may be related to between study difference in the dose and duration of supplementation, the source of NO_3^-, the modality of exercise, the protocol adopted or the training status of the participants.

Although the ~12-18% improvement in exercise tolerance demonstrated in Chapter 4 following the acute ingestion of 8.4 and 16.8 mmol NO_3^- may appear impressive, it is likely to translate into no more than a 1-2% improvement during a time trial exercise task in which a given distance is completed in the shortest possible time (Hopkins et al.

1999). This is consistent with the magnitude of improvement in endurance performance reported previously in moderately trained cyclists ($\dot{V}_{O2peak}$ = 56-58 ml·kg^{-1}·min^{-1}) during a 10 km cycling TT following 6 days of BR supplementation (Cermak et al. 2012a) and during 4 km and 16.1 km cycling TT after acute BR ingestion (Lansley et al. 2011a). A 1% improvement in endurance performance is highly meaningful in elite sport (Paton and Hopkins 2006) and the dose-response data from Chapter 4 indicate that the dose of NO_3^- administered may be an important factor in determining whether dietary NO_3^- can provide this benefit or not. In support, Hoon et al., (2014) recently reported an improvement in 2000-m rowing performance time with the acute ingestion of 8.4 mmol NO_3^- but not 4.2 mmol NO_3^- (administered as 140 ml and 70 ml concentrated BR, respectively).

There are a number of potential mechanisms responsible for the improvement in exercise tolerance following acute dietary NO_3^- supplementation. Dietary NO_3^- supplementation has been shown to preferentially increase perfusion and oxygenation during exercise in muscle groups containing principally type II muscle fibres (Ferguson et al. 2013a; Ferguson et al. 2014), while NO_2^- infusion has been reported to acutely increase blood flow in the exercising forearm (Cosby et al. 2003). It is therefore possible that an improvement in exercise tolerance may be mediated, in part, by an improved blood flow to muscle or to an NO-mediated enhancement of local matching of O_2 delivery to metabolic rate, particularly in type II muscle fibres which are progressively recruited during severe intensity exercise. The improvement in exercise tolerance may also be related to a reduction in the ATP and PCr cost of muscle force production (Bailey et al. 2010), possibly through Ca^{2+}-related improvements in type II muscle fibre contractility (Hernández et al. 2012). Although it is suggested that a structural modification may be required for an improvement in contractile function to occur (Hernández et al. 2012), and that this is unlikely to occur after acute ingestion, recent research by Coggan et al., (2014) has reported a significant improvement in contractile function at high contraction velocities in human skeletal muscle following acute ingestion of 11.2 mmol NO_3^-. It is possible that improvements in skeletal muscle perfusion and oxygenation, and contractility, particularly in type II fibres, may operate simultaneously and/or synergistically to improve exercise tolerance following acute dietary NO_3^- supplementation. However, further research is required to understand how dietary NO_3^- improves exercise tolerance as soon as 2.5 h post administration.

As discussed previously, Chapter 5 showed that a reduction in submaximal exercise $\dot{V}_{O2}$ following chronic dietary NO_3^- supplementation is preserved up to 24 h after the final ingestion of NO_3^- and thus, in the absence of an increase in plasma [NO_2^-]. However, it is currently unclear if the increase in plasma [NO_2^-] afforded by an acute bolus of dietary NO_3^- pre-exercise is advantageous during high-intensity exercise where the vascular effects of dietary NO_3^- may be beneficial and where the PO2 and pH decline more which provides better conditions for nitrite reduction to NO. Indeed, while improvements in contractile function following NO_3^- supplementation (Hernández et al. 2012; Coggan et al. 2014) may be maintained due to the preserved alteration of contractile protein content (Hernández et al. 2012), an improvement in perfusion and oxygenation in type II muscles (Ferguson et al. 2013a; Ferguson et al. 2014) are likely coupled closely to circulating NO bioavailability (Cosby et al. 2003; Wylie et al. 2013) and therefore may not be maintained when plasma [NO_2^-] returns to baseline. On the other hand, if a prolonged chronic NO_3^- supplementation does result in a greater storage of skeletal muscle tissue NO_2^- (see discussion above) and this tissue NO_2^- is mobilized during exercise (Piknova et al. 2015), then the vascular benefits of NO_3^- supplementation may also be preserved after NO_3^- supplementation is terminated and plasma [NO_2^-] returns to baseline. Consequently, further research is required to determine the impact of an acute bolus of dietary NO_3^- on high-intensity exercise performance after chronic NO_3^- supplementation.

In summary, Chapter 4 demonstrates that the acute ingestion of a low dose (≤4.2 mmol) of NO_3^- is not sufficient to improve exercise tolerance during severe-intensity exercise. In contrast, the acute ingestion of 8.4 mmol NO_3^- does improve exercise tolerance, but the acute ingestion of a higher dose (i.e. 16.8 mmol NO_3^-) does not provide a further benefit. Furthermore, it is also clear that the influence of dietary NO_3^- on $\dot{V}_{O2peak}$ and the $\dot{V}_{O2}$ slow component is controversial and requires further investigation.

Responders and non-responders

As with many other nutritional ergogenic aids (e.g. caffeine, beta alanine, creatine and sodium bicarbonate), research has outlined the potential for ‘responders’ and ‘non-responders’ to NO_3^- supplementation (Wilkerson et al. 2012; Christensen et al. 2013;

Boorsma et al. 2014). Understanding the mechanistic bases behind differences in the responsiveness of individuals to dietary NO_3^- supplementation is important in optimising the effectiveness of this supplement and nutritional advice for athletes. Some studies indicate that NO_3^- supplementation may be less effective in highly trained endurance athletes (Wilkerson et al. 2012; Christensen et al. 2013; Boorsma et al. 2014). Compared to less well-trained athletes, highly trained endurance athletes have a higher proportion of type I fibres, greater mitochondrial and capillary density and a higher baseline plasma [NO_2^-] due to greater training related NOS activity, all of which may reduce the potential benefits of NO_3^- supplementation (Wilkerson et al. 2012; Jones 2014). Importantly, Chapters 4 and 6 provide further insights into why there may be responders and non-responders to NO_3^- supplementation. In Chapter 4 the number of non-responders (in terms of exercise capacity) decreased as the dose of NO_3^- ingested increased. For example, there were three non-responders in the 4.2 mmol condition, two in the 8.4 mmol condition, and one in the 16.8 mmol condition. However, unlike in a previous study by Wilkerson et al (2012) the increase in plasma [NO_2^-] from baseline to pre-exercise for non-responders was not smaller than that measured in the other subjects who did respond, and the non-responders did not have a particularly high baseline plasma [NO_2^-]. Therefore, these results suggest that some individuals require larger acute NO_3^- doses than others to elicit any positive effect on exercise capacity, but that this does not appear to be related to the magnitude of increase in plasma [NO_2^-]. In other words, it appears that each individual may have an independent threshold which plasma [NO_2^-] must surpass before a beneficial effect is observed.

Another interesting observation with regard to responders and non-responders was made in Chapter 6. In this experiment, three participants of the 14 tested did not experience an improvement in Yo-Yo test performance with BR and, consistent with Chapter 4, these participants did not appear to have a particularly low rise in plasma [NO_2^-] following dietary NO_3^- supplementation. However, it was found that although the majority of participants exhibited a greater decline of plasma [NO_2^-] with BR compared to PL during the Yo-Yo IR1 test, this was not the case in two of three participants whose test performance did not improve with BR. In other words, it appears that two of the three non-responders may not have experienced an improvement in performance following BR because they did not ‘utilize’ the increased availability of plasma [NO_2^-].

When considered together, the results of the present thesis suggest that an individual's lack of responsiveness to dietary NO_3^- supplementation, in terms of improving exercise tolerance/performance, may be related to the administration of an insufficient dose and/or a limited ability to utilize elevated levels of plasma NO_2^- during exercise.

Dietary NO_3^- as an ergogenic aid to improve Intermittent Exercise Performance

At the onset of the series of experiments that comprise this thesis, the majority of literature on the performance benefits of dietary NO_3^- supplementation were focused on continuous endurance type exercise (Bailey et al. 2009; Lansley et al. 2011a; Cermak et al. 2012a; Wilkerson et al. 2012). Importantly, this neglected intermittent exercise which is a hallmark of many of the worlds most popular sports (e.g. association football, rugby union/league and field hockey), and a key part of the training programmes (i.e. high intensity interval training) used by endurance and team sport athletes. In an attempt to further explore the application of dietary NO_3^- as an ergogenic aid, and in light of the potential preferential effects of NO_3^- supplementation on type II muscle that is recruited during high-intensity intermittent exercise, Chapters 6 and 7 investigated the influence of dietary NO_3^- supplementation on high-intensity intermittent exercise performance.

The first available evidence to support the ergogenic potential of dietary NO_3^- in high-intensity intermittent exercise was provided by Bond and colleagues in 2012. These researchers observed a higher mean power output with NO_3^- supplementation in trained rowers during 6 x 500 m rowing ergometer repetitions (~90-s completion time per repetition) interspersed with 90 s recovery (Bond et al. 2012). Although promising, the exercise protocol adopted by Bond et al., (2012) was not applicable to the intermittent exercise undertaken by team sport athletes, i.e. brief high-intensity exercise bouts interspersed with short recovery periods. Therefore, Chapter 6 aimed to elucidate if dietary NO_3^- could improve performance during team sport specific intermittent exercise.

An important novel finding in Chapter 6 was that dietary NO_3^- supplementation improved performance by 4.2% (compared to a PL) during a Yo-Yo IR1 test, which is a test specifically designed to mimic the intermittent high-intensity running bouts in

association football match-play (Bangsbo et al. 2008). Importantly, performance during this test has been shown to: 1) discriminate between players of different fitness in various sports (Atkins 2006; Bangsbo et al. 2008; Veale et al. 2010; Vernillo et al. 2012); and 2) correlate closely with high-intensity running during soccer games (Krustrup et al. 2003), which is a key determinant of soccer performance (Mohr et al. 2003; Bradley et al., 2011). These results suggest that an improvement in Yo-Yo IR1 test performance may be expected to improve the amount of high intensity running during a team sport game. Therefore, the 4.2% improvement in Yo-Yo IR1 test performance following dietary NO_3^- supplementation in this Chapter indicated, for the first time, that dietary NO_3^- might be an effective ergogenic aid for team sport players. This finding is consistent with a recent study by Thompson et al., (2015) which reported an increase in total work done during a prolonged intermittent sprint test designed to reflect the metabolic demands of a hockey match. Specifically, dietary NO_3^- supplementation improved total work done in the first of two 40-min halves comprising repeated 2 min blocks of a 6-s sprint, 100-s active recovery, and 20 s of rest.

While intermittent exercise during team sport games typically consist of brief bouts of high intensity work interspersed with short recovery periods, other athletic events (e.g. track cycling) and exercise training interventions (e.g. high-intensity interval training) often consist of longer duration high intensity work with longer periods of rest (Burgomaster et al. 2006; Weston et al. 2014). As discussed in the literature review, the physiological demands of intermittent exercise can change considerably by alterations in: work and rest intensities; work and rest durations; work-to-rest ratio; and the number of work intervals. Consequently, the intermittent protocol itself may influence the ergogenic potential of dietary NO_3^- supplementation and therefore may contribute to the conflicting findings from studies investigating the effects of NO_3^- supplementation on intermittent exercise performance (e.g. Bond et al. 2012; Muggeridge et al. 2013; Martin et al. 2014; Thompson et al. 2015). To fully understand the ergogenic potential of dietary NO_3^- during intermittent exercise, Chapter 6 examined the influence of dietary NO_3^- supplementation on performance during three different intermittent exercise protocols. Specifically, performance was assessed in 24 x 6 s all-out sprints, interspersed by 24 s of recovery, 7 x 30 s all-out sprints interspersed by 4 min of recovery or 6 x 60 s self-paced maximal efforts interspersed with 60 s of recovery following 2-5 d of supplementation with concentrated BR. Importantly, the influence

of dietary NO_3^- supplementation on performance in all three of these tests was conducted using the same participant population, exercise modality and supplementation regime, in an attempt to limit the potential for these factors to confound the interpretation of the relative effects of dietary NO_3^- ingestion across the intermittent exercise protocols investigated.

The novel finding of Chapter 7 was that dietary NO_3^- supplementation improved the mean power output during the 24 x 6-s all-out sprint protocol, but not the 7 x 30-s all out sprint or 6 x 60-s self-paced maximal effort protocol. Specifically, performance (measured as MPO) during the 24 x 6-s protocol was improved by 7% in BR compared to PL. Furthermore, when the protocol was divided into 25% completion segments, MPO was improved with BR in sprints 1-6 (+ 7%) but not in sprints 7-12 (+4%; P = 0.18), 13-18 (+5%; P = 0.12) or 19-24 (+5%; P = 0.14). An important observation here is that dietary NO_3^- improved performance during the test that most closely resembles the intermittent exercise pattern typical of team sport play. These data are therefore consistent with those of Chapter 6, and other recent observations (Thompson et al., 2015). Together, Chapters 6 and 7 provide support for the use of dietary NO_3^- as an ergogenic aid for team sport players. However, it must be acknowledged that in Chapter 6 the study was performed using cycle ergometry and therefore further research is needed to determine if repeated running sprint performance is also improved after NO_3^- supplementation... Moreover, considering that in Chapters 6 and 7 the studies were conducted on only recreational team sport players additional research is required to examine the ergogenic potential of dietary NO_3^- in elite level team sport players (See Further Directions section for more discussion).

An important observation in Chapter 7 was that dietary NO_3^- supplementation did not improve performance during seven 30-s all-out sprints interspersed with 4 min of recovery or six 60-s maximal self-paced efforts interspersed with 1 min of recovery. Other studies have also reported no improvement in high-intensity intermittent exercise performance following NO_3^- supplementation (Christensen et al. 2013; Muggeridge et al. 2013; Martin et al. 2014). Since the beginning of the programme of research reported in this thesis, many studies have now investigated the influence of dietary NO_3^- on intermittent exercise performance. In addition to the findings presented in Chapter 6 and 7, there is currently evidence that dietary NO_3^- supplementation can improve (Bond

et al. 2012; Aucouturier et al. 2015; Thompson et al. 2015), compromise (Martin et al. 2014), or have no effect (Christensen et al. 2013; Muggeridge et al. 2013) on intermittent exercise performance. The intermittent exercise tests employed differs between these studies; moreover, interpretation of how dietary NO_3^- supplementation impacts intermittent exercise performance is further complicated by inter-study differences in participant training status, exercise modality and supplementation procedures, which could also contribute to the disparate results. A strength of Chapter 7 is that the influence of dietary NO_3^- supplementation on intermittent exercise performance was assessed in three different protocols in the same participant population (i.e. recreational team sport players) and following the same supplementation procedure, allowing for a more accurate assessment of how the exercise test impacts the relative efficacy of dietary NO_3^-. Therefore, the observation that dietary NO_3^- improved performance in the 24 x 6-s protocol but not the 7 x 30-s or 6 x 60-s protocol provides an important addition to the current knowledge of the ergogenic potential of dietary NO_3^- in intermittent exercise. Specifically, these results suggest that dietary NO_3^- may not improve performance during intermittent exercise with long duration (>30 s) and/or long recovery periods, at least during cycling exercise and in recreational team sport players.

As explained earlier, a previous study has reported an improvement in intermittent exercise with longer bouts of exercise and longer recovery periods during rowing ergometry following chronic NO_3^- supplementation (Bond et al. 2012), suggesting that there may be situations in which dietary NO_3^- can improve intermittent exercise with this activity pattern and/or exercise modality. A body of evidence exists to demonstrate that dietary NO_3^- supplementation is more effective at enhancing physiological responses and performance in type II compared to type I muscle (Hernández et al. 2012; Ferguson et al. 2013a; Ferguson et al. 2014), or in situations where type II muscle fibre recruitment is expected to be greater (Breese et al. 2013; Coggan et al. 2014; Bailey et al. 2015). Importantly, when compared to cycling, rowing ergometry results in the greater recruitment of the upper body musculature, which is suggested to contain a higher proportion of type II muscle fibres than the lower body. The reason for the inconsistency between Bond et al., (2012) and Chapter 7 may therefore be related to the exercise modality adopted (i.e. cycling vs. rowing ergometry) and its impact on muscle fibre recruitment. Interestingly, this hypothesis may also explain why NO_3^-

supplementation has been shown to improve performance during a kayak TT in elite level kayak athletes (Peeling et al. 2015) but not during cycling and running TT's in elite level cyclists, skiers or runners (Christensen et al. 2013; Boorsma et al. 2014; Sandbakk et al. 2015).

There are a number of potential mechanisms by which dietary NO_3^- supplementation may improve intermittent exercise performance. Fatigue development during intermittent exercise is linked, in part, to the depletion of muscle PCr (Gaitanos et al. 1993; Fulford et al. 2013) and the capacity to resynthesise this PCr during recovery periods (Bogdanis et al. 1995; Bogdanis et al. 1996; Mendez-Villanueva et al. 2012), particularly in the type II muscle fibres that are heavily recruited during this type of exercise (Green 1977; Essén 1978; Krustrup et al. 2004; Krustrup et al. 2009). Interestingly, supplementation with dietary NO_3^- has been reported to significantly lower the PCr cost of force production during high-intensity intermittent exercise (Fulford et al. 2013) which may delay the attainment of a critically low muscle [PCr] during intermittent exercise (Chidnok et al. 2013). Improvement in intermittent exercise performance following NO_3^- supplementation may also be related to enhanced force production in type II muscle (Hernández et al. 2012; Coggan et al. 2014) as a result of increased sarcoplasmic reticulum Ca^{2+} release (Hernández et al. 2012), and/or an improved perfusion and oxygenation of type II muscle (Ferguson et al. 2013a; Ferguson et al. 2014). For example, an increase in O_2 delivery to type II muscle may improve the resynthesis of PCr during recovery period (Paganini et al. 1997; Vanhatalo et al. 2011). It is possible that these mechanisms operate simultaneously or synergistically to enhance performance during high-intensity intermittent exercise.

Physiological measurements made during the Yo-Yo IR1 test in Chapter 6 provided insights into the potential mechanism responsible for improved intermittent exercise performance following dietary NO_3^- supplementation. In Chapter 6, blood [glucose] was significantly lower over the Yo-Yo IR1 test with BR compared to PL. This is the first (and currently only) report to show that dietary NO_3^- supplementation lowers blood [glucose] during exercise. A lower blood glucose concentration may be interpreted as an increase in skeletal muscle glucose uptake which has the potential to improve performance during the Yo-Yo IR1 test by sparing glycogen utilisation (Tsintzas and Williams 1998). Indeed, NO has been shown to play a key role in glucose uptake during

contraction in skeletal muscle (Merry et al., 2010; Bradley et al. 1999; McConell and Kingwell 2006; McConell et al. 2012), while NO_2^- has been reported to increase glucose transporter 4 (GLUT-4) translocation (Jiang et al. 2014). It is important to note that although recent research has shown that acute dietary NO_3^- ingestion (~8 mmol) does not improve skeletal muscle glucose uptake during submaximal exercise (Betteridge et al. 2016), this does not exclude the possibility that dietary NO_3^- supplementation can enhance glucose uptake during high-intensity intermittent exercise when P_{O2} and pH decline more, the reduction of NO_2^- to NO may be enhanced (compared to submaximal constant work rate exercise), and glycogen utilisation is greater, and/or when larger doses or a chronic supplementation procedure is adopted. Further research is required to investigate the effects of chronic dietary NO_3^- supplementation on glucose kinetics during high-intensity exercise.

Fatigue during intermittent exercise may also be related, in part, to reduced muscle excitability, due to a net loss of muscle K^+ (Nielsen and de Paoli 2007; McKenna et al. 2008). Interestingly, in Chapter 6 there was a trend ($P = 0.08$) for the rise in plasma $[K^+]$ during the Yo-Yo IR1 test to be attenuated with BR. These data suggest that BR supplementation may have reduced muscle K^+ efflux and accumulation in the extracellular fluids, and therefore contributed to the enhanced Yo-Yo IR1 test performance by preserving muscle excitability.

In summary, the results from Chapters 6 and 7 have made important novel contributions to further understanding of the ergogenic potential of dietary NO_3^- in intermittent exercise. Findings in both Chapters 6 and 7 provide evidence that short-term (36 h – 3 d) dietary NO_3^- supplementation can improve performance during intermittent exercise that closely resembles the exercise pattern typical of team sports game play. In doing so, these results provide support for the use of dietary NO_3^- as an ergogenic aid by team sport players competing at a non-elite level. While a number of possible mechanisms exist for this ergogenic effect, physiological measurements made in Chapter 6 suggest that an improvement in intermittent exercise may, in part, be related to an increase in glucose uptake and/or muscle excitability. Other important findings in Chapter 7 also indicate that chronic dietary NO_3^- supplementation may not be effective at improving intermittent cycling exercise with longer work bouts (i.e. >30 s) and longer recovery periods (i.e. >60 s). However, it should be acknowledged that the exercise modality,

supplementation period, and participant population tested may have a substantial influence on the ergogenic potential of dietary NO_3^- in intermittent exercise.

Future directions

Exploring the variability in plasma [NO_2^-] pharmacokinetics following acute dietary NO_3^- ingestion.

In Chapter 4, kinetic analysis of plasma [NO_2^-] pharmacokinetics following the acute ingestion of 16.8 mmol NO_3^- demonstrated a large between-participant variation in the time for plasma [NO_2^-] to reach peak concentration, and the magnitude of this peak increase. However, the measurements made in Chapter 4 did not allow for the cause of this variation to be ascertained. Considering that the acute effects of dietary NO_3^- on BP, exercise efficiency and performance are likely related to the reduction of NO_2^- to NO, or NO_2^- itself (Lundberg et al. 2010; Bailey et al. 2012), understanding this variability is likely to prove important in understanding the effectiveness of acute NO_3^- supplementation. The reduction of NO_3^- to NO_2^- is largely dependent on oral bacteria capable of NO_3^- reduction (Govoni et al. 2008). Therefore, research should focus on investigating how the pharmacokinetics of plasma [NO_2^-] are affected by inter-individual differences in microbiome as well as changes in factors that may alter the activity of the oral bacteria (e.g. temperature and pH). Moreover, investigating how normalising NO_3^- dose to body mass (as opposed to administering a set absolute NO_3^- load) influences the pharmacokinetics may also provide valuable information.

Dose-response in elite endurance athletes

In Chapter 4, the dose-response relationship between acute NO_3^- supplementation and the O_2 cost of submaximal exercise and exercise tolerance was examined. However, the participant cohort in this Chapter (and Chapter 5) was limited to healthy, recreationally-active participants. Studies suggest that highly trained/elite endurance athletes may not experience performance benefits from dietary NO_3^- supplementation (e.g. Wilkerson et al. 2012; Christensen et al. 2013; Porcelli et al. 2014), potentially due to a higher baseline plasma [NO_2^-], greater training related NOS activity, a higher proportion of type I fibres, and a greater mitochondrial and capillary density, compared to their recreationally active counterparts. However, it is possible that the dose-response

relationship is altered in this population, and elite athletes need more NO_3^- to elicit an ergogenic effect (cf. Boorsma et al. 2014). Furthermore, it is also possible that this population may benefit from additional interventions that may 'boost' NO production. For instance, Muggeridge et al., (2014) has reported that while acute NO_3^- supplementation alone did not improve 16.1 km cycling TT performance in trained cyclists, combining NO_3^- ingestion with ultra-violet (UV)-A irradiation further increased the rise in plasma [NO_2^-] (by decomposing photo reactive nitrogen oxides stored in dermal cells [Mowbray et al. 2009; Opländer et al. 2009]) and enhanced performance. Further research should be aimed at determining the influence of different NO_3^- doses, as well as the influence of NO_3^- supplementation combined with UVA irradiation (or other treatments found to 'boost' NO availability) on exercise performance in elite endurance athletes.

Exploring the mechanisms for, and duration of, a preserved reduction in submaximal exercise $\dot{V}O_2$ following chronic dietary NO_3^- supplementation.

A novel finding of Chapter 5 was that a reduction in submaximal exercise $\dot{V}O_2$ following ~4 weeks NO_3^- supplementation was observed 24 h after the latest ingestion of dietary NO_3^-, and thus in the absence of a significant rise in plasma [NO_2^-]. Although interesting, the measurements and study design in Chapter 5 did not allow this effect to be explored further. Further research should focus on the mechanism responsible for this preserved effect, the duration of supplementation required to elicit this preserved effect and how long the effect is preserved for. In regards to the mechanism, further research should explore whether the preserved reduction in submaximal $\dot{V}O_2$ is related to: 1) a maintained elevation in muscle NO availability (via the measurement of skeletal muscle tissue [NO_2^-]); and/or, 2) protein expression changes that occur and are maintained. In addition, further research should ascertain whether an improvement in high-intensity exercise performance following chronic dietary NO_3^- supplementation is also preserved after the cessation of supplementation.

Elite-level team sport athletes

Chapters 6 and 7 demonstrated that dietary NO_3^- supplementation can improve performance during intermittent exercise that closely resembles the exercise pattern typical of team sports game play. However, the participants in these experiments were

limited to recreational team-sport players. Further research is required to determine if dietary NO_3^- supplementation can improve high-intensity intermittent exercise performance in highly trained/elite team sport athletes. Although evidence for a performance-enhancing effect following dietary NO_3^- supplementation in highly trained/elite endurance athletes is weak (Wilkerson et al. 2012; Christensen et al. 2013; Porcelli et al. 2014), the type II fibre specific effects of dietary NO_3^- supplementation (Hernández et al. 2012; Ferguson et al. 2013a; Coggan et al. 2014; Ferguson et al. 2014) are likely to favour highly trained/elite team sport players who would be expected to have a greater proportion of type II muscle fibres than their endurance-trained counterparts (Tesch and Karlsson 1985).

Can dietary NO_3^- improve performance during a simulated team sport game?

The improvements in high-intensity intermittent performance observed during the Yo-Yo IR1 test (Chapter 6) and 24 x 6-s sprints (Chapter 7) provides evidence that dietary NO_3^- may improve intermittent exercise performance during team sport game play. Consistent with this, Thompson et al., (2015) recently reported an improvement in mean work done during an intermittent cycling test that aimed to mimic the physical demands and also duration of team sports match-play. However, further research is required to confirm if dietary NO_3^- can improve performance during a running test that also simulates the activity pattern, physical demands and typical distance covered during a team sport game. The Loughborough Intermittent Recovery Test (LIST; Nicholas et al. 2000) should be considered a valid test for this purpose.

Conclusion

Following reports that dietary NO_3^- supplementation can reduce the O_2 cost of submaximal exercise and improve endurance exercise performance (e.g. time-to-exhaustion tests and time-trials), the use of dietary NO_3^- as ergogenic aid has grown exponentially. By examining the influence of different dietary NO_3^- supplementation regimes, and exploring the potential of this natural supplement to improve performance during intermittent exercise, the current thesis has contributed novel information to optimise the use of dietary NO_3^- as an ergogenic aid not only for endurance exercise, but also individuals participating in invasion games.

Results in the current thesis demonstrate that 16.8 mmol NO_3^- is optimal for acutely reducing submaximal exercise $\dot{V}O_2$, while an optimal improvement in exercise tolerance is achieved with an acute dose of 8.4 mmol NO_3^-, with 16.8 mmol NO_3^- not providing a further benefit. Moreover, evidence is provided to suggest that low doses of dietary NO_3^- should not be expected to improve exercise performance or lower the O_2 cost of submaximal exercise, even when a chronic supplementation regime is adopted. Although an elevation in plasma [NO_2^-] has long been considered crucial for the effects of dietary NO_3^- supplementation to be elicited, results in this thesis showed that a lowering of submaximal exercise $\dot{V}O_2$ is preserved after chronic supplementation is ceased and plasma [NO_2^-] returns to baseline.

At the onset of this programme of research, dietary NO_3^- was reported as an aid to enhance performance mainly during continuous endurance exercise. The present thesis has presented data to indicate that this ergogenic effect also extends to intermittent exercise. Specifically, data presented herein show that dietary NO_3^- can improve performance during intermittent exercise consisting of brief bouts of high-intensity/all-out exercise, interspersed with short-recovery periods. Given that this activity pattern is consistent with that typically required during team-sport games, these data provide evidence that dietary NO_3^- may be an effective ergogenic aid for team sport players. However, dietary NO_3^- may not be effective at improving performance during cycling intermittent exercise that consists of longer work bouts and recovery periods.

In summary, the findings presented in this thesis suggest that the supplementation procedure, particularly the dose of NO_3^-, should be carefully considered by individuals wishing to benefit from the ergogenic potential of dietary NO_3^- and that dietary NO_3^- can be considered as a potential ergogenic aid for team sport players.

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