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FACTORS INFLUENCING DIETARY NITRATE METABOLISM IN HUMANS

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Submitted by Stuart Cocksedge to the University of Exeter as a thesis for the degree of Masters by Research in Sport and Health Sciences

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Abstract

Dietary nitrate (NO_3^-) supplementation, with NO_3^--rich beetroot juice (BR), increases nitric oxide (NO) bioavailability by the stepwise reduction of NO_3^- to nitrite (NO_2^-) and NO. This has been associated with a number of beneficial physiological and exercise performance effects, but understanding of NO_3^- metabolism is incomplete. The purpose of this thesis was to investigate some of the factors purported to influence dietary NO_3^- metabolism in humans. Specifically, the influence of oral cavity temperature and pH on dietary NO_3^- metabolism, and the influence of muscle oxygenation on exercise economy and tolerance were investigated following BR supplementation. **Chapter 3**: Salivary and plasma [NO_3^-] and [NO_2^-] were assessed at a neutral oral pH with a low ($Temp_{Lo}$-pH_{Norm}), intermediate ($Temp_{Mid}$-pH_{Norm}) and high ($Temp_{Hi}$-pH_{Norm}) oral temperature, and at an alkaline oral pH with a low ($Temp_{Lo}$-pH_{Hi}), intermediate ($Temp_{Mid}$-pH_{Hi}) and high ($Temp_{Hi}$-pH_{Hi}) oral temperature. Compared to the $Temp_{Mid}$-pH_{Norm} trial (976 ± 388 µM), mean salivary [NO_2^-] over the protocol was higher in the $Temp_{Mid}$-pH_{Hi} (1855 ± 423 µM), $Temp_{Hi}$-pH_{Norm} (1371 ± 653 µM), $Temp_{Hi}$-pH_{Hi} (1792 ± 741 µM), $Temp_{Lo}$-pH_{Norm} (1495 ± 502 µM) and $Temp_{Lo}$-pH_{Hi} (2013 ± 662 µM) conditions, with salivary [NO_2^-] also higher at a given oral temperature when oral pH was increased (P<0.05). The increase in mean salivary [NO_2^-] was positively correlated with the increase in salivary flow rate when all data were combined (r = 0.48, P<0.01). Plasma [NO_2^-] was higher 3 hours post BR consumption in the $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Hi} trials, but not the $Temp_{Lo}$-pH_{Norm} and $Temp_{Hi}$-pH_{Norm} trials, compared to the $Temp_{Mid}$-pH_{Norm} trial (P<0.05). **Chapter 4**: Work-to-work step cycle tests were completed to exhaustion (T_{lim}) in normobaric hypoxia (fraction of inspired oxygen; FiO_2: 15%), normoxia (FiO_2: 21%) and hyperoxia (FiO_2: 40%). Plasma [NO_2^-] was higher in all BR compared to all PL trials (P<0.05). Quadriceps tissue oxygenation index (TOI) was higher in normoxia compared to hypoxia (P<0.05) and higher in the hyperoxia compared to hypoxia and normoxia (P<0.05). T_{lim} was improved after BR compared to PL consumption (250 ± 44 vs. 231 ± 41 sec), with the magnitude of improvement being negatively correlated with quadriceps TOI at exhaustion (r = -0.78), in hypoxia (P<0.05). T_{lim} tended to be improved with BR in normoxia (BR: 364 ± 98 vs. PL: 344 ± 78 sec; P=0.087), but was not improved in hyperoxia (BR: 492 ± 212 vs. PL: 472 ±

196 sec; $P>0.05$). BR consumption increased peak oxygen uptake in hypoxia ($P<0.05$), but not normoxia or hyperoxia ($P>0.05$). Collectively, these findings improve our understanding of dietary NO_3^- metabolism and might help guide future studies assessing the efficacy of dietary NO_3^- supplementation on human health and performance.

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Symbols and abbreviations

Δ	Difference
ATP	Adenosine tri-phosphate
BR	NO_3^--rich beetroot juice
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
FiO_2	Fraction of inspired oxygen
GET	Gas exchange threshold
HCl	Hydrochloric acid
K_M	Michaelis-Menten constant
NaI	Sodium iodide
NaOH	Sodium hydroxide
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
O_2	Oxygen
PL	Placebo
PO_2	Partial pressure of oxygen
rpm	Revolutions per minute
VCl_3	Vanadium chloride
$\dot{V}CO_2$	Volume of carbon dioxide produced
V_E	Expired ventilation
V_{max}	Maximum velocity
$\dot{V}O_2$	Oxygen uptake
$\dot{V}O_{2\ peak}$	Peak oxygen uptake
$ZnSO_4$	Zinc sulphate

Declaration

The material contained within this thesis is original work conducted and written by the author.

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Chapter 1: Introduction and Literature Review

Nitric oxide

Nitric oxide (NO) is a gaseous signalling molecule that was discovered as an 'endothelium-derived relaxing factor' by Dr. Louis Ignarro, Dr. Robert Furchgott and Dr. Ferid Murad in the 1980's. NO is best known for its vasodilatory function (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Murad et al., 1978) but it also influences numerous physiological processes including mitochondrial respiration (Ghafourifar and Cadenas, 2005) and biogenesis (Nisoli et al., 2003), sarcoplasmic reticulum calcium handling (Viner, Williams and Schoneich, 2000), neurotransmission (Garthwaite, 2008) and skeletal muscle glucose uptake (Merry, Lynch and McConnell, 2010) and fatigue (Percival et al., 2010). NO has a circulatory half-life of ~0.1 seconds (Kelm and Schrader, 1990).

Nitric oxide production

NO was initially considered to be exclusively synthesised from the oxidation of L-arginine by the NO synthase (NOS) enzymes (i.e. endothelial NOS, neuronal NOS and inducible NOS; Stamler and Meissner, 2001) in a complex metabolic reaction (the NOS pathway) that requires several additional substrates/co-factors including O_2, nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin, haem and calmodulin (Alderton, Cooper and Knowles, 2001). The oxidation of NOS-derived NO was known to produce nitrate (NO_3^-) and nitrite (NO_2^-); specifically, NO_3^- is synthesised from the reaction of NO_2^- or NO with oxyhaemoglobin (Cooper, 1999) and NO_2^- is synthesised from the reaction of NO with O_2 (Ignarro et al., 1993) or when NO is oxidised by ceruloplasmin (Shiva et al., 2006). However, NO_3^- and NO_2^- were long considered inert by-products of NO metabolism (Moncada and Higgs, 1993).

It is now known that NO_3^- and NO_2^- can be reduced into bioactive NO (the NO_3^--NO_2^--NO pathway; Lundberg et al., 2004; Lundberg and Weitzberg, 2009). In addition to its endogenous synthesis, NO_3^- is naturally consumed as

part of the diet. ~80% of human dietary NO_3^- intake originates from vegetable sources (Gilchrist, Winyard and Benjamin, 2010; Hord, Tang and Bryan, 2009) with the remaining 20% derived from processed meats, where NO_3^- is used as a preservative, and drinking water. Vegetables with a 'very high' NO_3^- content (>250 mg/100 g fresh weight) include leafy greens (i.e. lettuce, spinach and rocket) and beetroot (Hord, Tang and Bryan, 2009). Following consumption, NO_3^- is rapidly absorbed in the stomach and small intestine (Florin, Neale and Cummings, 1990) and enters systemic circulation within ~60 min (Lundberg and Weitzberg, 2009) where it has a half-life of ~5 hr (McKnight et al., 1997). ~25% of the consumed NO_3^- is concentrated and secreted by the salivary glands (Lundberg and Weitzberg, 2009). This is then reduced to NO_2^- by commensal anaerobic bacteria located on the dorsal surface of the tongue, which contain the NO_3^- reductase enzymes required for NO_2^- production that humans lack (Duncan et al., 1995; Sasaki and Matano, 1979; Spiegelhalder, Eisenbrand and Preussmann, 1976). These enzymes have been suggested to have a high affinity for NO_3^-, with a maximum velocity (V_{max}) of ~2 µmol of $NO_3^-.mg^{-1}.min^{-1}$ and a Michaelis-Menten constant (K_M; the substrate concentration that provides half of the enzymes V_{max}) of <300 µM (Sparacino-Watkins, Stolz and Basu, 2014). When swallowed, NO_2^- reacts with gastric acid in the stomach to form a mixture of nitrogen oxides, including NO (Benjamin et al., 1994; Duncan et al., 1995). Alternatively, NO_2^- can be absorbed into circulation to increase plasma [NO_2^-]. Therefore, the reduction of NO_2^- to NO also occurs systemically (Zweier et al., 1995). This reaction can be catalysed by several enzymes including xanthine oxidase (Zhang et al., 1997), mitochondrial respiratory chain enzymes (Kozlov, Staniek and Nohl, 1999), cytochrome P-450 (Kozlov, Dietrich and Nohl, 2003), deoxyhaemoglobin (Cosby et al., 2003; Nagababu et al., 2003), deoxymyoglobin (Shiva et al., 2007; Rassaf et al., 2007) and even NOS (Vanin et al., 2007). NO_2^- can also be reduced non-enzymatically in the presence of protons, which is greatly enhanced by reductants such as polyphenols (Peri et al., 2005) and vitamin C (Carlsson et al., 2001). The NO_3^--NO_2^--NO pathway has been proposed to represent a means of NO synthesis when the activity of the NOS pathway is impaired, such as conditions of low O_2 availability (Bryan, 2006), and has become a major focus of NO physiology research in recent years.

Dietary nitrate supplementation

Plasma [nitrate] and [nitrite]

Human plasma [NO_3^-] and [NO_2^-] is subject to notable inter-individual variability due to differences in age, health, training status and nutritional intake, however, typical resting values have been suggested to be ~34 µmol (Jungersten et al., 1997) and ~305 nmol (Kleinbongard et al., 2003), respectively. Supplementing dietary NO_3^- intake with pharmacological (sodium NO_3^-; Larsen et al., 2007; 2010) and natural (NO_3^--rich beetroot juice; BR; Bailey et al., 2009; 2010) means has been demonstrated to increase plasma [NO_2^-]. This thesis will focus on natural dietary NO_3^- supplementation with BR.

Previous research has used a range of BR supplementation doses typically containing between 5.1 and 23 mmol NO_3^-. Wylie et al., (2013) investigated the dose-response relationship and pharmacokinetics and pharmacodynamics between acute BR supplementation (providing 4.2, 8.4 or 16.8 mmol NO_3^-) and resting plasma [NO_3^-] and [NO_2^-]. Plasma [NO_3^-] was increased by 130, 282 and 580 µM, while plasma [NO_2^-] was increased by 150, 291 and 425 nM, following 4.2, 8.4 and 16.8 mmol NO_3^- consumption, respectively. The peak change in plasma [NO_3^-] occurred 1 hour post-consumption following 4.2 and 8.4 mmol NO_3^- and 2 hour post-consumption following 16.8 mmol NO_3^-, while the peak change in plasma [NO_2^-] occurred 2 hour post-consumption following 4.2 and 8.4 mmol NO_3^- and 4 hour post-consumption following 16.8 mmol NO_3^-. Collectively, these findings indicate that BR supplementation dose-dependently increases plasma [NO_3^-] and [NO_2^-] with the delayed peak increase in plasma [NO_2^-], compared to plasma [NO_3^-], presumably consequent to the time requirement for the enterosalivary circulation and reduction, by commensal anaerobic bacteria, of NO_3^- to NO_2^-.

Physiological effects

Blood pressure

Three days of dietary NO_3^- supplementation with BR (providing 5.6 mmol.day^{-1} NO_3^-) was demonstrated to reduce systolic blood pressure by ~5 mmHg in healthy young adults (Bailey et al., 2009). Subsequent research identified that this magnitude of change was evident following a single dose (providing 5.2 mmol NO_3^-) and was maintained following both five and fifteen days of supplementation (Vanhatalo et al., 2010). The effect of BR supplementation on diastolic blood pressure appears to be less consistent, with results for (~4 mmHg reduction; Vanhatalo et al., 2010) and against (no change; Bailey et al., 2009) a reduction reported in normotensive participants. In hypertensive patients, four weeks of BR supplementation (providing 6.4 mmol.day^{-1} NO_3^-) was demonstrated to reduce systolic blood pressure by ~8 mmHg and diastolic blood pressure by ~2 mmHg (Kapil et al., 2015). Wylie et al., (2013) reported that systolic blood pressure was reduced dose dependently following acute BR supplementation providing 4.2 (~5 mmHg reduction) and 8.4 (~10 mmHg reduction) mmol NO_3^- with no additional benefit gained from the consumption of 16.8 mmol NO_3^- (~9 mmHg reduction), while diastolic blood pressure was reduced equally following the consumption of 8.4 and 16.8 mmol NO_3^- (~3 mmHg reduction) with no effect evident following the consumption of 4.2 mmol NO_3^-. The time to peak reduction in systolic and diastolic blood pressure (2-4 hours) occurred after the time to peak plasma [NO_3^-] and [NO_2^-] (~2 hours) following BR supplementation providing 8.4 mmol NO_3^- (Wylie et al., 2013). Therefore, the beneficial haemodynamic effect of dietary NO_3^- supplementation is believed to be associated with the stepwise reduction of NO_3^- to NO_2^- and NO. NO stimulates smooth muscle relaxation by the synthesis of cyclic guanosine monophosphate (cGMP; Murad, 1986).

Pulmonary and muscular oxygen uptake

Dietary NO_3^- supplementation with BR has been demonstrated to reduce the O_2 cost of moderate-intensity exercise by ~5% in healthy young adults following acute (Vanahtalo et al., 2010) and chronic (Bailey et al., 2009; Vanahatalo et al., 2010) consumption. Wylie et al., (2013) reported that steady-state O_2 uptake ($\dot{V}O_2$) was reduced dose-dependently following acute BR supplementation (providing 4.2, 8.4 or 16.8 mmol NO_3^-), with a peak reduction of ~50 ml.min^{-1}.

NO has been suggested to alter steady-state $\dot{V}O_2$ by reducing the adenosine triphosphate (ATP) cost of skeletal muscle force production (Bailey et al., 2010; Fulford et al., 2013), increasing the oxidative phosphorylation efficiency of the mitochondria (Larsen et al., 2011) and/or improving muscle blood flow (Bailey et al., 2009; Ferguson et al., 2013). Bailey et al., (2009) reported an improved muscle oxygenation following BR supplementation via the observation of an increased [total haemoglobin] and [oxyhaemoglobin] at baseline and over the first 120 sec of exercise in the *vastus lateralis* muscle using single-channel near infrared spectroscopy. Furthermore, [deoxyhaemoglobin], an index of muscle fractional O_2 extraction, was reduced by 13% during exercise following BR supplementation. The Fick equation denotes that for the same $\dot{V}O_2$, an increased O_2 delivery enables a reduced muscle fractional O_2 extraction. Therefore, the reduced $\dot{V}O_2$ following BR supplementation suggests that less O_2 extraction was required to sustain oxidative energy turnover during moderate-intensity exercise. However, while BR supplementation has been reported to lower the O_2 cost of sub-maximal exercise in health, recreationally active individuals, most studies conducted on well-trained athletes suggest it does not lower the O_2 cost of sub-maximal exercise in this population (Bescós et al., 2011; Carriker et al., 2016; Christensen, Nyberg and Bangsbo, 2013; Nyakayiru et al., 2017; Porcelli et al., 2015).

Exercise tolerance and performance

Previous research has demonstrated a ~15% improvement in continuous cycling (Bailey et al., 2009; Wylie et al., 2013) and running (Lansley et al., 2011b) exercise tolerance following BR supplementation. This magnitude of improvement has been suggested to equate to a ~1-2% improvement in athletic performance when using an ecologically valid test, such as a cycling time trial (Paton and Hopkins, 2006), which would be extremely meaningful in competition. Subsequently, a ~3% improvement in 4 and 16.1 km time trial performance by club-level cyclists (mean $\dot{V}O_{2\ peak}$ = 56 ± 6 ml.kg body mass.min^{-1}) was demonstrated following acute BR consumption (providing ~6.2 mmol NO_3^-; Lansley et al., 2011a), while a ~1% improvement in 10 km time trial performance by well-trained cyclists (mean $\dot{V}O_{2\ peak}$ = 58 ± 2 ml.kg body

mass.min^{-1}) was demonstrated following chronic BR supplementation (providing ~8 mmol.day^{-1} NO_3^- for 6 days; Cermak, Gibala and van Loon, 2012). However, not all studies have demonstrated an improved exercise performance in well-trained endurance athletes following BR supplementation (Lowings et al., 2017; McQuillan et al., 2016; Nyakayiru et al., 2017; Porcelli et al., 2015; Wilkerson et al., 2012). For example, Wilkerson et al., (2012) reported a non-significant 0.8% reduction in 50 mile time trial performance by well-trained cyclists (mean $\dot{V}O_{2\,peak}$ = 63 ± 8 ml.kg body mass.min^{-1}) following acute BR consumption (providing ~6.2 mmol NO_3^-).

Factors influencing dietary nitrate metabolism

Oral cavity temperature and pH

The reduction of NO_3^- to NO_2^- is a crucial limiting factor for dietary NO_3^- metabolism since humans lack the NO_3^- reductase enzymes required for NO_2^- production (Duncan et al., 1995; Lundberg et al., 2004). These enzymes are contained within commensal oral bacteria, which use NO_3^- as a terminal electron acceptor during anaerobic respiration (Duncan et al., 1995; Lundberg et al., 2004). Previous research has demonstrated that suppressing these oral bacteria attenuates the rise in salivary and plasma [NO_2^-] following BR supplementation (McDonagh et al., 2015; Woessner et al., 2016). The influence of facilitating the activity of the oral bacteria and the subsequent effect on salivary and plasma [NO_2^-] following dietary NO_3^- supplementation is currently unclear. Previous research has demonstrated a role for oral temperature and pH on [NO_2^-] following the incubation of NO_3^- solutions in the oral cavity, with optimal [NO_2^-] evident at a temperature attuned to a summer climate and a pH of 8 (Bojić, Bojić and Perović, 2004). The human oral cavity maintains a relatively constant temperature (34-36°C) and pH (~7; Marcotte and Lavoie, 1998). Therefore, elevating these conditions might augment the increment in salivary and plasma [NO_2^-] observed following BR supplementation, but this remains to be determined.

Muscle oxygenation

The reduction of NO_2^- to NO has been suggested to be facilitated under hypoxic conditions (Bryan, 2006) which might be evident in contracting skeletal muscle (Bailey et al., 2010; Richardson et al., 1999). This suggests a role for muscle oxygenation in the physiological effects induced by dietary NO_3^- supplementation, which might be greater in hypoxia (<21% oxygen; O_2) and lower in hyperoxia (>21% O_2) compared to normoxia (21% O_2).

The reduced fraction of inspired O_2 (FiO_2) in hypoxic air decreases arterial O_2 saturation and the intracellular partial pressure of O_2 (PO_2; Richardson et al., 1995). This stimulates local NO-mediated vasodilation to increase muscle blood flow and O_2 supply (Casey et al., 2010). Compared to normoxia, sub-maximal constant work rate exercise in hypoxia is performed at the same $\dot{V}O_2$ but with greater muscle metabolic perturbation, consequent to greater utilisation of the anaerobic reserves (i.e. phosphocreatine and glycogen) to sustain the required rate of ATP turnover (Hogan, Richardson and Haseler, 1999). The subsequent accumulation of fatigue-related metabolites (i.e. adenosine diphosphate, inorganic phosphate and hydrogen ions) contribute to an impaired exercise tolerance (Hogan, Richardson and Haseler, 1999). Dietary NO_3^- supplementation with BR has been demonstrated to reduce muscle metabolic perturbation (Vanhatalo et al., 2011), lower steady-state $\dot{V}O_2$ and speed $\dot{V}O_2$ kinetics (Kelly et al., 2014) and improve exercise tolerance (Kelly et al., 2014; Vanhatalo et al., 2011) in hypoxia, presumably due to an increased NO bioavailability.

The increased FiO_2 in hyperoxic air increases arterial O_2 saturation and the intracellular PO_2 (Collins et al., 2015) and has been associated with an improved sub-maximal (Adams and Welch, 1980) and maximal (Adams and Welch, 1980; Linossier et al., 2000) exercise capacity. This is likely mediated by an increased muscle $\dot{V}O_2$ (Astorino and Robergs, 2003) and greater maintenance of muscle contractile function (Linossier et al., 2000); mechanisms that are consistent with those induced by dietary NO_3^- supplementation in hypoxia. To date, no study has investigated the physiological effects induced by dietary NO_3^- supplementation during exercise in hyperoxia. However, a reduced time to fatigue was observed in mouse muscle fibres following incubation with sodium NO_2^- at a supra-physiological PO_2 (Nogueira et al., 2013). Therefore,

exercise performed in hyperoxia might be associated with a reduced stimulus for NO_2^- reduction to NO, thus attenuating the physiological effects induced by dietary NO_3^- supplementation. However, this remains to be identified.

Summary

Dietary NO_3^- supplementation can elicit several physiological effects, presumably by increasing NO bioavailability. These effects include: a reduced systolic blood pressure (Bailey et al., 2009; Vanhatalo et al., 2010; Webb et al., 2008; Wylie et al., 2013), due to an increased synthesis of cGMP (Murad, 1986); a reduced O_2 cost of moderate-intensity exercise (Bailey et al., 2009; Vanhatalo et al., 2010; Wylie et al., 2013), due to improvements in muscle contractile function (Bailey et al., 2010), mitochondrial efficiency (Larsen et al., 2011) and/or muscle blood flow (Bailey et al., 2009; Ferguson et al., 2013); and an improved tolerance to severe-intensity exercise (Bailey et al., 2009; Wylie et al., 2013). These effects are believed to be NO-mediated. The synthesis of NO by NO_3^- metabolism is critically dependent on the reduction of NO_3^- to NO_2^- in the mouth and the subsequent reduction of NO_2^- to NO in the stomach or systemically. Manipulating oral cavity temperature and pH has been suggested to aid NO_3^- reduction to NO_2^- while lowering muscle oxygenation has been suggested to aid the reduction of NO_2^- to NO. Therefore, these factors have the potential to increase NO signalling after BR supplementation.

Aims

The purpose of this thesis is to investigate the effect of manipulating oral temperature and pH, and muscle oxygenation, on dietary NO_3^- metabolism. This thesis aims to enhance existing knowledge of the conditions that might influence the efficacy of dietary NO_3^- supplementation to improve exercise physiology and performance. The following research questions will be addressed:

1) Does the manipulation of oral cavity temperature and pH influence the effectiveness of BR supplementation at increasing NO bioavailability in humans?
    a. Does an alkaline oral cavity pH increase the effectiveness of BR supplementation at elevating salivary and plasma [NO_2^-]?
    b. Does an increased oral cavity temperature increase the effectiveness of BR supplementation at elevating salivary and plasma [NO_2^-]?
    c. Does the combination of an alkaline oral cavity pH and an increased oral cavity temperature exert a synergistic increase in salivary and plasma [NO_2^-]?

2) Does altering the FiO_2 influence the effectiveness of BR supplementation at improving the physiological responses and tolerance to exercise?
    a. Does BR supplementation reduce the O_2 cost of moderate-intensity exercise and enhance severe-intensity exercise tolerance in normoxia, as previously reported (Bailey et al., 2009).
    b. Does BR supplementation reverse the detrimental effects of hypoxia on the physiological responses and tolerance to exercise, as previously reported (Kelly et al., 2014).
    c. Does BR supplementation induce physiological responses and alter the tolerance to exercise in hyperoxia?

Hypotheses

The following hypotheses will be tested:

1) Salivary and plasma [NO_3^-] and [NO_2^-] would be increased following BR supplementation, [NO_2^-] would be increased further when oral temperature and pH were elevated independently and the greatest [NO_2^-] would be achieved when oral temperature and pH were elevated simultaneously.

2) Following dietary NO_3^- supplementation, muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance would be augmented in normoxia, increased further in hypoxia and attenuated in hyperoxia.

Chapter 2: General methods

General experimental procedures

Ethics and participant consent

The two studies included in this thesis were approved by the University of Exeter's Sport and Health Sciences Ethics Committee prior to the onset of data collection. Participants provided their written informed consent after reading the Participant Information Sheet, which described in detail the experimental protocol and their specific involvement, and having any questions about the study answered.

Health and safety

All experimental procedures were conducted in accordance with the Sport and Health Sciences Health and Safety Code of Practise 2015-16. The researchers were vigilant of laboratory cleanliness, safety and suitability for the testing of human participants.

Participants

The participants recruited for the studies in Chapters 3 and 4 were male University of Exeter students (mean ± SD; age = 23 ± 3 years, height = 1.80 ± 0.06 m; body mass = 77.1 ± 9.4 kg). Participants were non-smokers and free from disease. No participant reported antibiotics use prior to the study or dietary supplement or antibacterial mouthwash use during the study. The effects of prior antibacterial mouthwash use on the oral microbiome would be transient, thus not likely to influence our findings. All participants were recreationally active but not highly trained. In both studies, participants were instructed to report to the laboratory in a rested and fully hydrated state, having not consumed food in the previous 3 hours, caffeine in the previous 12 hours or alcohol in the previous 24 hours. Strenuous exercise was to be avoided in the previous 24 hours. Experimental testing was performed at the same time of day

(±2 hours) for each participant to minimise the potential for diurnal variation to influence the results.

Supplementation

Dietary NO_3^- supplementation was administered in the form of BR, which was donated by James White Drinks (Ipswich, UK). In both studies, participants were instructed to consume either concentrated NO_3^--rich BR (providing ~6.2 mmol NO_3^- per 70 ml) or concentrated NO_3^--depleted BR as a placebo (PL; providing negligible NO_3^- content) in a repeated measures, crossover experimental design. The [NO_3^-] of three BR supplements was measured directly to determine the accuracy of the data reported by the manufacturer (Jajja et al., 2014). Similar to the findings of Jajja and colleagues, mean [NO_3^-] (6.5 ± 0.7 mmol) differed from that reported by the manufacturer. The PL supplement was manufactured by filtering the BR through a column containing Purolite A520E ion exchange resin prior to pasteurisation, which selectively removes the NO_3^- ions (Lansley et al., 2011a; 2011b). This procedure creates a PL supplement that is identical to the test supplement in appearance, taste and smell.

In Chapter 3, the supplements were either concentrated BR (1 x 70 ml) or concentrated PL (1 x 70 ml). Participants were instructed to consume the test supplement at the beginning of the experimental protocol. A washout period of at least 24 hours separated each experimental visit. In Chapter 4, the supplements were either concentrated BR (3 x 70 ml) or concentrated PL (3 x 70 ml). Participants were instructed to consume the test supplement 2.5 hours before the exercise test. A washout period of at least 48 hours separated each experimental visit (Lansley et al., 2011a; 2011b). In both chapters, participants were instructed to consume a NO_3^- and glucosinolate/thiocyante restricted diet throughout data collection and to replicate their dietary intake in the 24 hours prior to each experimental visit. The harmless, temporary side effects of BR consumption, including beeturia (red urine) and red stools, were communicated to participants.

Measurement procedures

Descriptive data

Prior to any experimental testing, each participant's age was recorded and their height and mass were measured using a Seca Stadiometer SEC-225 (Seca, Hamburg, Germany) and Seca Digital Column Scale SEC-170 (Seca, Hamburg, Germany), respectively.

Blood sample collection

For the studies in chapters 3 and 4, a Insyte-W cannula (Becton-Dickinson, Madrid, Spain) was inserted into a forearm vein by a phlebotomy-trained individual. This allowed for multiple blood samples to be drawn efficiently during the subsequent protocol. In chapter 3, the cannula was kept clear with the infusion of 0.9% saline at a rate of 10 $ml.h^{-1}$, which was administered with single-use 5 ml syringes (Terumo, Leuven, Belgium). 5 ml syringes were also used to draw the blood samples, following the removal of dead space content using a 2.5 ml single-use syringe (Terumo, Leuven, Belgium). The samples were immediately ejected into 5 ml Vacutainer lithium-heparin tubes (Becton-Dickinson, New Jersey, USA) and were subsequently centrifuged at 4000 revolutions per minute (rpm) and 4°C for 8 min. The plasma was then extracted and stored at -80°C for later analyses of NO_3^- and NO_2^-.

Measurement of nitrate and nitrite concentrations

The quantification of plasma [NO_3^-] and [NO_2^-] are technically challenging with considerable variability reported between the assays used. For example, the modified Griess reaction is an assay used frequently to measure [NO_2^-] (Tsikas, 2007). However, this method lacks the sensitivity to identify the nanomolar NO_2^- levels present in human plasma. Therefore, ozone-based chemiluminescence is considered the preferred assessment method (Higuchi and Motomizu, 1999).

All glassware, utensils and surfaces were regularly rinsed with deionised water to remove residual NO_3^- and NO_2^- before sample analyses. In chapter 3, plasma samples were deproteinized using zinc sulfate ($ZnSO_4$)/sodium hydroxide (NaOH) precipitation prior to determination of [NO_3^-]. Firstly, 500 µL of 0.18 N NaOH was added to 100 µL of sample followed by 5 min incubation at room temperature. Subsequently, samples were treated with 300 µL aqueous $ZnSO_4$ (5% w/v) and vortexed for 30 seconds before undergoing an additional 10 min incubation period at room temperature. Samples were then 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 chloride (VCl_3) in 1 M hydrochloric acid (HCl) within an air-tight purging vessel. Plasma samples were introduced to the vessel via 50 uL injections into the septum at the top of the vessel. The spectral emission of electronically excited nitrogen dioxide, derived from the reaction of NO with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers NOA 280i gas-phase chemiluminescence NO analyzer (Analytix, Durham, UK). The [NO_3^-] was determined by plotting signal (mV) area against a calibration plot of sodium NO_3^- standards. The [NO_2^-] of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and aqueous sodium iodide (NaI) (4% w/v) from sodium NO_2^- standards. 100 uL injections were used for plasma [NO_2^-] determination. After thawing at room temperature, saliva samples were centrifuged for 10 min at 14000 rpm and the supernatant was removed for subsequent analysis. The supernatant was diluted 100-fold with deionized water and [NO_3^-] and [NO_2^-] were determined from 50 uL injections using the same reagents described above for the plasma analyses. In chapter 4, plasma [NO_2^-] was determined using the same method as in chapter 3.

Exercise testing

In chapter 4, participants performed a cycle ramp incremental exercise test for the determination of their $\dot{V}O_{2\ peak}$ and gas exchange threshold (GET). All tests were performed on an Excalibur Sport cycle ergometer (Lode, Groningen, The

Netherlands). Participants were instructed to remain seated and maintain a pedal cadence of 80 rpm for as long as possible. The test began with a baseline stage of 20 W for 4 min. Following this, the workload was increased at a linear rate of 40 $W.min^{-1}$ and continued until the participant reached volitional exhaustion or the pedal cadence fell below 70 rpm for five consecutive seconds. Breath-by-breath pulmonary gas exchange data were collected continuously throughout using a Medgraphics Cardiorespiratory Diagnostics (Express Series, Gloucester, UK). The data were then averaged over consecutive 10 sec time periods with $\dot{V}O_{2\ peak}$ determined as the highest 30 sec average value. GET was derived from test data using the cluster of measurements previously employed by Bailey et al., (2009). Briefly, GET was determined as the first: disproportionate increase in the volume of carbon dioxide (CO_2) produced ($\dot{V}CO_2$), as determined by visual inspection of the individual data points of $\dot{V}O_2$ plotted against $\dot{V}CO_2$; increase in expired ventilation (V_E)/$\dot{V}O_2$ without an increase in $V_E/\dot{V}CO_2$; increase in end-tidal O_2 tension without a decline in end-tidal CO_2 tension.

'Work-to-work step' cycle exercise tests were then used to assess moderate-intensity exercise economy and severe-intensity exercise tolerance (Breese et al., 2011). All tests were performed on an Excalibur Sport cycle ergometer (Lode, Groningen, The Netherlands). Each participant's preferred saddle height and handle bar configuration was recorded and reproduced for subsequent tests. The test began with a baseline stage of 20 W for 3 min. Following this, immediate transitions were performed to 90% GET for 4 min and then 75% Δ (% difference between power output at the GET and $\dot{V}O_{2\ peak}$) until volitional exhaustion. Breath-by-breath pulmonary gas exchange data were collected continuously throughout using a Medgraphics Cardiorespiratory Diagnostics (Express Series, Gloucester, UK). The work rates were calculated from the individual's ramp test pulmonary gas exchange data, with consideration for the mean response time of $\dot{V}O_2$ during this mode of exercise (i.e. two-thirds of the ramp rate was subtracted from the power output value at GET and $\dot{V}O_{2\ peak}$; Whipp et al., 1981). Exercise tolerance was represented by the time to exhaustion following the transition to 75% Δ (Bailey et al., 2009).

Statistical methods

In chapters 3 and 4, all statistical analyses were performed using Statistical Package for Social Sciences version 23. The specific statistical analyses run in each study are outlined in their respective chapters. Prior to any analysis, data sets were screened for normality using appropriate procedures. Statistical significance was accepted at $P<0.05$. All data are presented as mean ± standard deviation unless otherwise stated.

Chapter 3: Influence of oral temperature and pH on dietary nitrate metabolism in healthy adults

Abstract

It has been reported that the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by oral NO_3^--reducing bacteria can be augmented by an increased oral temperature and pH. However, the independent and combined effect of increasing oral temperature and pH on dietary NO_3^- metabolism is unclear. This study tested the hypothesis that the elevated salivary and plasma [NO_2^-] observed following NO_3^- supplementation would be further enhanced by independently increasing oral temperature and pH, with a greater effect demonstrated by simultaneously increasing oral temperature and pH. Seven heathy adult males (mean ± SD, age 23 ± 4 yr, body mass 76 ± 11 kg) consumed 1 x 70 ml of beetroot juice concentrate (BR, providing ~6.5 mmol NO_3^-) on six separate laboratory visits. In a randomised crossover experimental design, salivary and plasma [NO_3^-] and [NO_2^-] were assessed at a neutral oral pH with a low ($Temp_{Lo}$-pH_{Norm}), intermediate ($Temp_{Mid}$-pH_{Norm}) and high ($Temp_{Hi}$-pH_{Norm}) oral temperature, and at an alkaline oral pH with a low ($Temp_{Lo}$-pH_{Hi}), intermediate ($Temp_{Mid}$-pH_{Hi}) and high ($Temp_{Hi}$-pH_{Hi}) oral temperature. Compared to the $Temp_{Mid}$-pH_{Norm} trial (976 ± 388 µM), mean salivary [NO_2^-] over the protocol was higher in the $Temp_{Mid}$-pH_{Hi} (1855 ± 423 µM), $Temp_{Hi}$-pH_{Norm} (1371 ± 653 µM), $Temp_{Hi}$-pH_{Hi} (1792 ± 741 µM), $Temp_{Lo}$-pH_{Norm} (1495 ± 502 µM) and $Temp_{Lo}$-pH_{Hi} (2013 ± 662 µM) conditions, with salivary [NO_2^-] also higher at a given oral temperature when oral pH was increased (P<0.05). The increase in mean salivary [NO_2^-] was positively correlated with the increase in salivary flow rate when all data were combined (r = 0.48, P<0.01). Plasma [NO_2^-] was higher 3 hours post BR consumption in the $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Hi} trials, but not the $Temp_{Lo}$-pH_{Norm} and $Temp_{Hi}$-pH_{Norm} trials, compared to the $Temp_{Mid}$-pH_{Norm} trial (P<0.05). Therefore, despite consuming the same NO_3^- dose, the increases in salivary and plasma [NO_2^-] varied depending on the temperature and pH of the oral cavity. These findings might have implications for enhancing dietary NO_3^- metabolism and its potential beneficial effect on human health and performance.

Introduction

Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) have classically been viewed as inert end-products of nitric oxide (NO) metabolism. However, a substantial body of evidence has emerged over the last two decades indicating that NO_3^- and NO_2^- can be chemically reduced into bioactive NO (Lundberg et al., 2004; Lundberg and Weitzberg, 2009; 2010). Therefore, increasing dietary NO_3^- consumption might enhance NO production. Supplementation with natural NO_3^--rich beetroot juice (BR) has been demonstrated to increase salivary (McDonagh et al., 2015) and plasma (Bailey et al., 2009; 2010; McDonagh et al., 2015; Webb et al., 2008) [NO_2^-]. This has been associated with the observation of NO-like physiological effects including reductions in resting blood pressure (Bailey et al., 2009; McDonagh et al., 2015; Webb et al., 2008) and the oxygen cost of moderate-intensity exercise (Bailey et al., 2009). As such, considerable research interest has been generated regarding the therapeutic potential of dietary NO_3^- supplementation for the treatment of cardiovascular diseases (reviewed by Lundberg et al., 2011) as well as the ergogenic implications for physical performance (reviewed by Bailey et al., 2012).

~80% of human dietary NO_3^- intake originates from vegetable sources including leafy greens and beetroot (Gilchrist et al., 2010; Hord et al., 2009). Following consumption, NO_3^- is rapidly absorbed in the stomach and small intestine (Florin et al., 1990) and enters systemic circulation within ~60 minutes (Lundberg and Weitzberg, 2009). The salivary glands concentrate and secrete ~25% of the ingested NO_3^- (Lundberg and Weitzberg, 2009), which is then reduced to NO_2^- by commensal bacteria located on the dorsal surface of the tongue (Duncan et al., 1995; Sasaki and Matano, 1979; Spiegelhalder et al., 1976). When swallowed, NO_2^- reacts with gastric acid in the stomach to form a mixture of nitrogen oxides including NO (Benjamin et al., 1994; Duncan et al., 1995).

The reduction of NO_3^- to NO_2^- is a crucial limiting factor for dietary NO_3^- metabolism because humans lack the NO_3^- reductase enzymes required for NO_2^- production (Duncan et al., 1995; Lundberg et al., 2004). Commensal oral bacteria, such as the *Veillonella* genus (Doel et al., 2005; Hyde et al., 2014),

contain these enzymes and use NO_3^- as a terminal electron acceptor during anaerobic respiration (Duncan et al., 1995; Lundberg et al., 2004). This promotes a symbiotic relationship between the bacteria and its host; one that is characterised by the reduction of NO_3^- to NO_2^-. Importantly, the use of antibacterial mouthwash abolishes the oral microbiota, thus attenuating the rise in salivary and plasma [NO_2^-] following BR supplementation (McDonagh et al., 2015; Woessner et al., 2016). Conversely, NO_2^- production, following the incubation of NO_3^- solutions in the oral cavity, was facilitated by increments in temperature and pH with peak bioconversion occurring at a temperature attuned to a summer climate and a pH of 8 (Bojić, Bojić and Perović, 2004; Xu, Xu and Verstraete, 2000). Considering that the human oral cavity maintains a relatively constant temperature (34-36°C) and pH (~7; Marcotte and Lavoie, 1998), it might be possible for NO_2^- production following BR supplementation to be altered by manipulating these conditions. To date, no study has investigated the reduction of NO_3^- to NO_2^- following dietary NO_3^- supplementation and changes in oral temperature and pH. Increased salivary [NO_3^-] after dietary NO_3^- consumption might enhance NO production and its associated health and performance benefits.

The purpose of this study was to investigate how changes in oral temperature and pH influence the ability of acute BR supplementation to increase salivary and plasma [NO_2^-] in humans. It was hypothesised that: 1) salivary and plasma [NO_3^-] and [NO_2^-] would increase following BR supplementation; 2) salivary and plasma [NO_2^-] would be increased further when oral temperature and pH were elevated independently; and 3) a greater salivary and plasma [NO_2^-] would be achieved when oral temperature and pH were elevated simultaneously.

Methods

Seven healthy adult males (mean ± SD; age = 23 ± 4 years, height = 1.79 ± 0.06 m, body mass = 75.7 ± 10.5 kg) volunteered to participate in this experiment. Participants were all non-smokers and were not current users of any medication. Each participant was familiarised with the laboratory testing protocol prior to data collection. The procedures received ethical approval from

the Institutional Research Ethics Committee. Written informed consent was obtained prior to any involvement in the experiment and following detailed explanations of the procedures, associated risks and potential benefits. For each visit, participants were instructed to report to the laboratory in a rested and fully hydrated state, having not consumed food in the previous 3 hours, caffeine in the previous 12 hours or alcohol in the previous 24 hours. Strenuous exercise was also avoided in the 24 hours preceding each visit. Participants kept a food diary in the 24 hours preceding their first experimental visit, avoiding foods high in dietary NO_3^- and glucosinolate/thiocyanate, and replicated this consumption in the 24 hours prior to their subsequent visits. The use of antibacterial mouthwash was prohibited to preserve the commensal bacteria in the oral cavity (Govoni et al., 2008). All experimental visits were performed in an air-conditioned laboratory (20°C) at the same time of day (±2 hours) to minimise the influence of diurnal biological variation.

Experimental design

Participants reported to the laboratory on seven occasions over a 4-7 week period. The first visit was used to familiarise the individual with the experimental testing procedures including venous cannulation, saliva sampling, oral temperature assessment and mouth rinse administration. The experimental conditions were completed on visits two to seven. On each occasion a single dose (1 x 70 ml) of NO_3^--rich BR concentrate (Beet it, James White Drinks, Ipswich, UK), containing ~6.5 mmol NO_3^-, was consumed and the temperature and pH of the oral cavity were manipulated to assess the effect of these variables on dietary NO_3^- metabolism. Temperature of the oral cavity was manipulated using a combination of breathing techniques, hot water bottles and/or gel packs. The three temperature conditions were: 1) 'low', which consisted of breathing through the mouth ($Temp_{Lo}$); 2) 'intermediate', which consisted of breathing through the nose with the mouth closed ($Temp_{Mid}$); and 3) 'high', which consisted of breathing though the nose with the mouth closed while wearing two fleeced, hot water bottles, containing boiling water, applied anti-parallel around the neck and a heated gel pack (Reliance Medical, Cheshire, UK) strapped to the left cheek ($Temp_{Hi}$). Specifically, the apex of the

inferior hot water bottle was in contact with the posterior surface of the neck while the apex of the superior hot water bottle was in contact with the anterior surface of the neck and the upper surface rested on the submandibular space. The gel pack was heated for 45 sec in a microwave oven immediately prior to application. pH of the oral cavity was manipulated using mouth rinses. Here, 30 ml of either neutral (tap water; pH 7.3 ± 0.1; pH_{Norm}) or alkaline (tap water titrated using food-grade sodium bicarbonate; 500 ml water to 10 g sodium bicarbonate; pH 8.1 ± 0.1; pH_{Hi}) test solution was swilled for 2 min before being fully expectorated. Therefore, the six experimental conditions were: 1) intermediate oral temperature, neutral oral pH ($Temp_{Mid}$-pH_{Norm}); 2) intermediate oral temperature, high oral pH ($Temp_{Mid}$-pH_{Hi}); 3) high oral temperature, neutral oral pH ($Temp_{Hi}$-pH_{Norm}); 4) high oral temperature, high oral pH ($Temp_{Hi}$-pH_{Hi}); 5) low oral temperature, neutral oral pH ($Temp_{Lo}$-pH_{Norm}); and 6) low oral temperature, high oral pH ($Temp_{Lo}$-pH_{Hi}). A test order was assigned using a randomised crossover design and each visit was separated by at least 24 hours.

Procedures

Upon arrival at the laboratory, participants were provided with a standardised, low dietary NO_3^- and glucosinolate/thiocyanate, breakfast consisting of 54 g of Oats So Simple porridge oats (Quaker Oats, Chicago, USA), prepared with 180 ml of tap water, and one 20 g sachet of Lyle's Golden Syrup (Tate & Lyle, London, UK). Following this, an Insyte-W cannula (Becton-Dickinson, Madrid, Spain) was inserted into a forearm vein by a phlebotomy-trained individual and was kept clear with the infusion of 0.9% saline at a rate of 10 $ml.h^{-1}$ for the duration of the protocol. The baseline blood sample was then drawn (*see Measurements*). Subsequently, baseline oral temperature was recorded (*see Measurements*) for 10 min with participants breathing through their nose with a closed mouth. The baseline saliva sample was collected (*see Measurements*) during the last 2 min of the baseline oral temperature measurement. The temperature manipulation was then applied with the hot water bottles/gel packs replaced every 30 min in the $Temp_{Hi}$-pH_{Norm} and $Temp_{Hi}$-pH_{Hi} trials to maintain the elevated oral temperature. The temperature probe was removed after 5 min

with subsequent measurements taken for 5.5 min every 30 min. The first mouth rinse was then performed with subsequent rinses occurring every 7.5 min. Pilot testing revealed this to be the required frequency of rinsing to maintain the elevated oral pH. The test supplement was then consumed and each hour, for the next 3 hours, a blood and saliva sample were collected. At the 2:30 hour mark, the time taken for peak NO_3^- reduction following BR consumption (Wylie et al., 2013), the temperature and pH manipulations ceased.

Measurements

Venous blood samples (~5 ml) were drawn into lithium-heparin tubes (Becton-Dickinson, New Jersey, USA) and immediately centrifuged at 4000 rpm and 4°C for 10 min. Following this, the plasma was extracted and stored at -80°C for later analysis of [NO_3^-] and [NO_2^-] (*see below*). For the collection of whole, unstimulated, saliva samples, participants first swallowed their saliva content and then drooled into a sample tube until the base was full (~1.2 ml). The sample was immediately analysed for temperature and pH, using a S_I series pH meter (Sentron, Leek, The Netherlands), and volume, using a S1 Pipet Filler and serological pipette (Thermo Scientific, Massachusetts, USA), before storage at -80°C for later analyses of [NO_3^-] and [NO_2^-] (*see below*). Oral temperature was measured by placing a temperature probe (Carefusion, Illinois, USA) under the tongue. The probe was connected to a Squirrel SQ2020 Series Data Logger (Grant, Cambridgeshire, UK), which captured data at 0.33 Hz. Upon completion of each mouth rinse, the solution was fully expectorated and the volume of the mixture was recorded to provide an indication of how much NO_2^--rich saliva had been lost. In the $Temp_{Mid}$-pH_{Norm} and $Temp_{Mid}$-pH_{Hi} trials, mouth rinses were heated to 35.0 ± 1.2 °C to maintain oral temperature, whereas mouth rinses were administered at room temperature (20.7 ± 0.1 °C) in the $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} trials to lower oral temperature, and heated to 40.4 ± 0.7 °C to increase oral temperature in the $Temp_{Hi}$-pH_{Norm} and $Temp_{Hi}$-pH_{Hi} trials.

All glassware, utensils and surfaces were rinsed with deionised water to remove residual NO_3^- and NO_2^- before sample analyses. Plasma samples were deproteinized using zinc sulfate (ZnSO4)/sodium hydroxide (NaOH)

precipitation prior to determination of [NO_3^-]. Firstly, 500 µL of 0.18 N NaOH was added to 100 µL of sample followed by 5 min incubation at room temperature. Subsequently, samples were treated with 300 µL aqueous ZnSO4 (5% w/v) and vortexed for 30 seconds before undergoing an additional 10 min incubation period at room temperature. Samples were then 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 chloride (VCl_3) in 1 M hydrochloric acid (HCl) within an air-tight purging vessel. Plasma samples were introduced to the vessel via 50 uL injections into the septum at the top of the vessel. The spectral emission of electronically excited nitrogen dioxide, derived from the reaction of NO with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers NOA 280i gas-phase chemiluminescence nitric oxide analyzer (Analytix, Durham, UK). The [NO_3^-] was determined by plotting signal (mV) area against a calibration plot of sodium nitrate standards. The [NO_2^-] of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and aqueous sodium iodide (NaI) (4% w/v) from sodium nitrite standards. 100 uL injections were used for plasma [NO_2^-] determination. After thawing at room temperature, saliva samples were centrifuged for 10 min at 14000 rpm and the supernatant was removed for subsequent analysis. The supernatant was diluted 100-fold with deionized water and [NO_3^-] and [NO_2^-] were determined from 50 uL injections using the same reagents described above for the plasma analyses.

Data analysis

Oral temperature at each time point was taken as the mean temperature over the final 5 min of the recording. Data for oral temperature were then analysed as a condition mean, which was taken as the mean temperature of all time points. Data for salivary pH was analysed as the mean salivary pH between the baseline, 1 and 2 hours post BR consumption samples. Salivary flow rate was calculated by dividing the volume added to the mouth rinse solutions by the collection time, based on the procedure used by Walsh et al., (2004).

Statistical analysis

A two-way (condition × time) repeated measures ANOVA was used to determine the independent and combined effects of manipulating oral temperature and pH on salivary and plasma [NO_3^-] and [NO_2^-]. A one-way repeated measures ANOVA was used to determine the effects of oral temperature and pH manipulation on the mean salivary and plasma [NO_3^-] and [NO_2^-], oral temperature, salivary pH and salivary flow rate across the experimental conditions. Where the analysis revealed a significant main or interaction effect, Fishers Least Significant Difference tests were used to determine the origin of such effects. Pearson's product moment correlation coefficient was used to assess the relationship between changes in variables across conditions. Statistical analyses were performed using SPSS version 23.0 (IBM, Chicago, USA) with significance set at $P<0.05$. All results are presented as means ± SD unless stated otherwise.

Results

Self-reported compliance to the avoidance of potential confounding factors (i.e. restricting dietary intake of NO_3^- and glucosinolate/thiocyanate and not using antibacterial mouthwash) was 100%. BR consumption was well tolerated by all participants with only minor, non-harmful side effects, including beeturia (red urine) and red stools, reported. This is consistent with previous studies using a similar dose of BR consumption (Bailey et al., 2009; Wylie et al., 2013).

Oral temperature

The influence of BR consumption and oral temperature and pH manipulation on oral temperature is presented in Figure 1. There was a main effect for condition on oral temperature ($P<0.01$). Oral temperature was higher in the $Temp_{Mid}$-pH_{Norm} and $Temp_{Mid}$-pH_{Hi} trials compared to the $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-

pH_{Hi} trials, and higher in the $Temp_{Hi}$-pH_{Norm} and $Temp_{Hi}$-pH_{Hi} trials compared to the $Temp_{Mid}$-pH_{Norm}, $Temp_{Mid}$-pH_{Hi}, $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} trials (P<0.01 for all comparisons).

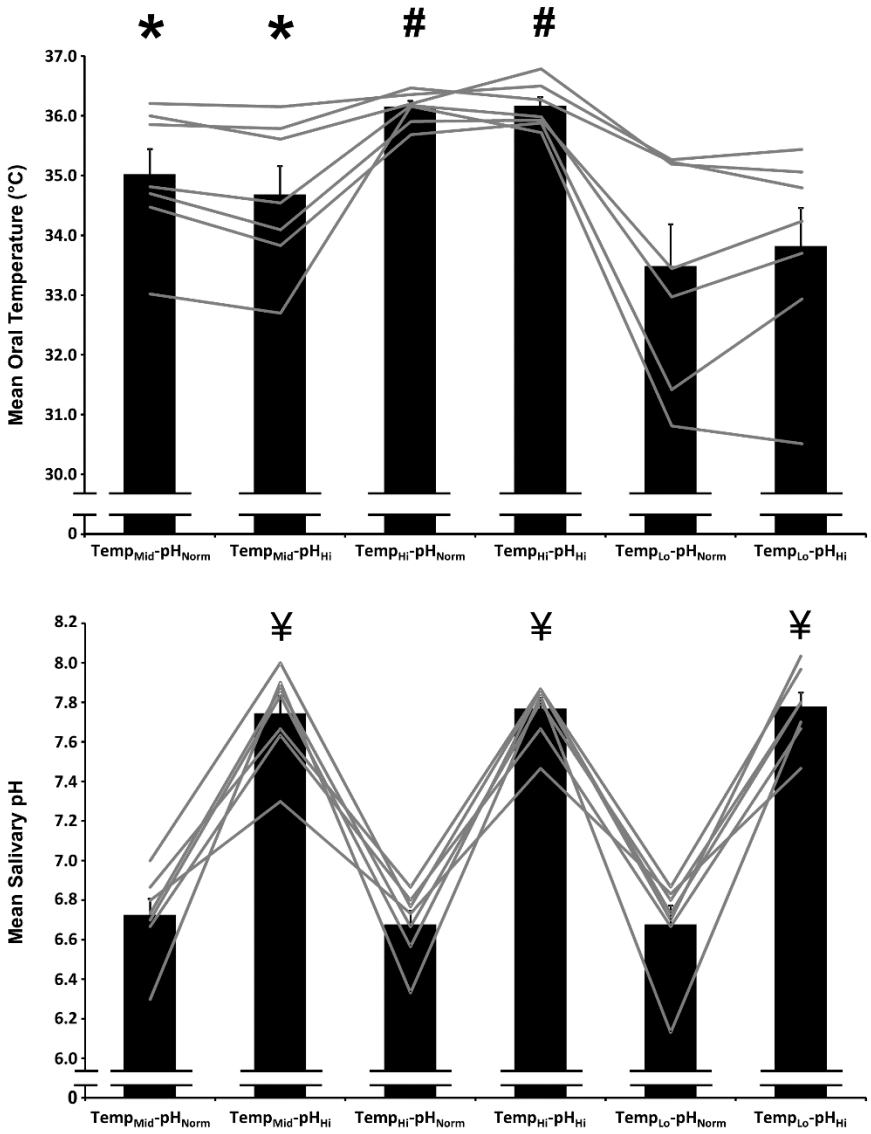

Figure 1. Oral temperature (upper panel) and salivary pH (lower panel) after acute nitrate-rich beetroot juice consumption in intermediate temperature-neutral pH ($Temp_{Mid}$-pH_{Norm}), intermediate temperature-high pH ($Temp_{Mid}$-pH_{Hi}), high temperature-neutral pH ($Temp_{Hi}$-pH_{Norm}), high temperature-high pH ($Temp_{Hi}$-pH_{Hi}), low temperature-neutral pH ($Temp_{Lo}$-pH_{Norm}) and low temperature-high pH ($Temp_{Lo}$-pH_{Hi}) conditions. The oral temperature and salivary pH data are expressed as the mean response of all samples collected during the oral temperature and pH manipulations. The filled bars represent the group mean ± SEM responses while the solid grey lines represent the individual responses in each experimental condition. * indicates higher than $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} (P<0.05). # indicates higher than $Temp_{Mid}$-pH_{Norm}, $Temp_{Mid}$-pH_{Hi}, $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} (P<0.05). ¥ indicates higher than $Temp_{Mid}$-pH_{Norm}, $Temp_{Hi}$-pH_{Norm} and $Temp_{Lo}$-pH_{Norm} (P<0.05).

Salivary pH

The influence of BR consumption and oral temperature and pH manipulation on salivary pH is presented in Figure 1. There was a main effect for condition on salivary pH (P<0.01). Salivary pH was higher in $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Hi} trials compared to the $Temp_{Mid}$-pH_{Norm}, $Temp_{Hi}$-pH_{Norm} and $Temp_{Lo}$-pH_{Norm} trials (P<0.01 for all comparisons).

Salivary flow rate

The influence of BR consumption and oral temperature and pH manipulation on salivary flow rate is presented in Figure 2. There was a main effect for condition on salivary flow rate (P<0.05). Salivary flow rate was higher in the $Temp_{Hi}$-pH_{Hi} trial than the $Temp_{Hi}$-pH_{Norm} trial (P<0.05), higher in the $Temp_{Lo}$-pH_{Hi} trial than the $Temp_{Mid}$-pH_{Norm}, $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Norm} and $Temp_{Lo}$-pH_{Norm} trials (P<0.05) and tended to be higher in the $Temp_{Lo}$-pH_{Hi} trial than the $Temp_{Hi}$-pH_{Hi} trial (P=0.086).

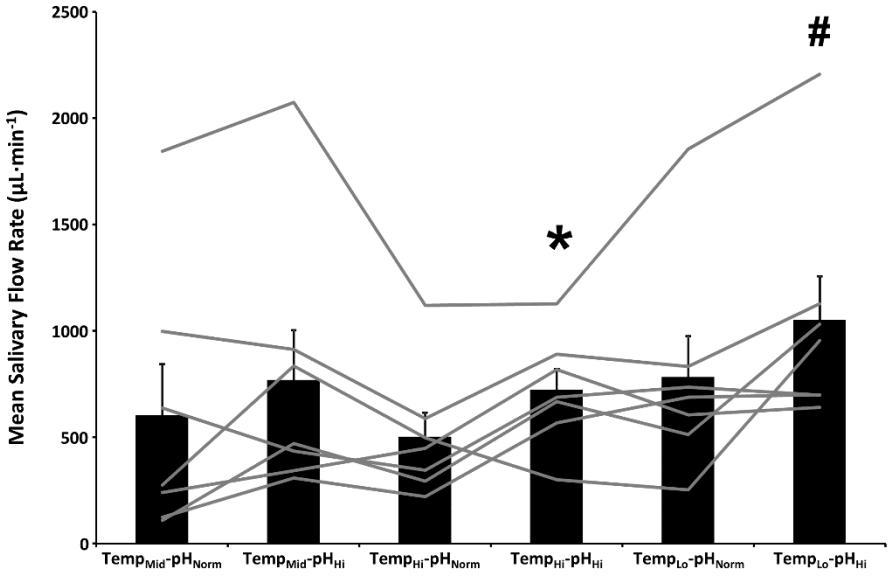

Figure 2. Salivary flow rate after acute nitrate-rich beetroot juice consumption in intermediate temperature-neutral pH ($Temp_{Mid}$-pH_{Norm}), intermediate temperature-high pH ($Temp_{Mid}$-pH_{Hi}), high temperature-neutral pH ($Temp_{Hi}$-pH_{Norm}), high temperature-high pH ($Temp_{Hi}$-pH_{Hi}), low temperature-neutral pH ($Temp_{Lo}$-pH_{Norm}) and low temperature-high pH ($Temp_{Lo}$-pH_{Hi}) conditions. The data are expressed as the mean response of all samples collected during the oral temperature and pH manipulations. The filled bars represent the group mean ± SEM responses while the solid grey lines represent the individual responses in each experimental condition. *

indicates higher than $Temp_{Hi}$-pH_{Norm} (P<0.05). # indicates higher than $Temp_{Mid}$-pH_{Norm}, $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Norm} and $Temp_{Lo}$-pH_{Norm} (P<0.05).

Salivary [nitrate]

The influence of BR consumption and oral temperature and pH manipulation on absolute salivary [NO_3^-] at baseline and 1, 2 and 3 hours post BR consumption, and mean salivary [NO_3^-] 1-3 hours post BR consumption, are presented in Figure 3. There was a main effect for time (P<0.001) with absolute salivary [NO_3^-] increasing above baseline in all experimental conditions. Absolute salivary [NO_3^-] was lower in the $Temp_{Mid}$-pH_{Hi} trial compared to the $Temp_{Hi}$-pH_{Norm}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Norm} trials 1 hour following BR consumption (P<0.05). The mean salivary [NO_3^-] 1-3 hours post BR consumption was also lower in the $Temp_{Mid}$-pH_{Hi} trial compared to the $Temp_{Hi}$-pH_{Norm}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Norm} trials (P<0.05).

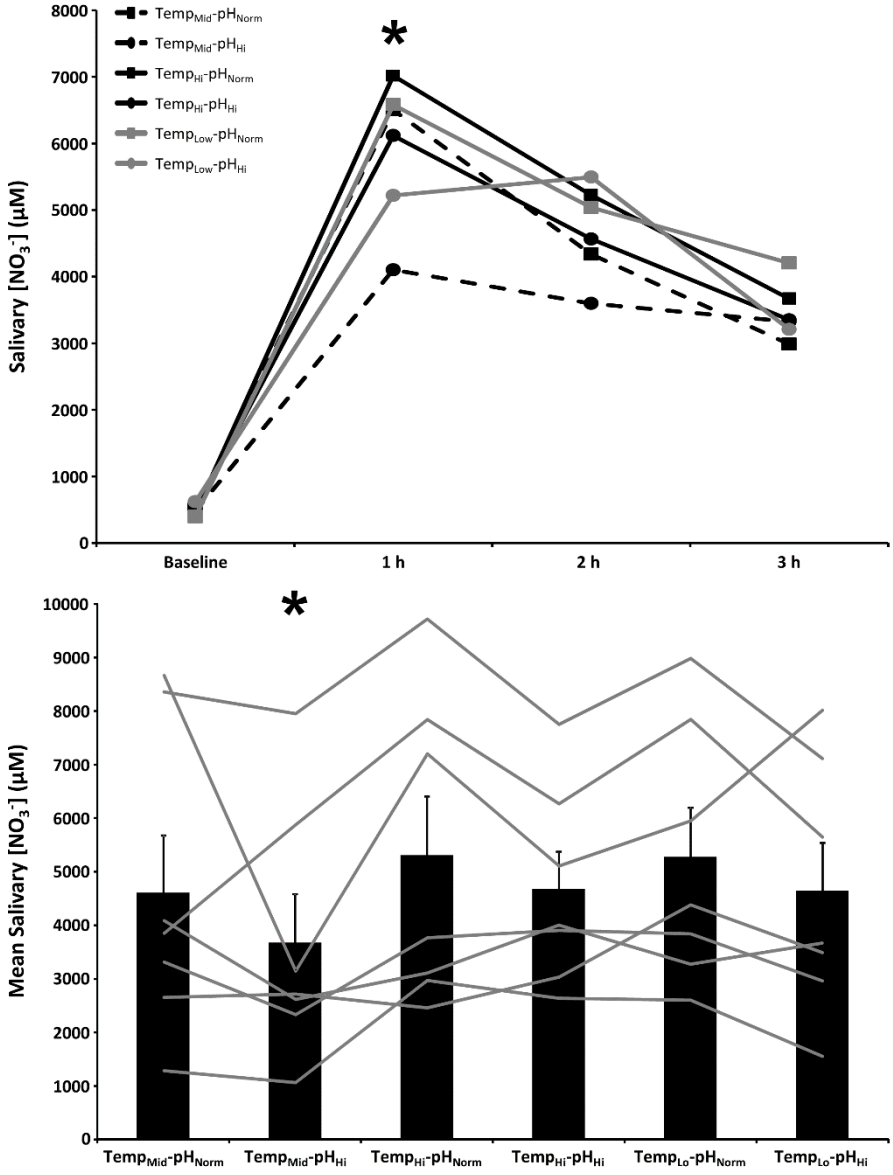

Figure 3. Salivary nitrate concentration ([NO_3^-]) across the protocol (upper panel) and the mean salivary [NO_3^-] at hours 1-3 of the protocol (lower panel) after acute NO_3^--rich beetroot juice consumption in intermediate temperature-neutral pH ($Temp_{Mid}$-pH_{Norm}), intermediate temperature-high pH ($Temp_{Mid}$-pH_{Hi}), high temperature-neutral pH ($Temp_{Hi}$-pH_{Norm}), high temperature-high pH ($Temp_{Hi}$-pH_{Hi}), low temperature-neutral pH ($Temp_{Lo}$-pH_{Norm}) and low temperature-high pH ($Temp_{Lo}$-pH_{Lo}) conditions. Salivary [NO_3^-] values across the protocol (upper panel) are expressed as group mean values with error bars omitted for clarity. The filled bars represent the group mean ± SEM responses while the solid grey lines represent the individual responses in each experimental condition for mean salivary [NO_3^-] at hours 1-3 of the protocol (lower panel). * indicates $Temp_{Mid}$-pH_{Hi} lower than $Temp_{Hi}$-pH_{Norm}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Norm} (P<0.05).

Salivary [nitrite]

The influence of BR consumption and oral temperature and pH manipulation on absolute salivary [NO_2^-] at baseline and 1, 2 and 3 hours post BR consumption,

and mean salivary [NO_2^-] 1-3 hours post BR consumption, are presented in Figure 4. There were main effects for time (P<0.001) and condition (P<0.01), as well as a condition × time interaction effect (P<0.05), with absolute salivary [NO_2^-] increasing above baseline in all experimental conditions. Absolute salivary [NO_2^-] was higher 1 and 2 hours post BR consumption in the $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi}, $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} trials compared to the $Temp_{Mid}$-pH_{Norm} trial (P<0.05), and higher than the $Temp_{Mid}$-pH_{Norm} trial in all other experimental conditions 3 hours post BR consumption (P<0.05). There was a main effect for condition on the mean salivary [NO_2^-] 1-3 hours post BR consumption (P<0.01) with mean salivary [NO_2^-] higher than the $Temp_{Mid}$-pH_{Norm} trial in all other experimental conditions (P<0.05), higher than the $Temp_{Hi}$-pH_{Norm} trial in the $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Hi} trials (P<0.05) and higher than the $Temp_{Lo}$-pH_{Norm} trial in the $Temp_{Lo}$-pH_{Hi} trial (P<0.05). When all data were pooled, salivary flow rate was positively correlated with the absolute salivary [NO_2^-] 1 hour (r = 0.32, P<0.05), 2 hours (r = 0.5, P<0.01) and 3 hours (r = 0.47, P<0.01) post BR consumption, and the mean salivary [NO_2^-] 1-3 hours post BR consumption (r = 0.48, P<0.01).

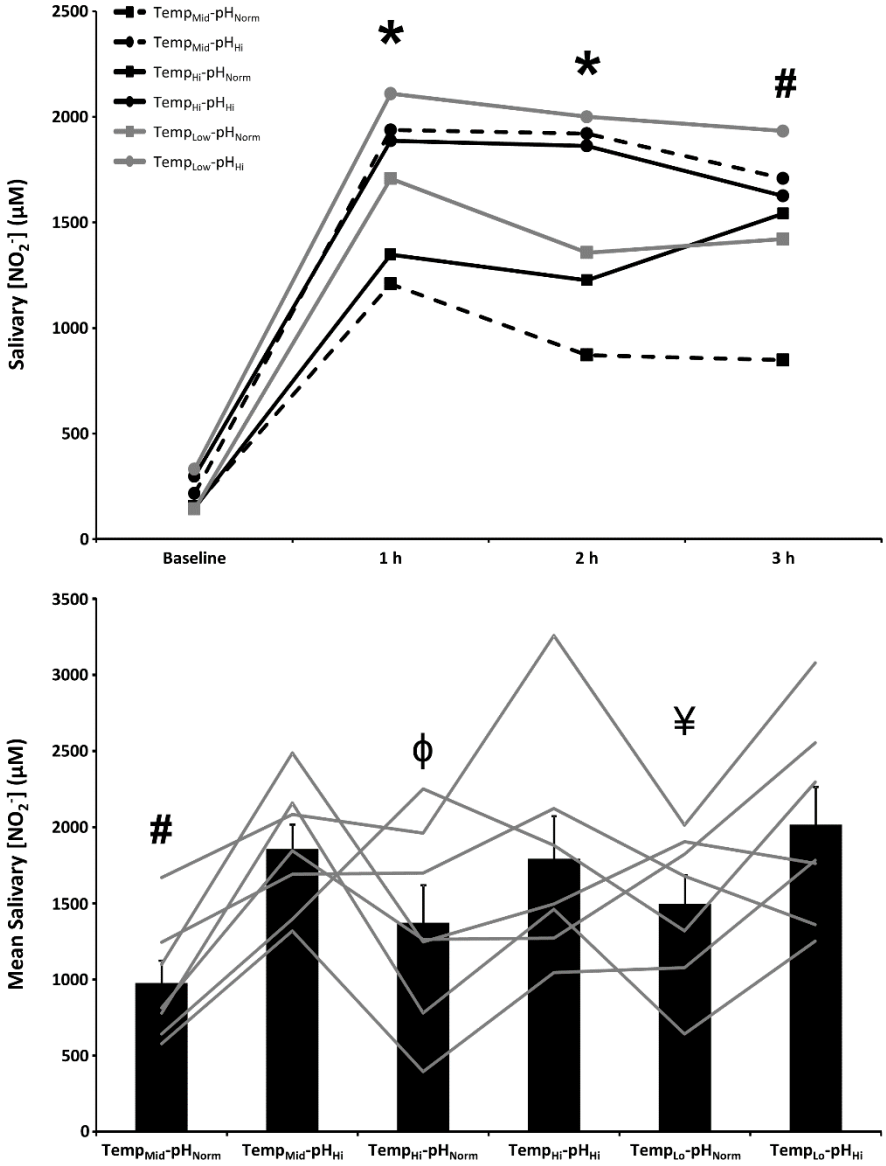

Figure 4. Salivary nitrite concentration ([NO_2^-]) across the protocol (upper panel) and the mean salivary [NO_2^-] at hours 1-3 of the protocol (lower panel) after acute nitrate-rich beetroot juice consumption in intermediate temperature-neutral pH ($Temp_{Midm}$-pH_{Norm}), intermediate temperature-high pH ($Temp_{Mid}$-pH_{Hi}), high temperature-neutral pH ($Temp_{Hi}$-pH_{Norm}), high temperature-high pH ($Temp_{Hi}$-pH_{Hi}), low temperature-neutral pH ($Temp_{Lo}$-pH_{Norm}) and low temperature-high pH ($Temp_{Lo}$-pH_{Lo}) conditions. Salivary [NO_2^-] values across the protocol (upper panel) are expressed as group mean values with error bars omitted for clarity. The filled bars represent the group mean ± SEM responses while the solid grey lines represent the individual responses in each experimental condition for mean salivary [NO_2^-] at hours 1-3 of the protocol (lower panel). * indicates $Temp_{Mid}$-pH_{Norm} lower than $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi}, $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} (P<0.05). # indicates $Temp_{Mid}$-pH_{Norm} lower than $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Norm}, $Temp_{Hi}$-pH_{Hi}, $Temp_{Lo}$-pH_{Norm} and $Temp_{Lo}$-pH_{Hi} (P<0.05). ϕ indicates lower than $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Hi} (P<0.05). ¥ indicates lower than $Temp_{Lo}$-pH_{Hi} (P<0.05).

Plasma [nitrate]

The influence of BR consumption and oral temperature and pH manipulation on absolute plasma [NO_3^-] at baseline and 1, 2 and 3 hours post BR consumption, and mean plasma [NO_3^-] 1-3 hours post BR consumption, are presented in Figure 5. There was a main effect for time (P<0.001) with absolute plasma [NO_3^-] increasing above baseline in all experimental conditions. There were no between condition differences in absolute plasma [NO_3^-] at 1, 2 and 3 hours post BR consumption or the mean plasma [NO_3^-] 1-3 hours post BR consumption (P>0.05).

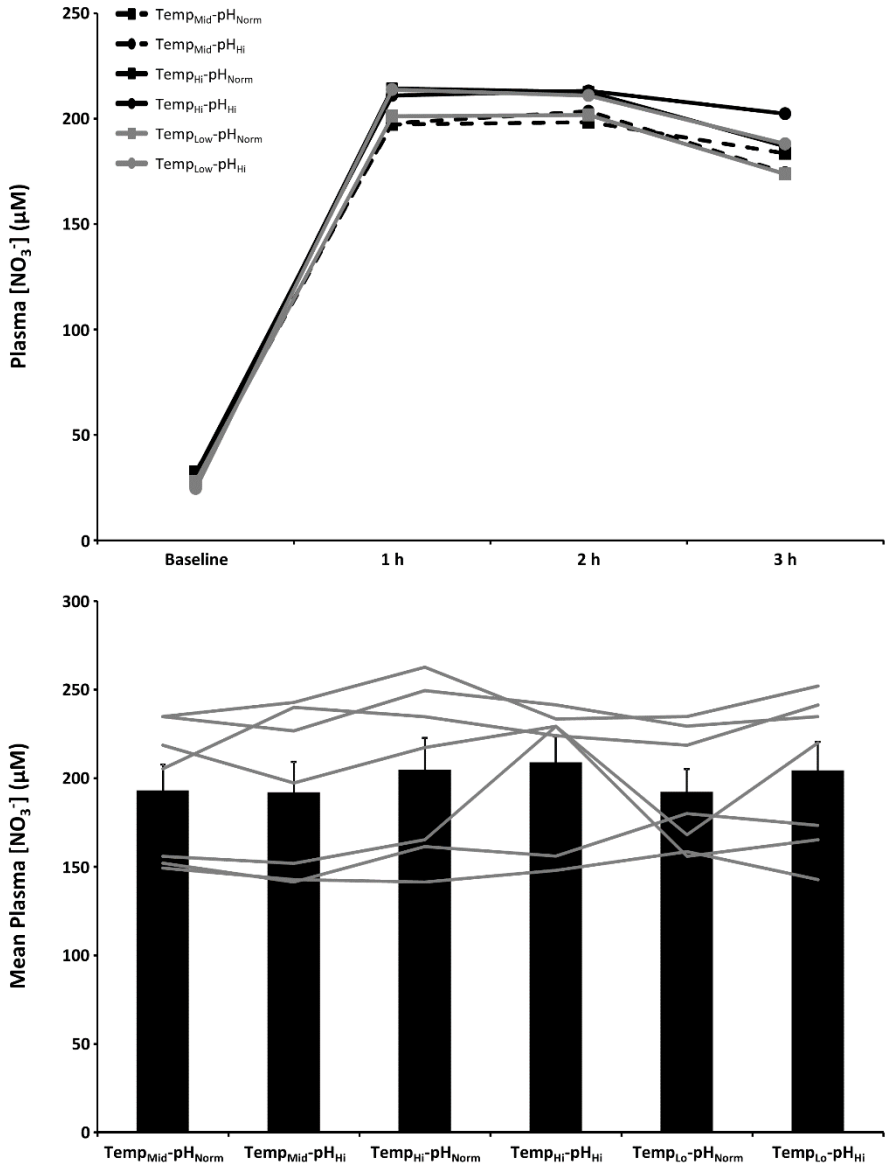

Figure 5. Plasma nitrate concentration ([NO_3^-]) across the protocol (upper panel) and the mean plasma [NO_3^-] at hours 1-3 of the protocol (lower panel) after acute NO_3^--rich beetroot juice consumption in intermediate temperature-neutral pH (Temp$_{Mid}$-pH$_{Norm}$), intermediate temperature-high pH (Temp$_{Mid}$-pH$_{Hi}$), high temperature-neutral pH (Temp$_{Hi}$-pH$_{Norm}$), high temperature-high pH (Temp$_{Hi}$-pH$_{Hi}$), low temperature-neutral pH (Temp$_{Lo}$-pH$_{Norm}$) and low temperature and pH (Temp$_{Lo}$-pH$_{Lo}$) conditions. Plasma [NO_3^-] values across the protocol (upper

panel) are expressed as group mean values with error bars omitted for clarity. The filled bars represent the group mean ± SEM responses while the solid grey lines represent the individual responses in each experimental condition for mean plasma [NO_3^-] at hours 1-3 of the protocol (lower panel).

Plasma [nitrite]

The influence of BR consumption and oral temperature and pH manipulation on absolute plasma [NO_2^-] at baseline and 1, 2 and 3 hours post BR consumption, and mean plasma [NO_2^-] 1-3 hours post BR consumption, are presented in Figure 6. There were main effects for time (P<0.001) and condition (P<0.05), with absolute plasma [NO_2^-] increasing above baseline in all experimental conditions. Absolute plasma [NO_2^-] was higher 3 hours post BR consumption in the $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi} and $Temp_{Lo}$-pH_{Hi} trials compared to the $Temp_{Mid}$-pH_{Norm} trial (P<0.05). There was a main effect for condition on the mean plasma [NO_2^-] 1-3 hours post BR consumption (P<0.01) with mean plasma [NO_2^-] higher than the $Temp_{Mid}$-pH_{Norm} in the $Temp_{Hi}$-pH_{Hi} trial (P<0.05).

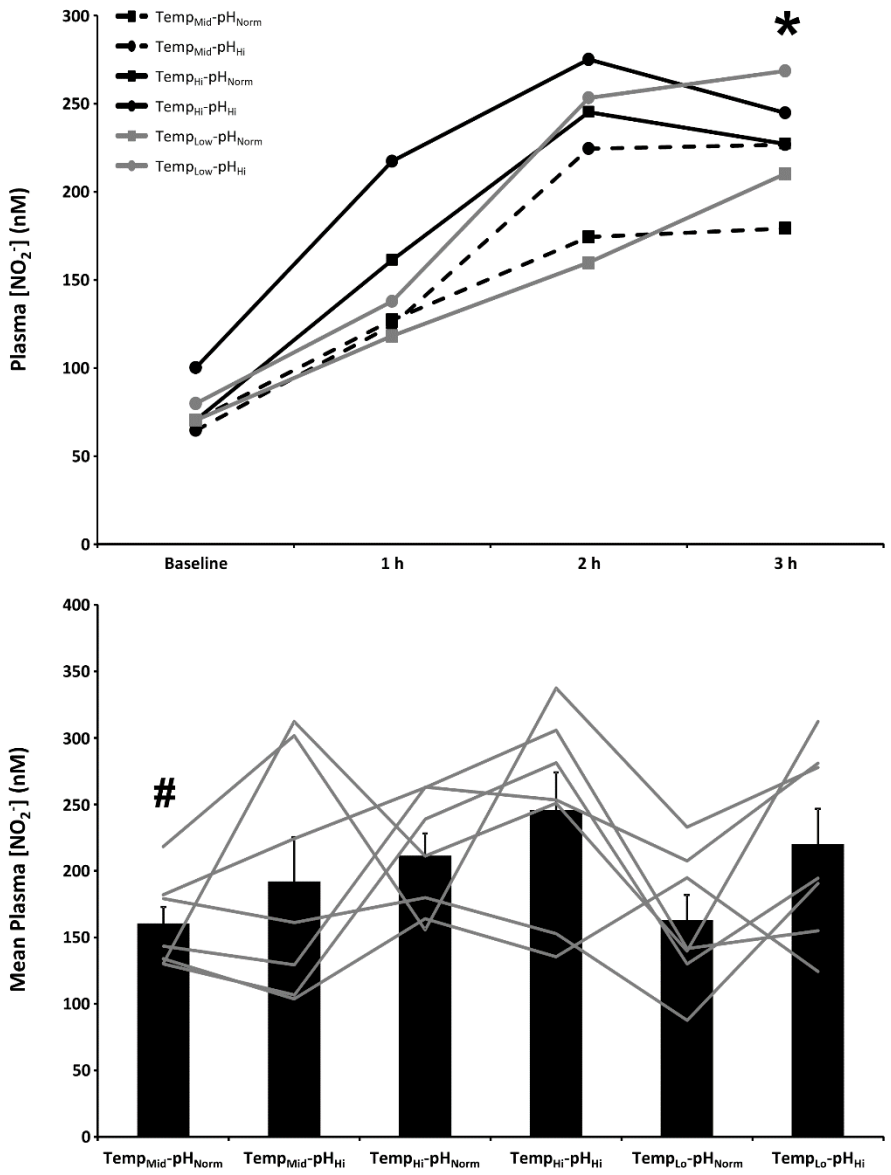

Figure 6. Plasma nitrite concentration ([NO_2^-]) across the protocol (upper panel) and the mean plasma [NO_2^-] at hours 1-3 of the protocol (lower panel) after acute nitrate-rich beetroot juice consumption in intermediate temperature-neutral pH ($Temp_{Mid}$-pH_{Norm}), intermediate temperature-high pH ($Temp_{Mid}$-pH_{Hi}), high temperature-neutral pH ($Temp_{Hi}$-pH_{Norm}), high temperature-high pH ($Temp_{Hi}$-pH_{Hi}), low temperature-neutral pH ($Temp_{Lo}$-pH_{Norm}) and low temperature and pH ($Temp_{Lo}$-pH_{Lo}) conditions. Plasma [NO_2^-] values across the protocol (upper panel) are expressed as group mean values with error bars omitted for clarity. The filled bars represent the group mean ± SEM responses while the solid grey lines represent the individual responses in each experimental condition for mean plasma [NO_2^-] at hours 1-3 of the protocol (lower panel). * indicates $Temp_{Mid}$-pH_{Norm} lower than $Temp_{Mid}$-pH_{Hi}, $Temp_{Hi}$-pH_{Hi}, and $Temp_{Lo}$-pH_{Hi} (P<0.05). # indicates lower than $Temp_{Hi}$-pH_{Hi} (P<0.05).

Discussion

This is the first study to investigate how changes in oral temperature and pH influence the ability of BR supplementation to increase salivary and plasma

[NO_2^-]. Salivary and plasma [NO_3^-] and [NO_2^-] were all increased following BR supplementation, but the main novel findings from this study were that salivary [NO_2^-] was increased further when oral temperature and pH were manipulated with the peak increase evident when oral temperature was lowered and oral pH was elevated. Plasma [NO_2^-] was higher 3 hours post BR consumption in the alkaline pH conditions compared to the $Temp_{Mid}$-pH_{Norm} trial. These findings indicate that salivary and plasma [NO_2^-] following dietary NO_3^- consumption can be influenced by oral temperature and pH manipulation which might have implications for the physiological effects induced by dietary NO_3^- supplementation.

Oral temperature and pH

The experimental manipulations impacted upon oral temperature and pH. Mean oral temperature in the high, intermediate and low temperature conditions was 36.2 ± 0.3°C, 34.9 ± 1.2°C and 33.6 ± 1.7°C, respectively, while mean salivary pH in the alkaline and neutral pH conditions was 7.8 ± 0.2 and 6.7 ± 0.2, respectively. An increased oral temperature and pH has previously been suggested to elevate NO_3^- reduction to NO_2^-, with a pH of ~8 demonstrated to be optimal (Bojić, Bojić and Pervović, 2004). Therefore, the salivary pH of 7.8 in the alkaline conditions might allow for an optimal salivary [NO_2^-] following dietary NO_3^- supplementation with BR.

Salivary flow rate

NO_3^- metabolism might be influenced by changes in salivary flow rate. An increased salivary flow rate would be expected to elevate NO_3^- secretion by the salivary glands thus providing more substrate for reduction to NO_2^-. This might increase salivary [NO_2^-]. Simultaneous, but not independent, oral temperature and pH manipulation impacted upon salivary flow rate, which was greater in the alkaline pH condition for both the high ($Temp_{Hi}$-pH_{Hi}: 723 ± 261 µL.min^{-1} vs. $Temp_{Hi}$-pH_{Norm}: 501 ± 300 µL.min^{-1}) and low ($Temp_{Lo}$-pH_{Hi}: 1051 ± 543 µL.min^{-1} vs. $Temp_{Lo}$-pH_{Norm}: 689 ± 584 µL.min^{-1}) temperature conditions and tended to

be greater in the $Temp_{Lo}$-pH_{Hi} trial compared to the $Temp_{Hi}$-pH_{Hi} trial (Δ = 329 ± 282 µL.min^{-1}). Therefore, salivary [NO_2^-] following dietary NO_3^- consumption and oral temperature and pH manipulation might vary due to the differences elicited in salivary flow rate. The measurement of salivary flow rate, in the present study, builds upon previous research that did not account for this factor when investigating oral NO_3^- reduction to NO_2^- following temperature and pH manipulations (Bojić, Bojić and Pervović, 2004).

Salivary and plasma [nitrate] and [nitrite]

Consistent with previous research, BR supplementation increased salivary [NO_3^-] and [NO_2^-] (McDonagh et al., 2015; Woessner et al., 2016) and plasma [NO_3^-] (McDonagh et al., 2015; Wylie et al., 2013) and [NO_2^-] (Bailey et al., 2009; 2010; Vanhatalo et al., 2010; 2011; Webb et al., 2008; Wylie et al., 2013). Salivary [NO_3^-] increased 26.8-, 17.6- and 11.8-fold, salivary [NO_2^-] increased 12.6-, 8.8- and 8.6-fold, plasma [NO_3^-] increased 5.1-, 5.1- and 4.6-fold and plasma [NO_2^-] increased 0.8-, 1.5- and 1.6-fold at hours 1-3 post consumption in the $Temp_{Mid}$-pH_{Norm} trial, respectively. The magnitudes of change in plasma [NO_3^-] and [NO_2^-] conform with the dose-dependent elevations reported following acute BR supplementation by Wylie et al., (2013). The delayed time to peak plasma [NO_2^-] highlights the importance of dietary NO_3^- on the enterosalivary circuit and, therefore, the metabolism of NO_3^- by commensal oral bacteria (Duncan et al., 1995; Sasaki and Matano, 1979; Spiegelhalder et al., 1976).

Following BR consumption, oral temperature and pH manipulation had no effect on salivary [NO_3^-] but salivary [NO_2^-] was increased further in the independently ($Temp_{Mid}$-pH_{Hi}: Δ = 1076 ± 423 nM; 90%; $Temp_{Hi}$-pH_{Norm}: Δ = 592 ± 653 nM; 41%) and simultaneously ($Temp_{Hi}$-pH_{Hi}: Δ = 1012 ± 741 nM; 84%) elevated oral temperature and pH conditions and in the low oral temperature conditions ($Temp_{Lo}$-pH_{Norm}: Δ = 715 ± 502 nM; 53% $Temp_{Lo}$-pH_{Hi}: Δ = 1234 ± 662 nM; 106%) compared to the $Temp_{Mid}$-pH_{Norm} trial. Plasma [NO_3^-] also remained unaltered following BR consumption and the experimental manipulations, however plasma [NO_2^-] was increased further than the $Temp_{Mid}$-pH_{Norm} trial in the independently elevated oral pH condition ($Temp_{Mid}$-pH_{Hi}: Δ =

192 ± 89 nM; 20%), the simultaneously elevated oral temperature and pH condition ($Temp_{Hi}$-pH_{Hi}: Δ = 246 ± 75 nM; 53%) and in the $Temp_{Lo}$-pH_{Hi} trial (Δ = 220 ± 71 nM; 37%). Therefore, despite consuming the same NO_3^- dose, the increases in salivary and plasma [NO_2^-] varied depending on the temperature and pH of the oral cavity. The greatest increase in salivary [NO_2^-] was observed in the $Temp_{Lo}$-pH_{Hi} trial. This is likely due to salivary flow rate, which was positively correlated with salivary [NO_2^-] (r = 0.48). Despite the larger salivary [NO_2^-] in the $Temp_{Lo}$-pH_{Hi} trial, the greatest increase in plasma [NO_2^-] occurred in the $Temp_{Hi}$-pH_{Hi} trial. It is plausible that this measurement was confounded by the mouth rinse protocol, which resulted in 46% more NO_2^--rich saliva being expectorated following the mouth rinses and sample collections in the $Temp_{Lo}$-pH_{Hi} trial compared to the $Temp_{Hi}$-pH_{Hi} trial. This would likely impair the characteristic rise in plasma [NO_2^-] demonstrated following dietary NO_3^- supplementation (Lundberg and Govoni, 2004; Webb et al., 2008). Furthermore, the oral temperature manipulation might have elevated core temperature, which is known to increase eNOS activity and NO release (Harris et al., 2003). This would be expected to increase plasma [NO_2^-] independently of dietary NO_3^- metabolism. A limitation of the present study is that core temperature was not measured. Future research should build upon these limitations when administering oral temperature and pH manipulations following dietary NO_3^- supplementation.

The results herein build upon previous research that demonstrated an increased [NO_2^-] following both NO_3^- consumption and oral pH manipulation using a pH-regulating chewing gum (van Maanen, van Geel and Kleinjans, 1996) and the incubation of an alkaline NO_3^- test solution in the mouth (Bojić, Bojić and Pervović, 2004). Collectively, the findings suggest that NO_3^- reductase activity in the human oral cavity is sensitive to pH, salivary flow rate and, to a lesser extent, temperature. It is important to note that seasonal variations in oral cavity NO_3^- reductase activity has the potential to confound these findings. This might have influenced the results reported by Bojić, Bojić and Pervović (2004) due to their experimental manipulations being performed outdoors in a summer and winter climate. In the present study, the experimental manipulations were performed in a temperature-controlled laboratory during the winter and spring months, therefore, reducing this factor. Oral temperature and pH manipulation

following dietary NO_3^- supplementation might have implications for future BR supplementation recommendations, might contribute to understanding of the underlying physiology for responders and non-responders to dietary NO_3^- supplementation, and might enhance the cardiovascular health and physical performance benefits demonstrated at a given dietary NO_3^- dose. Therefore, further research is warranted.

Conclusion

The increase in salivary and plasma [NO_2^-] following acute BR supplementation was influenced by oral temperature and pH manipulation. Peak salivary [NO_2^-] was evident in the low oral temperature and alkaline oral pH condition, with the prior suggesting a key role for salivary flow rate in human NO_3^- metabolism. Plasma [NO_2^-] was also higher in the alkaline oral pH conditions. These results extend our understanding of dietary NO_3^- metabolism following BR supplementation. Further research is required to build upon the findings of this study. Specifically, investigating the impact of oral temperature and pH manipulation on plasma [NO_2^-] and the physiological effects induced by dietary NO_3^- supplementation might be fruitful areas of study.

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Chapter 4: Effect of dietary nitrate supplementation on oxygen uptake kinetics and exercise tolerance: influence of muscle oxygenation

Abstract

We tested the hypothesis that dietary nitrate (NO_3^-) supplementation would improve muscle oxygenation, oxygen uptake ($\dot{V}O_2$) kinetics and exercise tolerance (T_{lim}) in normoxia and that these improvements would be augmented in hypoxia and attenuated in hyperoxia. In a randomized, cross-over study, ten healthy males completed work-to-work step cycle tests to exhaustion following acute consumption of 3 x 70 ml NO_3^--rich beetroot juice (BR; 19.5 mmol NO_3^-) or NO_3^--depleted beetroot juice placebo (PL; 0.12 mmol NO_3^-). These tests were completed in normobaric hypoxia (FiO_2: 15%), normoxia (FiO_2: 21%) and hyperoxia (FiO_2: 40%). Pulmonary $\dot{V}O_2$ and quadriceps tissue oxygenation index (TOI), derived from multi-channel near-infrared spectroscopy, were measured during all trials. Plasma [nitrite] was higher in all BR compared to all PL trials (P<0.05). Quadriceps TOI was higher in normoxia compared to hypoxia (P<0.05) and higher in the hyperoxia compared to hypoxia and normoxia (P<0.05). T_{lim} was improved after BR compared to PL consumption (250 ± 44 vs. 231 ± 41 sec), with the magnitude of improvement being negatively correlated with quadriceps TOI at exhaustion (r = -0.78), in hypoxia (P<0.05). T_{lim} tended to be improved with BR in normoxia (BR: 364 ± 98 vs. PL: 344 ± 78 s; P=0.087), but was not improved in hyperoxia (BR: 492 ± 212 vs. PL: 472 ± 196 s; P>0.05). BR consumption increased peak $\dot{V}O_2$ in hypoxia (P<0.05), but not normoxia or hyperoxia (P>0.05). Therefore, NO_3^- supplementation is more likely to improve T_{lim} and peak $\dot{V}O_2$ as skeletal muscles become increasingly hypoxic.

Introduction

Nitric oxide (NO) is a gaseous signalling molecule with physiological functions including the regulation of blood flow, muscle contractility and mitochondrial respiration (Stamler and Meissner, 2001). NO is endogenously

synthesised from the oxidation of L-arginine by the NO synthase (NOS) enzymes (the NOS pathway; Stamler and Meissner, 2001) or from the stepwise reduction of inorganic nitrate (NO_3^-) to nitrite (NO_2^-) and NO (the NO_3^--NO_2^--NO pathway; Lundberg et al., 2004; Lundberg and Weitzberg, 2009). NO_3^- is consumed as part of the human diet, with ~80% of its intake originating from vegetable sources including leafy greens and beetroot (Gilchrist et al., 2010; Hord et al., 2009). ~25% of the consumed NO_3^- is concentrated and secreted by the salivary glands (Lundberg and Weitzberg, 2009), which is then reduced to NO_2^- by commensal anaerobic bacteria located on the dorsal surface of the tongue (Duncan et al., 1995; Sasaki and Matano, 1979; Spiegelhalder et al., 1976). When swallowed, NO_2^- reacts with gastric acid in the stomach to form a mixture of nitrogen oxides, including NO (Benjamin et al., 1994; Duncan et al., 1995). Alternatively, NO_2^- can be absorbed into circulation where its reduction to NO is catalysed by several enzymes (i.e. xanthine oxidase; Zhang et al., 1998) or reduced non-enzymatically in the presence of protons (Carlsson et al., 2001; Peri et al., 2005). The NO_3^--NO_2^--NO pathway has been proposed to represent a means of NO synthesis when the activity of the NOS pathway is impaired, such as conditions of low oxygen (O_2) availability (Bryan et al., 2006) which might be evident in contracting skeletal muscle (Bailey et al., 2010; Richardson et al., 1999), and has become a major focus of NO physiology research in recent years.

An abundance of evidence exists to support the use of dietary NO_3^- supplementation, including NO_3^--rich beetroot juice (BR), to increase plasma [NO_2^-] (Bailey et al., 2009; 2010; Vanhatalo et al., 2010; 2011; Webb et al., 2008; Wylie et al., 2013). This has been associated with lower pulmonary O_2 uptake ($\dot{V}O_2$; Bailey et al., 2009; Vanhatalo et al., 2010; Wylie et al., 2013) and greater indexes of *vastus* lateralis muscle oxygenation during moderate-intensity exercise (Bailey et al., 2009) and an enhanced tolerance to severe-intensity exercise (Bailey et al., 2009; Wylie et al., 2013) in healthy young adults performing in conditions of normal O_2 availability (~21% O_2; normoxia). These changes have been suggested to be caused by NO-mediated improvements in muscle contractile function (Bailey et al., 2010), mitochondrial efficiency (Larsen et al., 2011) and/or muscle blood flow (Ferguson et al., 2013) and might be more pronounced in conditions of reduced O_2 availability (<21% O_2; hypoxia)

whereby local NO-mediated vasodilation increases muscle blood flow to restore sufficient O_2 supply (Casey et al., 2010). Indeed, BR supplementation has been shown to lower steady-state $\dot{V}O_2$ and speed $\dot{V}O_2$ kinetics during moderate-intensity exercise and enhance severe-intensity exercise tolerance in hypoxia (Kelly et al., 2014). To date, no study has investigated the physiological and tolerance changes induced by dietary NO_3^- supplementation during exercise completed in conditions of increased O_2 availability (>21% O_2; hyperoxia). However, a reduced time to fatigue was observed in mouse muscle fibres following incubation with sodium NO_2^- at a supra-physiological partial pressure of O_2 (PO_2; Nogueira et al., 2013). Considering that the increased fraction of inspired oxygen (FiO_2) in hyperoxic air is associated with a greater intracellular PO_2 (Collins et al., 2015), exercise performed in hyperoxia might be associated with a reduced stimulus for NO_2^- reduction to NO, thus attenuating the physiological effects induced by dietary NO_3^- supplementation. However, this remains to be identified.

The dietary NO_3^- supplementation studies by Bailey et al., (2009) and Kelly et al., (2014) used single-channel near infrared spectroscopy (NIRS) systems to measure the muscle oxygenation status of the *vastus lateralis*. The use of a multi-channel system, that comprises multiple source/detector pairings, would enable the investigation of regional differences within a single muscle (i.e. proximal vs. distal) and/or wider differences within a muscle group (i.e. *vastus lateralis* vs. *rectus femoris* within the quadriceps muscle group). To date, no study has used a multi-channel NIRS system to investigate the changes in quadriceps muscle group oxygenation induced by dietary NO_3^- supplementation and exercise in hypoxia, normoxia or hyperoxia.

The purpose of this study was to investigate how dietary NO_3^- supplementation influenced muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance in hypoxia, normoxia and hyperoxia. Specifically, the alterations in plasma [NO_2^-], arterial SpO_2, multi-channel NIRS-derived quadriceps TOI, pulmonary $\dot{V}O_2$ and time to exhaustion (T_{lim}) were investigated following acute BR supplementation and work-to-work step cycling exercise in hypoxia, normoxia and hyperoxia. It was hypothesised that muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance would be augmented in normoxia, increased further in hypoxia and attenuated in hyperoxia following BR supplementation.

Methods

Ten healthy males (mean ± SD; age = 23 ± 3 years, height = 1.80 ± 0.07 m, body mass = 78.2 ± 8.9 kg) volunteered to participate in this study. None of the participants were tobacco smokers or users of any medication. All participants were fully familiarised with the laboratory testing protocol prior to data collection. The procedures received ethical approval from the Institutional Research Ethics Committee. Written informed consent was obtained prior to data collection and following detailed explanations of the experimental procedures, associated risks and potential benefits of participation. For each visit, participants were instructed to arrive at the laboratory in a rested and fully hydrated state, 3 hours post-prandial, having not consumed caffeine in the previous 12 hours or alcohol in the previous 24 hours. Strenuous exercise was also avoided in the 24 hours preceding each visit. Each participant performed their experimental visits at the same time of day (±2 hours) to minimise the influence of diurnal biological variation on physiological responses and exercise performance. The use of antibacterial mouthwash was prohibited to preserve the NO_3^--reducing commensal oral bacteria (Woessner et al., 2016). Participants recorded their food intake in the 24 hours preceding their first experimental visit, avoiding foods high in dietary NO_3^- and glucosinolate/thiocyante, and replicated this consumption in the 24 hours preceding each subsequent visit.

Experimental design

Participants reported to the laboratory on nine occasions over a 3 to 5 week period. Following an initial ramp incremental exercise test and familiarisation trials, participants completed work-to-work step cycle exercise tests for the determination of plasma [NO_2^-] and $\dot{V}O_2$ kinetics, quadriceps oxygenation and exercise tolerance. The experimental trials were completed after acute consumption of either NO_3^--rich beetroot juice concentrate (BR; Beet it, James White Drinks, Ipswich, UK) or NO_3^--depleted beetroot juice concentrate as a placebo (PL; Beet it, James White Drinks, Ipswich, UK) in normobaric hypoxia

(BR-Hypo, PL-Hypo), normoxia (BR-Norm, PL-Norm) and hyperoxia (BR-Hyper, PL-Hyper). These conditions were administered double blind in a randomised crossover experimental design.

Procedures

During the first visit to the laboratory, a ramp incremental exercise test was performed for the determination of $\dot{V}O_{2\ peak}$ and gas exchange threshold (GET). All exercise tests were performed on an electrically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Following a baseline cycle at 20 W for 3 min, the work rate was increased at a linear rate of 40 $W.min^{-1}$ until the limit of tolerance. Participants cycled at a self-selected cadence (between 70-90 rpm) and this pedal rate along with saddle and handle bar configuration was recorded and reproduced in subsequent tests. Breath-by-breath pulmonary gas exchange data were collected continuously during the incremental tests and subsequently averaged over consecutive 10 sec periods using a Medgraphics Cardiorespiratory Diagnostics (Express Series, Gloucester, UK). $\dot{V}O_{2\ peak}$ was determined as the highest 30 sec average value achieved before the participant's volitional exhaustion. The GET was determined from a cluster of measurements including: 1) the first disproportionate increase in carbon dioxide (CO_2) production ($\dot{V}CO_2$) from visual inspection of individual plots of $\dot{V}CO_2$ vs. $\dot{V}O_2$; 2) an increase in expired ventilation ($\dot{V}_E$) /$\dot{V}O_2$ with no increase in $\dot{V}_E$ /$\dot{V}CO_2$; and 3) an increase in end-tidal O_2 tension with no fall in end-tidal CO_2 tension. The data collected during the incremental test was used to calculate the work rates which were employed during the subsequent work-to-work step tests. Specifically, the work rates that would require 95% of the $\dot{V}O_2$ at GET (moderate-intensity exercise) and 75% of the difference between the $\dot{V}O_2$ at the GET and $\dot{V}O_{2peak}$ (75% Δ; severe-intensity exercise) were calculated, with consideration for the mean response time of $\dot{V}O_2$ during incremental ramp exercise (i.e. two-thirds of the ramp rate was subtracted from the power output at the GET and $\dot{V}O_{2\ peak}$; Whipp et al., 1981).

Participants completed a work-to-work step exercise test in each of the remaining eight visits. Visits two and three were used to familiarise participants

with the exercise test while breathing normoxic and hypoxic inspirates, respectively. The experimental conditions were then completed on visits four to nine. Upon arrival at the laboratory, a Insyte-W cannula (Becton-Dickinson, Madrid, Spain) was inserted into a forearm vein, by a phlebotomy-trained individual, to enable the collection of frequent blood samples during and immediately after the exercise test. The participant was then transferred to the cycle ergometer where the mouthpiece, fingertip pulse oximeter and three sets of NIRS probes were fitted (see *Measurements* section below). Subsequently, participants underwent a 5 min seated rest while breathing the test specific inspirate (see *Inspirate Generation* section below) immediately followed by the work-to-work exercise test. The test began with 2 min of low-intensity 'baseline' cycling at 20 W before an abrupt transition to a moderate-intensity constant work rate equivalent to 95% GET (U → M). Following 4 min of moderate-intensity cycling, the work rate was abruptly increased to a severe-intensity constant work rate equivalent to 75% Δ (M → S). The severe-intensity exercise bout was continued until the limit of tolerance, which was recorded when the pedal rate fell 10 rpm below the previously established self-selected cadence.

Participants were required to consume 3 x 70 ml beetroot juice concentrate 2.5 hours prior to arrival at the laboratory for all experimental tests. BR (which provided ~19.5 mmol NO_3^-) was consumed for the BR-Norm, BR-Hypo and BR-Hyper trials while PL (which provided ~0.1 mmol NO_3^-) was consumed for the PL-Norm, PL-Hypo and PL-Hyper trials (Wylie et al., 2013). Each experimental test was separated by a washout period of at least 48 hours (Lansley et al., 2011).

Inspirate generation

The test inspirates administered in this study were generated using an Altitude Air Generator 12 (Colorado Altitude Training, Louisville, USA). The generator comprised two outlets, one which expelled O_2-enriched air and one which expelled O_2-depleted air. Air from these outlets were delivered, via an extension conduit, to a 1000 L Douglas Bag (Cranlea & Co., Birmingham, UK), which acted as a reservoir and mixing chamber. To generate the hypoxic and hyperoxic inspirates, the O_2-enriched and O_2-depleted air was mixed until the

required O_2 percentage was achieved. To generate the normoxic inspirate, the O_2-enriched and O_2-depleted air was delivered to the Dougas Bag in equal parts. The O_2 and CO_2 concentration of the inspirate was recorded prior to each test using a 5200 High Accuracy Paramagnetic O_2 and CO_2 Analyser (Servomex, Crowborough, UK). This analyser was calibrated prior to each test with a 16.0% O_2, 8.0% CO_2 and 76.0% nitrogen gas mix (BOC Special Gases, Guildford, UK). The mean O_2 % in the PL-Norm, BR-Norm, PL-Hypo, BR-Hypo, PL-Hyper and BR-Hyper trials was 21.0 ± 0.2, 21.0 ± 0.1, 14.9 ± 0.1, 14.9 ± 0.1, 40.1 ± 0.1 and 40.0 ± 0.1, respectively. The Douglas bag comprised a separate outlet tube that connected to a two-way breathing valve system (Cranlea, Birmingham, UK). The two-way valve was connected to the mouthpiece which provided a constant, unidirectional flow rate and ensured that no re-breathing of expired air occurred.

Measurements

Venous blood samples were collected at the end of unloaded and moderate-intensity cycling, 120 sec following the onset of severe-intensity cycling and at the point of exhaustion during severe-intensity cycling. Samples (~5 ml) were drawn into lithium-heparin tubes (Becton-Dickinson, New Jersey, USA) and immediately centrifuged at 4000 rpm and 4°C for 10 min, within 2 min of collection. The plasma was subsequently extracted and immediately stored at -80°C for later analyses of [NO_2^-] via ozone-based chemiluminescence, as used previously (Wylie et al., 2013).

Arterial O_2 saturation (SpO_2) was measured continuously at 0.5 Hz using a Rad-87 pulse oximeter (Masimo, Irvine, USA) attached to the right index finger with the data file exported for later analysis. Quadriceps tissue oxygenation index (TOI) was measured continuously at three sites using two spatially-resolved NIRS systems with data sampled at 1 Hz. The TOI of the *rectus femoris* was assessed using a NIRO 200 tissue oxygenation spectrometer (Hamamatsu Photonics KK, Hamamatsu City, Japan) while the TOI of the *vastus lateralis* was assessed using a NIRO 200NX tissue oxygenation spectrometer (Hamamatsu Photonics KK, Hamamatsu City, Japan). Both systems comprised an emission probe that irradiated laser beams

and a detection probe. Three different wavelength laser diodes provided the light source (775, 810 and 850 nm in the NIRO 200 and 735, 810 and 850 in the NIRO 200NX) and the light returning from the tissue was detected by a photomultiplier tube in the spectrometer. The optodes were placed in a holder, which was secured to the skin with adhesive. The *rectus femoris* probe was placed at 50% of the distance between the patella and the greater trochanter. The proximal *vastus lateralis* probe was attached at 70%, with the distal *vastus lateralis* probe attached at 30%, of the distance between the patella and the greater trochanter. To secure the holder and wires in place, an elastic bandage was wrapped around the subject's leg. The wrap helped to minimize the possibility that extraneous light could influence the signal and also ensured that the optodes did not move during exercise. Indelible pen marks were made around the holder to enable precise reproduction of the placement in subsequent tests. The inter-optode distance (3 cm) and optical pathlength factor (18.6 cm) were consistent between measurement sites.

During all exercise tests, pulmonary gas exchange and ventilation were measured continuously using a Medgraphics CPX Express stress testing system (MGC Diagnostics, Saint Paul, USA). Participants wore a nose clip and breathed through a low-dead-space, low-resistance mouthpiece and preVent pneumotach flowmeter assembly (MGC Diagnostics, Saint Paul, USA). The inspired and expired gas volume and gas concentration signals were continuously sampled, the latter using galvanic (O_2) and non-dispersive infrared (CO_2) analysers (MGC Diagnostics, Saint Paul, USA) via a capillary line connected to the mouthpiece. The gas analysers 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, USA).

Data analysis

The breath-by-breath $\dot{V}O_2$ data from each trial were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than four standard deviations from the local mean were removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values and time-aligned to the start of exercise. A

single-exponential model without time delay, with the fitting window commencing at *t* = 0 sec (equivalent to the mean response time, MRT) was used to characterise the kinetics of the overall $\dot{V}O_2$ response during the U→M and M→S step work rate increments as described in the following equation:

$$\dot{V}O_2(t) = \dot{V}O_{2\ baseline} + A(1-e^{-(t/MRT)}) \quad \text{(Eqn. 1)}$$

where $\dot{V}O_2$ (*t*) represents the absolute $\dot{V}O_2$ at a given time; $\dot{V}O_{2\ baseline}$ represents the mean $\dot{V}O_2$ measured over the final 60 sec of baseline; and A and MRT represent the amplitude and MRT, respectively, describing the overall increase in $\dot{V}O_2$ above baseline. An iterative process was used to minimise the sum of the squared errors between the fitted function and the observed values. We quantified the $\dot{V}O_2$ MRT with the fitting window constrained to the end of the U→M work rate increment and to 180 sec of the M→S work rate increment. The absolute $\dot{V}O_2$ at the end (mean over the final 60 sec) of the U→M and M→S work rate increments, and at 180 sec (± 15 sec) of M→S were also calculated, as was the change (Δ) in $\dot{V}O_2$ between baseline and end-exercise in the U→M and M→S work rate increments and between baseline and 180 sec of the M→S work rate increment.

The TOI responses at the *rectus femoris* and proximal and distal *vastus lateralis* were averaged prior to analysis. The absolute TOI at the end (mean over the final 60 sec) of the unloaded baseline, the U→M and M→S work rate increments, and at 180 sec (± 15 sec) during the M→S work rate increment were subsequently calculated. The SpO_2 data from the start of unloaded cycling up to 120 sec of severe-intensity cycling exercise were averaged for each experimental condition to provide an overall SpO_2 profile for each trial.

Statistical analysis

A two-way, supplement (PL and BR) × inspirate (hypoxia, normoxia and hyperoxia), repeated-measures ANOVA was employed to assess differences in plasma [NO_2^-], $\dot{V}O_2$ kinetics, SpO_2, TOI and exercise tolerance across the

experimental conditions. Significant effects were further explored using post-hoc Fisher's LSD *t*-tests. Relationships between the outcome variables were assessed using Pearson's correlation coefficient (*r*). Statistical analyses were performed using SPSS version 23.0 (IBM, Chicago, USA) with significance accepted when $P<0.05$. Data are presented as mean ± SD, unless stated otherwise.

Results

The BR and PL supplements and hypoxic and hyperoxic inspirates administered in this study were well tolerated with no side-effects reported. All participants self-reported that their dietary and exercise habits were consistent across the duration of the study. Participants attained a peak work rate of 379 ± 40 W and $\dot{V}O_2$ of 3.82 ± 0.50 $L \cdot min^{-1}$ (49 ± 5 $ml \cdot kg^{-1} \cdot min^{-1}$) during the ramp incremental test. The work rates applied during the moderate-intensity and severe-intensity step cycle tests were 106 ± 14 and 292 ± 34 W, respectively.

Plasma [nitrite]

Plasma [NO_2^-] data are presented in Figure 1. Plasma [NO_2^-] was higher at the end of baseline cycling, moderate-intensity cycling, 120 sec of severe-intensity cycling and at exhaustion in the BR-Hypo (410 ± 220, 385 ± 212, 334 ± 216 and 231 ± 130 nM, respectively), BR-Norm (398 ± 191, 368 ± 130, 390 ± 147 and 246 ± 77 nM, respectively) and BR-Hyper (359 ± 104, 343 ± 88, 369 ± 113 and 247 ± 74 nM, respectively) trials compared to the PL-Hypo (107 ± 92, 97 ± 87, 110 ± 82 and 99 ± 80 nM, respectively), PL-Norm (89 ± 44, 91 ± 42, 85 ± 27 and 80 ± 32 nM, respectively) and PL-Hyper (89 ± 40, 82 ± 37, 61 ± 24 and 59 ± 19 nM, respectively) trials ($P<0.01$); however, there were no differences between the BR-Hypo, BR-Norm and BR-Hyper trials or between the PL-Hypo, PL-Norm and PL-Hyper trials at specific time points ($P>0.05$). In the BR, but not PL trials, plasma [NO_2^-] was lower at exhaustion compared to the end of baseline cycling, moderate-intensity cycling and 120 sec of severe-intensity cycling ($P<0.05$).

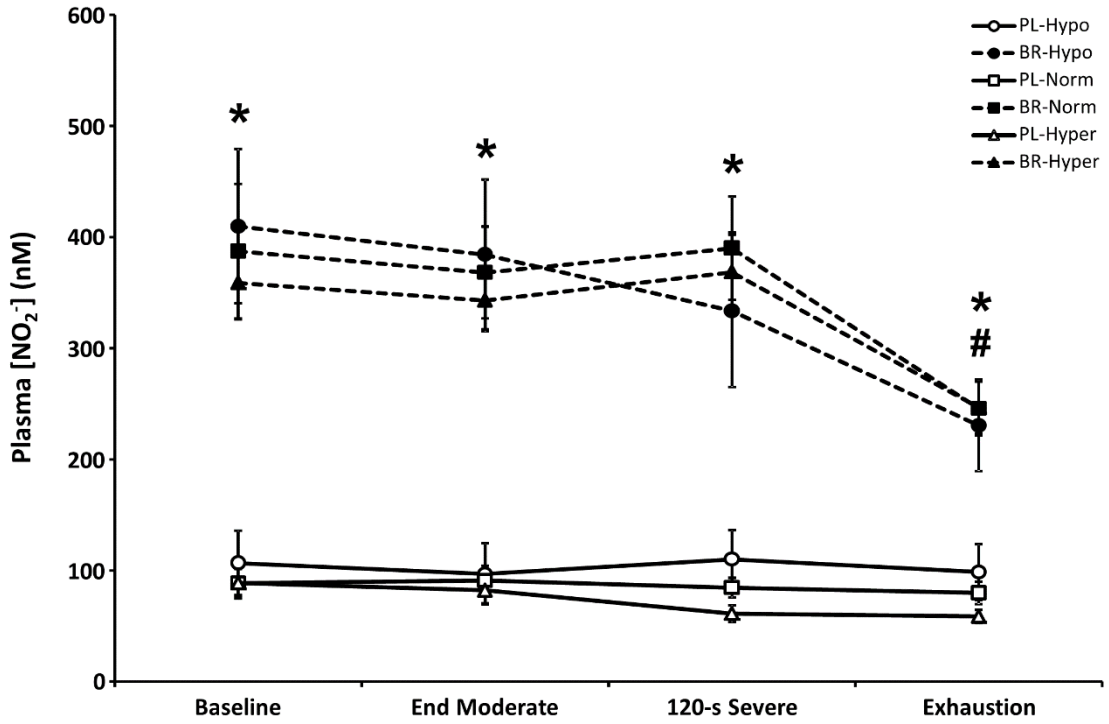

Figure 1. Plasma nitrite concentration ([NO_2^-]) following nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper), and following nitrate-rich beetroot juice (BR) in hypoxia (BR-Hypo), normoxia (BR-Norm) and hyperoxia (BR-Hyper). Plasma was sampled at the end of unloaded baseline cycling at 20 W, moderate-intensity cycling, 120 sec of severe-intensity cycling and at the point of exhaustion during severe-intensity cycling. Data are presented as group mean ± SEM. * indicates BR-Hypo, BR-Norm, and BR-Hyper trials are higher than PL-Hypo and PL-Norm and PL-Hyper (P<0.05). # indicates different from baseline, end moderate and 120 sec severe in the BR-Hypo, BR-Norm, and BR-Hyper trials (P<0.05).

Arterial oxygen saturation

The mean SpO_2 data are presented in Figure 2. Mean SpO_2 was lower in the hypoxic trials compared to the normoxic and hyperoxic trials (P<0.01), but not different between the normoxic and hyperoxic trials (P>0.05).

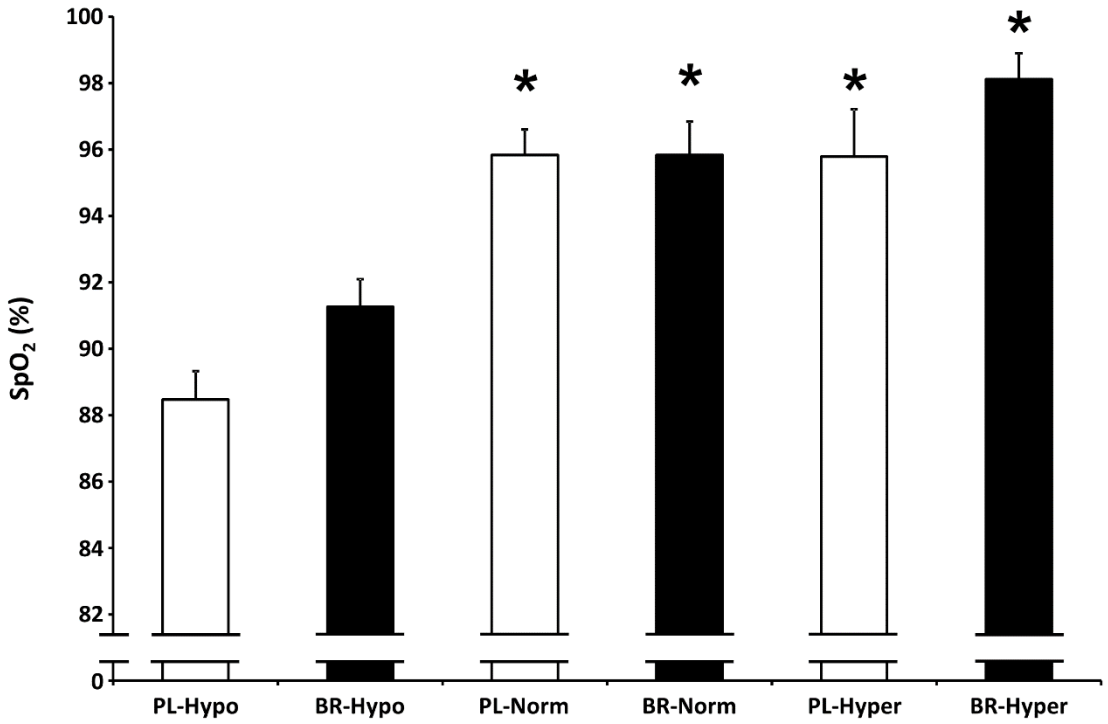

Figure 2. Arterial oxygen saturation (SpO_2) following nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper), and following nitrate-rich beetroot juice (BR) in hypoxia (BR-Hypo), normoxia (BR-Norm) and hyperoxia (BR-Hyper). The open bars represent the group mean ± SEM SpO_2 from the PL trials while the filled bars represent the group mean ± SEM SpO_2 from the BR trials. Data represent the mean SpO_2 up 120 sec of severe-intensity cycling exercise. * indicates different from PL-Hypo and BR-Hypo (P<0.05).

Tissue oxygenation index

Quadriceps TOI data are presented in Figure 3. Quadriceps TOI was higher during moderate-intensity and severe-intensity cycling exercise in the PL-Norm trial compared to the PL-Hypo trial (P<0.05) and in the PL-Hyper trial compared to the PL-Hypo and PL-Norm trials (P<0.05; Figure 3). There were no differences in quadriceps TOI between the PL-Hypo and BR-Hypo trials or between the PL-Hyper and BR-Hyper trials (P>0.05), but quadriceps TOI was higher during baseline (71 ± 3 vs. 69 ± 3 %) and at moderate-intensity (67 ± 5 vs. 65 ± 4 %) cycling exercise in the BR-Norm trial compared to the PL-Norm trial (P<0.05; Figure 4).

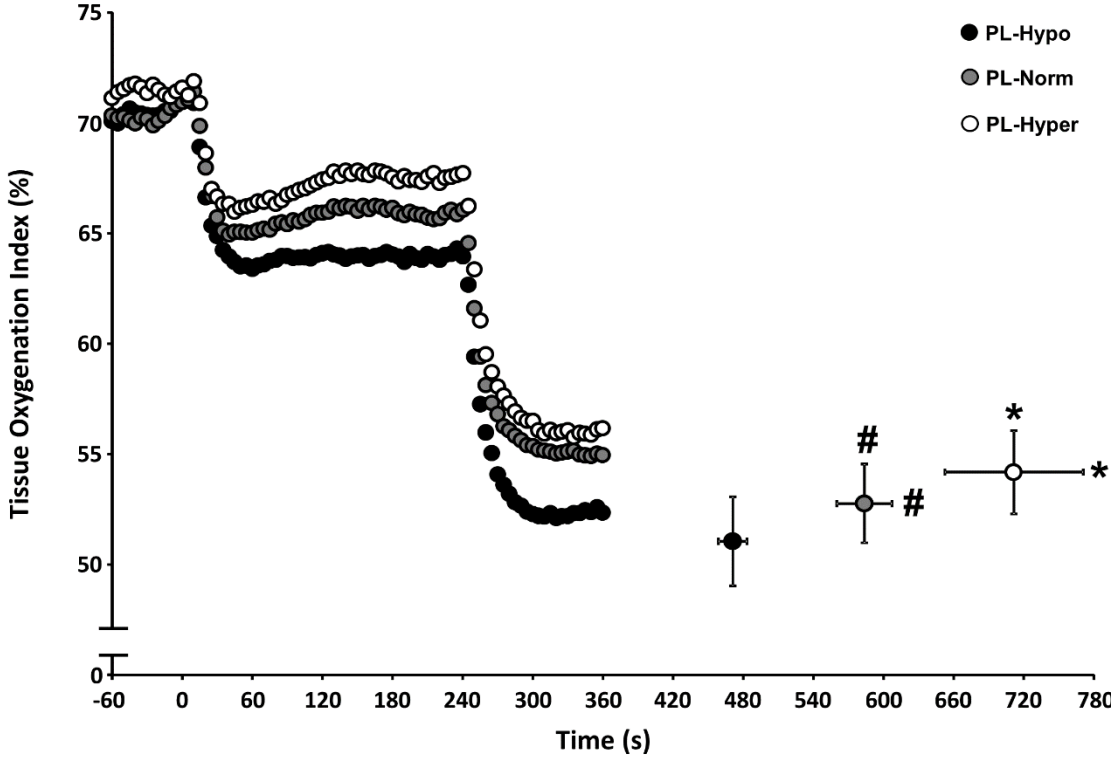

Figure 3. Quadriceps tissue oxygenation index (TOI) responses following nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper). TOI data during baseline cycling at 20 W (-60-0 sec), moderate-intensity cycling (0-240 sec) and severe-intensity cycling (240-360 sec) are group mean responses displayed as 5 sec averages. TOI data at exhaustion during severe-intensity cycling exercise represent the mean TOI over the final 60 sec of exercise and are presented as group mean ± SEM. # indicates different from PL-Hypo and BR-Hypo (P<0.05). * indicates different from PL-Norm, BR-Norm, PL-Hypo and BR-Hypo (P<0.05).

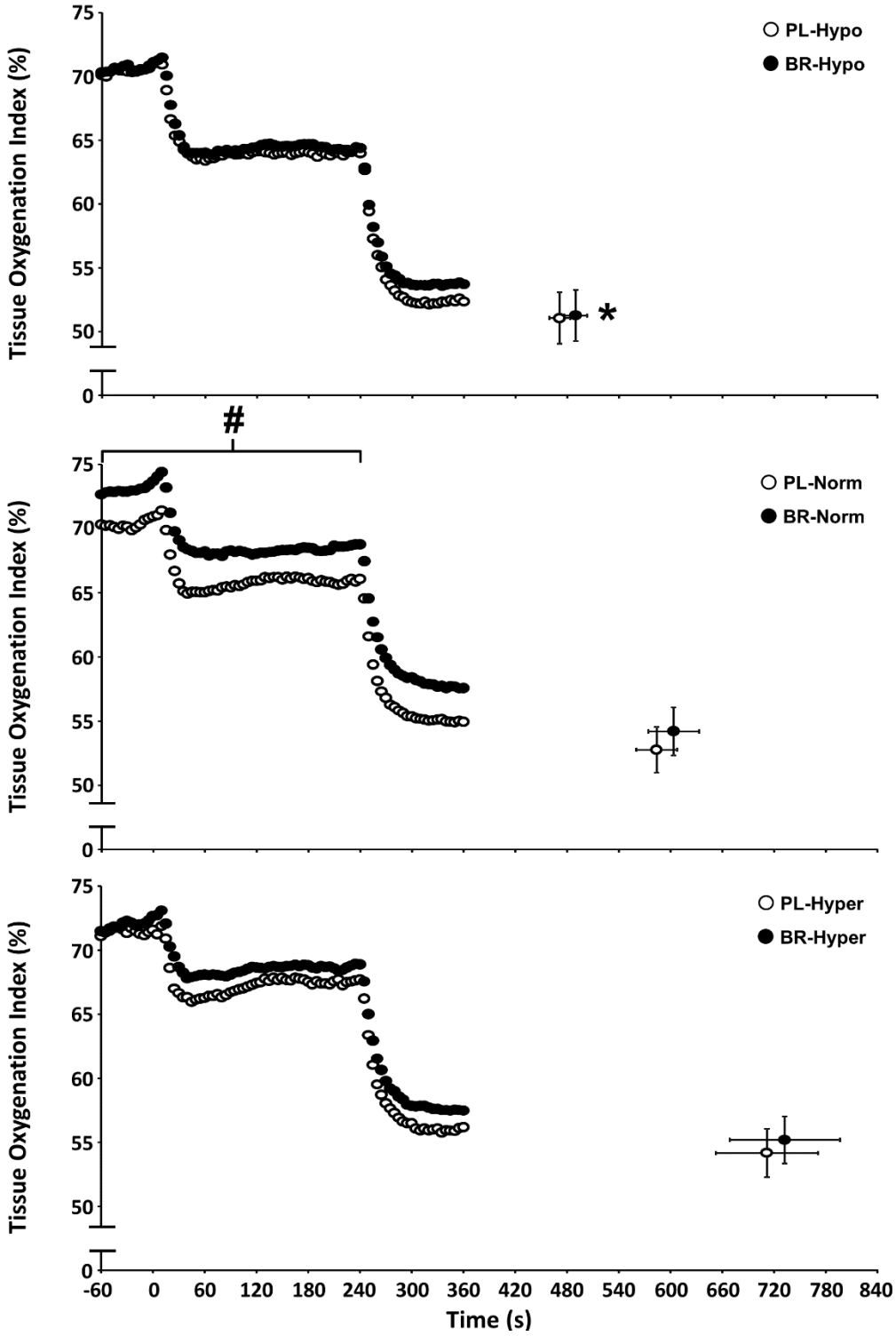

Figure 4. Quadriceps tissue oxygenation index (TOI) responses following nitrate-depleted beetroot juice (PL) and nitrate-rich beetroot juice (BR) in hypoxia (upper panel; PL-Hypo and BR-Hypo, respectively), normoxia (middle panel; PL-Norm and BR-Norm, respectively) and hyperoxia (lower panel; PL-Hyper and BR-Hyper, respectively). TOI data during baseline cycling at 20 W (-60-0 sec), moderate-intensity cycling (0-240 sec) and severe-intensity cycling (240-360 sec) are group mean responses displayed as 5 sec averages. TOI data at exhaustion during severe-intensity cycling exercise represent the mean TOI over the final 60 sec of exercise and are presented as group mean ± SEM. * indicates different from PL-Hypo (P<0.05). # indicates different from PL-Norm (P<0.05).

Pulmonary oxygen uptake kinetics

The group mean pulmonary $\dot{V}O_2$ data are presented in Table 1 with the $\dot{V}O_2$ responses from a representative individual and the group mean end-exercise $\dot{V}O_2$ shown in Figure 5. There were no differences in the absolute $\dot{V}O_2$ during baseline, moderate-intensity cycling or the first 180 sec of severe-intensity cycling between any of the experimental conditions (P>0.05). The $\dot{V}O_2$ MRT was longer following the onset of severe-intensity cycling initiated from a moderate-intensity baseline compared to moderate-intensity cycling initiated from an unloaded baseline (P<0.01; Table 1). However, the $\dot{V}O_2$ MRT during moderate-intensity and severe-intensity exercise were not different between experimental conditions (P>0.05). The change in $\dot{V}O_2$ over the first 180 sec of severe-intensity cycling and end-exercise $\dot{V}O_2$ during severe-intensity cycling were not different between the PL-Norm and PL-Hyper conditions (P>0.05) but were lower in the PL-Hypo trial compared to both the PL-Norm and PL-Hyper trials (P<0.05; Table 1). There were no differences in the change in $\dot{V}O_2$ over the first 180 sec or end-exercise $\dot{V}O_2$ during severe-intensity cycling between the BR and PL conditions in normoxia and hyperoxia (P>0.05), but these variables were both higher after BR supplementation in hypoxia compared to PL supplementation in hypoxia (P<0.05; Table 1; Figure 5).

Table 1. Pulmonary oxygen uptake kinetics following a step increment in work rate from unloaded to moderate-intensity cycling and moderate-intensity to severe-intensity cycling after nitrate-rich and nitrate-depleted beetroot juice in hypoxia, normoxia and hyperoxia.

	PL-Hypo	BR-Hypo	PL-Norm	BR-Norm	PL-Hyper	BR-Hyper
			U→M			
Baseline $\dot{V}O_2$ (L·min^{-1})	0.90 ± 0.14	0.87 ± 0.09	0.89 ± 0.09	0.89 ± 0.12	0.86 ± 0.09	0.90 ± 0.20
End $\dot{V}O_2$ (L·min^{-1})	1.65 ± 0.23	1.60 ± 0.20	1.64 ± 0.20	1.61 ± 0.22	1.62 ± 0.16	1.60 ± 0.25
∆ End-Baseline $\dot{V}O_2$ (L·min^{-1})	0.75 ± 0.11	0.72 ± 0.14	0.75 ± 0.16	0.72 ± 0.13	0.76 ± 0.15	0.69 ± 0.12
Mean response time (sec)	49 ± 15	39 ± 10	43 ± 9	47 ± 13	41 ± 14	41 ± 9
			M→S			
Baseline $\dot{V}O_2$ (L·min^{-1})	1.65 ± 0.23	1.60 ± 0.20	1.64 ± 0.20	1.61 ± 0.22	1.62 ± 0.16	1.60 ± 0.25
180 sec $\dot{V}O_2$ (L·min^{-1})	3.27 ± 0.35	3.35 ± 0.42	3.46 ± 0.47	3.49 ± 0.46	3.48 ± 0.25	3.40 ± 0.48

Δ 180 sec-Baseline $\dot{V}O_2$ ($L \cdot min^{-1}$)	1.62 ± 0.22	1.75 ± 0.24¥	1.81 ± 0.27#	1.88 ± 0.30#	1.86 ± 0.14#	1.80 ± 0.25#
Mean response time (sec)	64 ± 17*	62 ± 13*	68 ± 11*	67 ± 11*	61 ± 17*	60 ± 16*
End $\dot{V}O_2$ ($L \cdot min^{-1}$)	3.28 ± 0.33	3.43 ± 0.36¥	3.82 ± 0.53#	3.83 ± 0.44#	3.82 ± 0.35#	3.82 ± 0.58#
Δ End-Baseline $\dot{V}O_2$ ($L \cdot min^{-1}$)	1.64 ± 0.20	1.83 ± 0.21¥	2.18 ± 0.35#	2.21 ± 0.29#	2.20 ± 0.30#	2.22 ± 0.39#

Data are presented as mean ± SD. PL-Hypo, acute ingestion of a nitrate-depleted beetroot juice concentrate and inhalation of a hypoxic inspirate; BR-Hypo, acute ingestion of a nitrate-rich beetroot juice concentrate and inhalation of a hypoxic inspirate; PL-Norm, acute ingestion of a nitrate-depleted beetroot juice concentrate and inhalation of a normoxic inspirate; BR-Norm, acute ingestion of a nitrate-rich beetroot juice concentrate and inhalation of a normoxic inspirate; PL-Hyper, acute ingestion of a nitrate-depleted beetroot juice concentrate and inhalation of a hyperoxic inspirate; BR-Hyper, acute ingestion of a nitrate-rich beetroot juice concentrate and inhalation of a hyperoxic inspirate; U→M, a step increment from an unloaded baseline to a moderate-intensity work rate; M→S, a step increment from a moderate-intensity baseline work rate to a severe-intensity work rate; $\dot{V}O_2$, pulmonary oxygen uptake; Δ, difference; * different from U→M value (P<0.05); # different from PL-Hypo and BR-Hypo (P<0.05); ¥ different from PL-Hypo (P<0.05).

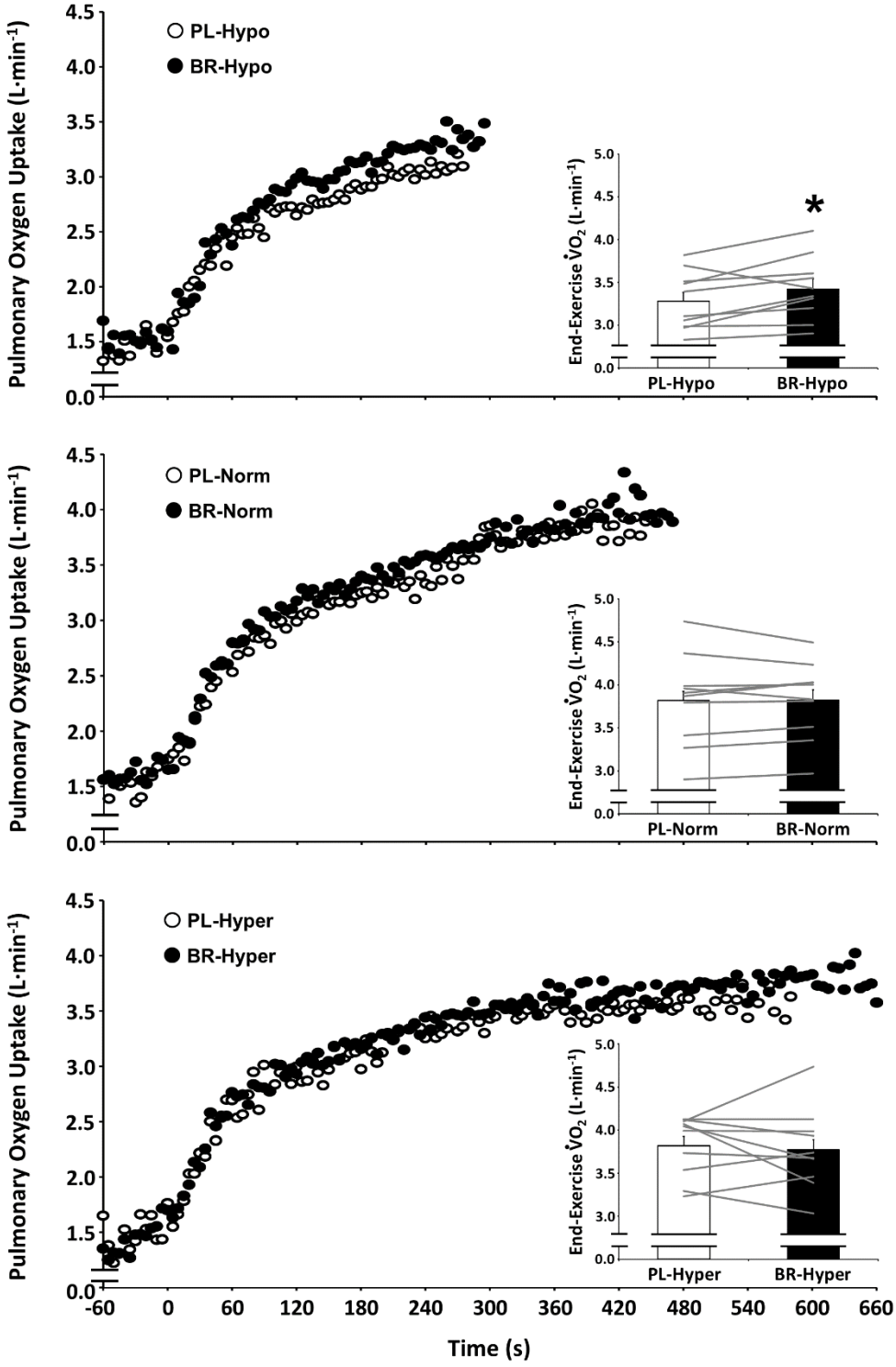

Figure 5. Pulmonary oxygen uptake ($\dot{V}O_2$) responses following nitrate-depleted beetroot juice (PL) and nitrate-rich beetroot juice (BR) in hypoxia (upper panel; PL-Hypo and BR-Hypo, respectively), normoxia (middle panel; PL-Norm and BR-Norm, respectively) and hyperoxia (lower panel; PL-Hyper and BR-Hyper, respectively). $\dot{V}O_2$ data during unloaded baseline cycling at 20 W (-60-0 sec), moderate-intensity cycling (0-240 sec) and severe-intensity cycling (240-360 sec) are displayed as 5 sec averages from a representative subject. Insets present end-exercise $\dot{V}O_2$ (mean over the final 60 sec of severe-intensity cycling) following PL and BR supplementation in hypoxia, normoxia and hyperoxia. The open bars represent the group mean ± SEM end-exercise $\dot{V}O_2$ in the PL trials, while the filled bars represent the group mean ± SEM plasma end-exercise $\dot{V}O_2$ in the BR trials. The solid grey lines represent the individual changes

in end-exercise $\dot{V}O_2$ following BR supplementation at a given fraction of inspired O_2. * indicates different from PL-Hypo (*P*<0.05).

Exercise tolerance

T_{lim} was lower in the normoxic trials (PL-Norm: 344 ± 78 sec; BR-Norm: 364 ± 98 sec) compared to the hyperoxic trials (PL-Hyper: 472 ± 196 sec; BR-Hyper: 492 ± 212 sec) and lower in the hypoxic trials (PL-Hypo: 231 ± 41 sec; BR-Hypo: 250 ± 44 sec) compared to the normoxic and hyperoxic trials (*P*<0.05; Figure 6). T_{lim} was not different between BR and PL in the hyperoxic trials (*P*>0.05), tended to be higher with BR in the normoxic trials (*P*=0.087) and was increased with BR in the hypoxic trials (*P*<0.05; Figure 6). The improved exercise tolerance after BR supplementation in hypoxia was negatively correlated with the end-exercise quadriceps TOI in the PL-Hypo trial (*r* = -0.78, *P*<0.05; Figure 7).

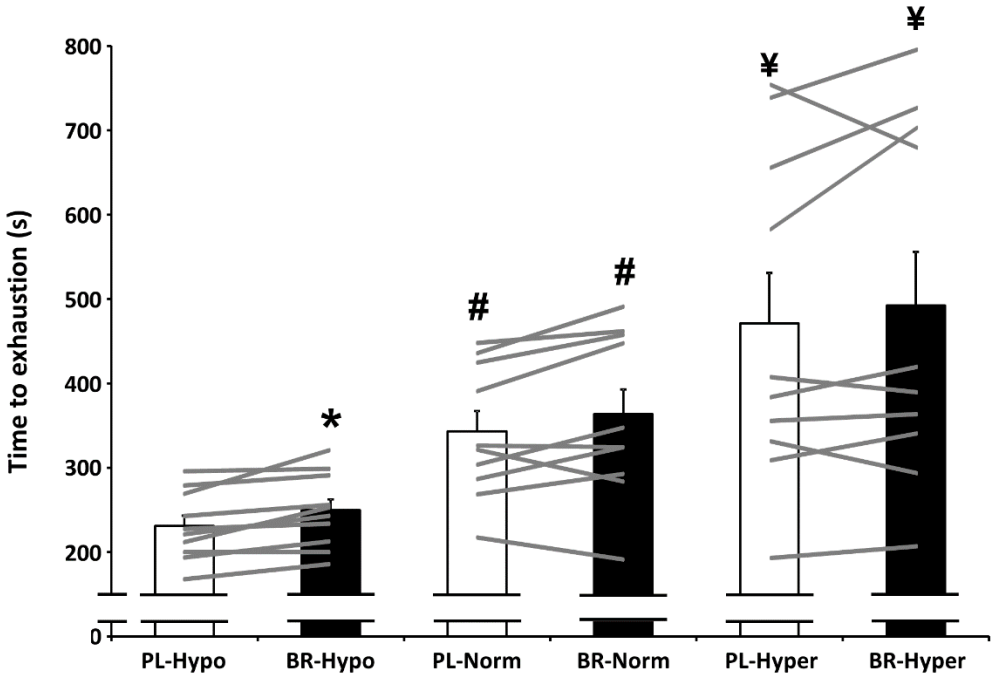

Figure 6. Time to exhaustion (T_{lim}) following nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper), and following nitrate-rich beetroot juice (BR) in hypoxia (BR-Hypo), normoxia (BR-Norm) and hyperoxia (BR-Hyper). The open bars represent the group mean ± SEM T_{lim} from the PL trials while the filled bars represent the group mean ± SEM T_{lim} from the BR trials. The solid grey lines represent the individual changes in T_{lim} following BR supplementation at a given fraction of inspired O_2. * indicates different from PL-

Hypo (P<0.05). # indicates different from PL-Hypo and BR-Hypo (P<0.05). ¥ indicates different from PL-Norm, BR-Norm, PL-Hypo and BR-Hypo (P<0.05).

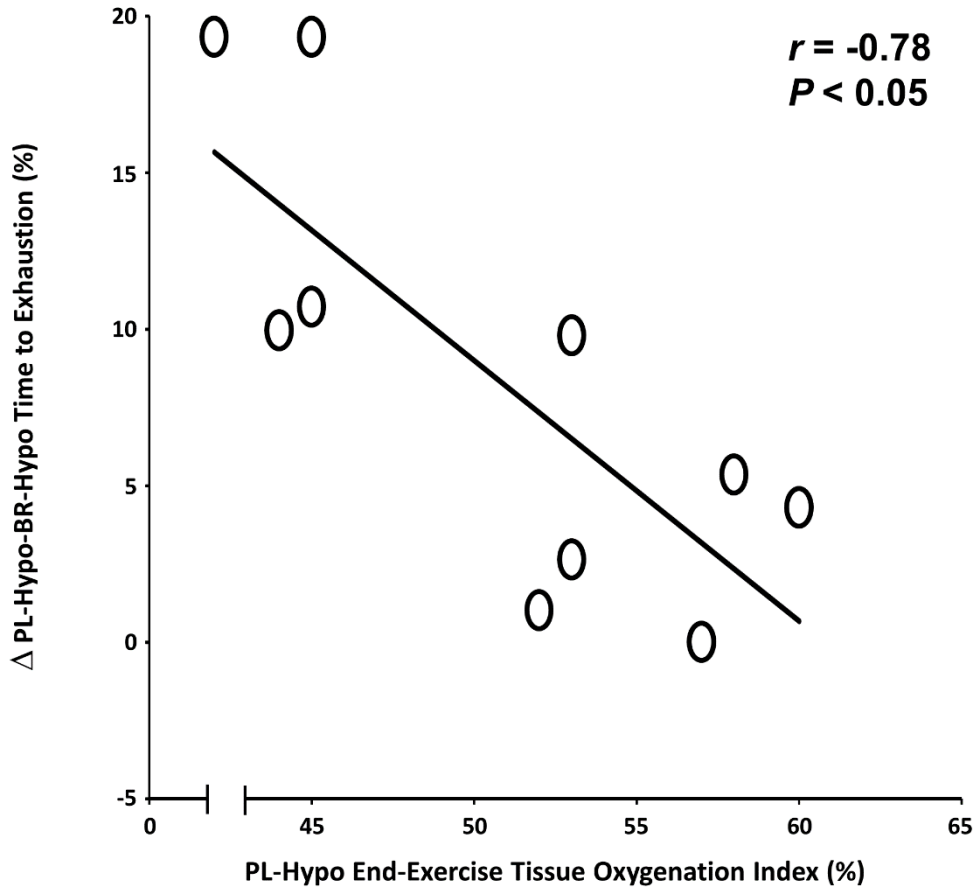

Figure 7. The relationship between end-exercise tissue oxygenation index (TOI; mean over the final 60 sec of severe-intensity cycling) following nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo) and the change (Δ) in time to exhaustion (T_{lim}) between the PL and nitrate-rich beetroot juice (BR) trials in hypoxia (PL-Hypo and BR-Hypo, respectively). Note that end-exercise TOI in PL-Hypo was negatively correlated with Δ T_{lim} between the PL-Hypo and BR-Hypo trials.

Discussion

This study investigated how dietary NO_3^- supplementation influenced muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance in hypoxia, normoxia and hyperoxia. Plasma [NO_2^-] was increased in all BR compared to all PL trials but was not influenced by the FiO_2 at rest, during exercise or at exhaustion. Multi-channel NIRS-derived quadriceps TOI was positively associated with the FiO_2 and was higher during baseline and moderate-intensity cycling exercise in normoxia following BR supplementation. T_{lim} was increased in hypoxia, with the

magnitude of improvement being negatively correlated with quadriceps TOI at exhaustion, tended to be improved in normoxia and remained unaltered in hyperoxia following BR supplementation. Peak $\dot{V}O_2$ was greater following BR supplementation in hypoxia but remained unaltered in normoxia and hyperoxia. These findings suggest that the potential for dietary NO_3^- supplementation to improve the physiological responses and tolerance to exercise is greater as skeletal muscle becomes increasingly hypoxic.

Plasma [nitrite]

Consistent with previous research (Bailey et al., 2009; 2010; Vanhatalo et al., 2010; 2011; Webb et al., 2008; Wylie et al., 2013), plasma [NO_2^-] was increased in all BR compared to all PL trials. The increment in plasma [NO_2^-] due to BR supplementation was not significantly different at baseline between hypoxia (347 ± 157 nM), normoxia (299 ± 147 nM) and hyperoxia (270 ± 64 nM) and plasma [NO_2^-] remained unaltered after 4 min of moderate-intensity and 2 min of severe-intensity exercise in all conditions. At exhaustion, plasma [NO_2^-] was lowered in each of the BR trials. This is likely due to an elevated rate of NO_2^- reduction to NO, which is facilitated in hypoxia and acidosis (Lundberg and Govoni, 2004); conditions that might be evident in contracting skeletal muscle (Bailey et al., 2010; Richardson et al., 1999). No additional influence was observed due to the FiO_2 at exhaustion. Similar [NO_2^-] kinetics were reported by Kelly et al., (2014) who investigated the variations between hypoxia and normoxia. However, the authors observed a significant reduction in plasma [NO_2^-] after 1 min of severe-intensity exercise in hypoxia following BR supplementation which was not evident in the present study. This might be explained by the larger NO_3^- dose (19.5 mmol) administered herein.

Influence of fraction of inspired oxygen on arterial and muscle oxygenation

Administration of the hypoxic (15% O_2), normoxic (21% O_2) and hyperoxic (40% O_2) inspirates influenced arterial SpO_2 and quadriceps TOI. SpO_2 was lower in hypoxia (88 ± 4%) than in normoxia (95 ± 3%) but was not different between

normoxia and hyperoxia (95 ± 6%). These changes in SpO_2 would be expected to impact upon the O_2 diffusion cascade, therefore, altering muscle oxygenation during exercise. Consequently, TOI was higher during moderate- (65 ± 4 vs. 64 ± 4%) and severe- (54 ± 6 vs. 51 ± 7%) intensity exercise in normoxia compared to hypoxia, and was greater still during moderate- (67 ± 5%) and severe- (56 ± 7%) intensity exercise in hyperoxia.

Influence of nitrate-rich beetroot juice supplementation on the physiological responses to moderate-intensity exercise

Acute BR supplementation had no effect on pulmonary $\dot{V}O_2$ but did influence quadriceps TOI, as investigated using multi-channel NIRS, during moderate-intensity exercise in normoxia. The unaltered $\dot{V}O_2$ is consistent with the observations of Breese et al., (2013) who employed a work-to-work step cycling exercise protocol, with a moderate-intensity bout that also lasted for 4 min, and a chronic BR supplementation procedure (providing ~8 $mmol.day^{-1}$ NO_3^- for 6 days). However, reductions in sub-maximal $\dot{V}O_2$ have previously been reported following a single step transition cycling bout lasting for 5-6 min and both a chronic (providing ~11 $mmol.day^{-1}$ NO_3^- for 6 days; 5% reduction in $\dot{V}O_2$; Bailey et al., 2009) and acute (providing 16.8 mmol NO_3^-; 3% reduction in $\dot{V}O_2$; Wylie et al., 2013) BR supplementation procedure. These discrepancies might be explained by the duration of the exercise protocol, such that the 4 min bout employed in the present study might not have been sufficient to detect a change in $\dot{V}O_2$. An extended duration was prevented due to the further step increment that was completed until the limit of tolerance and the possibility of this resulting in the 1000 L capacity of the Douglas bag being reached. This was a limitation of the present study and, consequently, is an area for further research. Also, chronic, rather than acute, BR supplementation might have been more effective. Despite the unaltered $\dot{V}O_2$, TOI was higher during baseline (71 ± 3 vs. 69 ± 3 %) and moderate-intensity (67 ± 5 vs. 65 ± 4 %) cycling exercise, indicating an improved muscle oxygenation (Ferrari, Mottola and Quaresima, 2004). This finding builds upon the single-channel work of Bailey et al., (2009), who observed an increased total [haemoglobin] and [oxyhaemoglobin], signifying an increased muscle O_2 delivery, and a reduced [deoxyhaemoglobin], signifying a

reduced muscle O_2 extraction, in the *vastus lateralis* during moderate-intensity exercise. This physiological response is explained by the Fick equation which states that for the same $\dot{V}O_2$ an increased muscle O_2 delivery allows for a reduced muscle O_2 extraction. An increased muscle O_2 delivery following dietary NO_3^- consumption is presumably the result of greater vasodilation, caused by increased NO_2^- reduction to NO.

Acute BR supplementation had no effect on pulmonary $\dot{V}O_2$ and quadriceps TOI in hypoxia or hyperoxia. Hypoxia is associated with increased NO_2^- reduction to NO (Bryan, 2006) whereas the combination of hypoxia and muscular contraction is known to create a strong stimulus for vasodilation to ensure sufficient muscle O_2 delivery (Calbet et al., 2009; Casey et al., 2010). Subsequent research has demonstrated a reduced $\dot{V}O_2$ during baseline (10%) and moderate-intensity (7%) cycling exercise in hypoxia with a trend towards an improved *vastus lateralis* TOI (3.6%) following BR supplementation (providing 8.4 $mmol.day^{-1}$ NO_3^- for 3 days; Kelly et al., 2014). The contradictory findings observed in the present study might be explained by the variation in supplementation procedure employed, such that the acute dose of BR administered might not have been sufficient to elicit a change in $\dot{V}O_2$ or TOI. In hyperoxia, the unaltered $\dot{V}O_2$ and TOI observed following BR supplementation, which was investigated for the first time in this study, suggests that O_2 availability to the active musculature was sufficient prior to dietary NO_3^- consumption to meet the demands for oxidative energy turnover during moderate-intensity exercise with this FiO_2. This observation supports previous research which demonstrated that O_2 delivery to the active musculature is greater than utilisation during submaximal exercise in hyperoxia (Evans et al., 2001).

Influence of nitrate-rich beetroot juice supplementation on the physiological responses and tolerance to severe-intensity exercise

Acute BR supplementation had no effect on quadriceps TOI but did influence pulmonary $\dot{V}O_2$ and exercise tolerance during severe-intensity exercise in hypoxia. T_{lim} was improved by 8%, while the change in $\dot{V}O_2$ over the first 180 sec of severe-intensity cycling exercise was increased by 8%, end-

exercise $\dot{V}O_2$ was increased by 4% and TOI remained unaltered. The elevated $\dot{V}O_2$ suggests that muscle O_2 delivery was increased following dietary NO_3^- consumption, which would be expected to enhance oxidative energy production. This would then spare the finite anaerobic reserves (i.e. phosphocreatine and glycogen), thus delaying the development of local muscle fatigue and prolonging exercise tolerance (Vanhatalo et al., 2011). Furthermore, the strong, negative correlation between the change in T_{lim} due to BR supplementation and end-exercise TOI following PL supplementation shows that participants with a lower muscle oxygenation status at the limit of tolerance had a greater ergogenic effect from dietary NO_3^- consumption. A lower oxygenation status would be accompanied by an increased O_2 pressure gradient between the contracting myocytes and the surrounding capillaries. This would likely stimulate local NO_2^- reduction to NO and, subsequently, NO-mediated vasodilation, thus elevating muscle O_2 delivery. The increased availability of O_2 would then allow for greater oxidative energy production, culminating in a prolonged tolerable duration of exercise. The magnitude of increment in T_{lim} following BR supplementation is similar to that reported previously by Kelly et al., (2014; 9%) and might translate to a ~1% improvement in athletic performance when using an ecologically valid test, such as a cycling time-trial (Paton and Hopkins, 2006). Therefore, BR supplementation might have ergogenic potential for athletes who experience a reduced skeletal muscle oxygenation status when performing at altitude.

Acute BR supplementation tended to improve exercise tolerance, while pulmonary $\dot{V}O_2$ and quadriceps TOI remained unaltered, in normoxia and had no effect in hyperoxia. In normoxia, T_{lim} was non-significantly improved by 5% which contradicts the 22% improvement reported by Breese et al., (2013), who also used the work-to-work step cycling exercise protocol. This discrepancy might be explained by the variation in supplementation procedures employed by the two studies. In hyperoxia, the unaltered T_{lim} builds upon previous research that demonstrated a reduced time to fatigue in intact mouse muscle fibres following incubation with sodium NO_2^- at a supra-physiological PO_2 (Nogueira et al., 2013). However, the 40% O_2 inspirate used in the present study is less than that administered by Nogueira et al., 2013. Therefore, further research is required to investigate how dietary NO_3^- supplementation influences muscle

oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance when inhaling 100% O_2. The unaltered $\dot{V}O_2$ and TOI might be explained by previous observations that hyperoxia is associated with an increased muscle O_2 delivery (Astorino and Robergs, 2003) and greater maintenance of muscle contractile function (Linossier et al., 2000) during maximal exercise; physiological responses that are consistent with those induced by dietary NO_3^- supplementation in hypoxia. This would be expected to attenuate the stimulus for local NO_2^- reduction to NO and provides a possible explanation for the lack of effect observed following BR supplementation in hyperoxia.

Conclusion

Plasma [NO_2^-] was increased by acute BR supplementation but was not different between hypoxia, normoxia or hyperoxia at rest, during exercise or at exhaustion. Multi-channel, NIRS-derived, quadriceps TOI was higher, while pulmonary $\dot{V}O_2$ remained unaltered, during baseline and moderate-intensity cycling exercise in normoxia following BR supplementation, which suggests an elevated muscle oxygenation consequent to an enhanced NO-mediated vasodilation. T_{lim}, the change in $\dot{V}O_2$ over the first 180 sec and end-exercise $\dot{V}O_2$ were all increased, while TOI remained unaltered, during severe-intensity exercise in hypoxia following BR supplementation. This is indicative of an increased muscle O_2 delivery which might have increased oxidative energy production, thus sparing the finite anaerobic reserves and improving exercise tolerance. T_{lim} tended to be increased in normoxia but was unaltered in hyperoxia following BR supplementation. These findings extend our understanding of the effect of dietary NO_3^- supplementation on muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance in hypoxia, normoxia and hyperoxia.

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Chapter 5: General discussion

The aim of this thesis was to enhance existing knowledge of the conditions that might influence the efficacy of dietary NO_3^- supplementation to improve exercise physiology and performance. To achieve this, two studies were performed independently to explore the reduction of NO_3^- to NO_2^- and NO_2^- to NO, respectively. These studies addressed the following research questions:

1) Does the manipulation of oral cavity temperature and pH influence the effectiveness of BR supplementation at increasing NO bioavailability in humans?

3) Does altering the FiO_2 influence the effectiveness of BR supplementation at improving the physiological responses and tolerance to exercise?

Summary of findings

Influence of oral temperature and pH on dietary nitrate metabolism in healthy adults

In chapter 3, the influence of oral cavity temperature and pH manipulation on the effectiveness of BR supplementation at increasing NO bioavailability was investigated for the first time. Salivary and plasma [NO_3^-] and [NO_2^-] were all increased following BR supplementation. Salivary [NO_2^-] was increased further when oral temperature and pH were elevated independently (41 and 90%, respectively) and concurrently (84%), however, the peak increase (116%) was evident when oral temperature was lowered (34°C) and oral pH was elevated (7.9). Plasma [NO_2^-] was also increased further 3 hours post BR consumption in the alkaline pH conditions.

Effect of dietary nitrate supplementation on oxygen uptake kinetics and exercise tolerance: influence of muscle oxygenation

In chapter 4, the influence of BR supplementation on muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance in hypoxia, normoxia and hyperoxia was investigated. Plasma [NO_2^-] was increased following BR supplementation but was not influenced by FiO_2 at rest, during exercise or at exhaustion. Multi-channel NIRS-derived quadriceps TOI was positively associated with the FiO_2 and was higher during baseline (3%) and moderate-intensity (3%) cycling exercise in normoxia following BR supplementation. T_{lim} was increased (8%) in hypoxia, with the magnitude of improvement being negatively correlated with quadriceps TOI at exhaustion, tended to be improved (5%) in normoxia and remained unaltered in hyperoxia following BR supplementation. Peak $\dot{V}O_2$ was greater (4%) following BR supplementation in hypoxia but remained unaltered in normoxia and hyperoxia.

Nitric oxide bioavailability

Consistent with previous research, the evidence in chapters 3 and 4 demonstrates that dietary NO_3^- supplementation with BR increases salivary [NO_3^-] and [NO_2^-] (McDonagh et al., 2015; Woessner et al., 2016) and plasma [NO_3^-] (McDonagh et al., 2015; Wylie et al., 2013) and [NO_2^-] (Bailey et al., 2009; 2010; Vanhatalo et al., 2010; 2011; Webb et al., 2008; Wylie et al., 2013). This suggests that NO bioavailability would also be greater following BR supplementation. The increase in resting plasma [NO_2^-] occurred following ~6.5 (142 nM; chapter 3) and ~19.5 (388 nM; chapter 4) mmol NO_3^- consumption, which identifies that the magnitude of improvement is determined by the dose of NO_3^- consumed. This finding supports the work of Wylie et al., (2013) who demonstrated dose-dependent increases in plasma [NO_2^-] following acute BR supplementation with 4.2, 8.4 and 16.8 mmol NO_3^-. The dietary NO_3^- doses used in chapters 3 and 4 were selected based on previous research which demonstrated that a larger dose is required to elicit improvements in exercise economy and tolerance compared to plasma and salivary [NO_3^-] and [NO_2^-] (McDonagh et al., 2015; Wylie et al., 2013). In chapter 3, the increase in resting salivary and plasma [NO_3^-] and [NO_2^-], and the delayed time to peak plasma [NO_2^-], following BR supplementation highlights the importance of dietary NO_3^-

on the enterosalivary circuit and, therefore, the metabolism of NO_3^- by commensal oral bacteria (Duncan et al., 1995; Sasaki and Matano, 1979; Spiegelhalder, Eisenbrand and Preussmann, 1976).

Factors influencing dietary nitrate metabolism

Oral cavity temperature and pH

Salivary and plasma [NO_3^-] remained unaltered following acute dietary NO_3^- supplementation with BR and oral temperature and pH manipulation. Salivary [NO_2^-] was increased further in the independently ($Temp_{Mid}$-pH_{Hi}: 90%; $Temp_{Hi}$-pH_{Norm}: 41%) and concurrently ($Temp_{Hi}$-pH_{Hi}: 84%) elevated oral temperature and pH conditions and in the low oral temperature conditions ($Temp_{Lo}$-pH_{Norm}: 53% $Temp_{Lo}$-pH_{Hi}: 106%) compared to the $Temp_{Mid}$-pH_{Norm} trial. Plasma [NO_2^-] was increased further than the $Temp_{Mid}$-pH_{Norm} trial in the independently elevated oral pH condition ($Temp_{Mid}$-pH_{Hi}: 20%), the concurrently elevated oral temperature and pH condition ($Temp_{Hi}$-pH_{Hi}: 53%) and in the $Temp_{Lo}$-pH_{Hi} trial (37%). This evidence indicates that dietary NO_3^- metabolism is influenced by oral cavity temperature and pH and builds upon previous research that demonstrated an increased [NO_2^-] following both NO_3^- consumption and oral pH manipulation using a pH-regulating chewing gum (van Maanen, van Geel and Kleinjans, 1996) and the incubation of an alkaline NO_3^- test solution in the mouth (Bojić, Bojić and Pervović, 2004).

Muscle oxygenation

In chapter 4, the increased plasma [NO_2^-] observed following acute dietary NO_3^- supplementation with BR was not significantly influenced by the FiO_2 at rest, during ‘work-to-work step’ cycling exercise to moderate- and severe-intensity constant work rates or at exhaustion. This evidence indicates that muscle oxygenation does not influence dietary NO_3^- metabolism and builds upon previous research, that demonstrated no variation in plasma [NO_2^-] kinetics between normoxia and hypoxia (Kelly et al., 2014), by showing, for the first time, that a similar effect is apparent in hyperoxia.

Applications

The studies in this thesis provide evidence that dietary NO_3^- supplementation increases the potential for O_2- and NOS-independent NO generation in healthy young adults. Increased NO bioavailability has previously been associated with a number of physiological benefits and ergogenic effects. For example, dietary NO_3^- consumption has been reported to lower systolic blood pressure at rest, reduce pulmonary $\dot{V}O_2$ and muscle fractional O_2 extraction during moderate-intensity exercise and to improve tolerance to severe-intensity exercise (Bailey et al., 2009).

In chapter 3, the elevated salivary and plasma [NO_2^-] demonstrated following acute BR supplementation and oral temperature and pH manipulation might have implications for future BR supplementation recommendations, might contribute to understanding of the underlying physiology for responders and non-responders to dietary NO_3^- supplementation, and might enhance the cardiovascular health and physical performance benefits demonstrated at a given dietary NO_3^- dose.

In chapter 4, the improved T_{lim} in hypoxia following acute BR supplementation might be of interest to athletes and coaches involved in short-duration, severe-intensity cycling exercise performed at moderate altitude. BR supplementation was demonstrated to be particularly effective for individuals who experience a reduced skeletal muscle oxygenation status when performing in hypoxia. The development of specific pre-competition nutritional plans involving the consumption of dietary NO_3^- might improve athletic performance.

Limitations and directions for future research

Despite the same dietary NO_3^- dose being consumed in chapter 3, the increases in salivary and plasma [NO_2^-] varied depending on the temperature and pH of the oral cavity. The greatest increase in salivary [NO_2^-] was observed in the $Temp_{Lo}$-pH_{Hi} trial. This was attributed to an elevated salivary flow rate which would likely result in greater NO_3^- secretion by the salivary glands thus

providing more substrate for reduction to NO_2^-. However, despite the larger salivary [NO_2^-] in the $Temp_{Lo}$-pH_{Hi} trial, the greatest increase in plasma [NO_2^-] occurred in the $Temp_{Hi}$-pH_{Hi} trial. It was suggested that this measurement might have been confounded by the mouth rinse protocol, which resulted in 46% more NO_2^--rich saliva being expectorated following the mouth rinses and sample collections in the $Temp_{Lo}$-pH_{Hi} trial compared to the $Temp_{Hi}$-pH_{Hi} trial. This would likely impair the characteristic rise in plasma [NO_2^-] demonstrated following dietary NO_3^- supplementation (Lundberg and Govoni, 2004; Webb et al., 2008). Furthermore, the oral temperature manipulation might have elevated core temperature, which is known to increase endothelial NOS activity and NO release (Harris et al., 2003). This would be expected to increase plasma [NO_2^-] independently of dietary NO_3^- metabolism. Future research should build upon these limitations by controlling for potential confounding factors when investigating the factors that influence dietary NO_3^- metabolism.

Conclusion

Investigation of the cardiovascular health and physical performance benefits of dietary NO_3^- supplementation is a current hot topic in nutrition. This thesis aimed to contribute to the available literature by investigating the factors that influence dietary NO_3^- metabolism in humans. The collected data suggests that oral cavity temperature and pH manipulation influences dietary NO_3^- metabolism in humans and that the performance benefit of NO_3^- supplementation is influenced by muscle oxygenation.

The study in chapter 3 demonstrates, for the first time, that salivary [NO_2^-] following acute BR supplementation is elevated by a decreased oral temperature and an increased oral pH. This finding might have implications for enhancing some of the beneficial effects of dietary NO_3^- supplementation on human cardiovascular health and physical performance. The study in chapter 4 demonstrates that severe-intensity exercise tolerance is improved to a greater extent as skeletal muscle becomes increasingly hypoxic. This finding suggests that acute BR supplementation might have applications as an ergogenic aid for the performance of short-duration, severe-intensity cycling exercise in moderate hypoxia.

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